REVIEW ARTICLE

Grb10 and Grb14: enigmatic regulators of insulin action – and more?

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The Grb proteins (growth factor receptor-bound proteins) Grb7, Grb10 and Grb14 constitute a family of structurally related multi-domain adapters with diverse cellular functions. Grb10 and Grb14, in particular, have been implicated in the regulation of insulin receptor signalling, whereas Grb7 appears predominantly to be involved in focal adhesion kinase-mediated cell migration. However, at least *in vitro*, these adapters can bind to a variety of growth factor receptors. The highest identity within the Grb7/10/14 family occurs in the C-terminal SH2 (Src homology 2) domain, which mediates binding to activated receptors. A second well-conserved binding domain, BPS [between the PH (pleckstrin homology) and SH2 domains], can act to enhance binding to the IR (insulin receptor). Consistent with a putative adapter function, some non-receptor-binding partners, including protein kinases, have also been identified. Grb10 and Grb14 are widely, but not uniformly, expressed in mammalian tissues, and there are various isoforms of Grb10. Binding of Grb10 or Grb14 to autophosphorylated IR *in vitro* inhibits tyrosine kinase activity towards other substrates, but studies on cultured cell lines have been con-

flicting as to whether Grb10 plays a positive or negative role in insulin signalling. Recent gene knockouts in mice have established that Grb10 and Grb14 act as inhibitors of intracellular signalling pathways regulating growth and metabolism, although the phenotypes of the two knockouts are distinct. Ablation of Grb14 enhances insulin action in liver and skeletal muscle and improves whole-body tolerance, with little effect on embryonic growth. Ablation of Grb10 results in disproportionate overgrowth of the embryo and placenta involving unidentified pathways, and also impacts on hepatic glycogen synthesis, and probably on glucose homeostasis. This review discusses the extent to which previous studies *in vitro* can account for the observed phenotype of knockout animals, and considers evidence that aberrant function of Grb10 or Grb14 may contribute to disorders of growth and metabolism in humans.

Key words: adapter protein, cell growth, cell signalling, Type II diabetes, tyrosine kinase.

INTRODUCTION

The Grb (growth factor receptor-bound) proteins Grb7/10/14 are cellular adapter proteins lacking intrinsic enzymatic activity, which share a common multidomain structure. These adapters can bind to numerous receptor (and non-receptor) tyrosine kinases, as well as several intracellular proteins, and have been suggested to function in diverse cellular processes, including the regulation of cellular growth and metabolism, apoptosis and cell migration (as reviewed previously [1–7]). Grb10 and Grb14, in particular, have been implicated in binding to, and regulating signals from, the IR (insulin receptor) and IGFR (type 1 insulin-like growth factor receptor), and this review will focus on studies in this area.

Each member of the Grb7/10/14 family was originally identified by CORT (cloning of receptor targets) screens of cDNA expression libraries using the autophosphorylated C-terminus of the EGFR (epidermal growth factor receptor) as a probe to identify interacting proteins. Grb10, initially discovered in a screen of an NIH-3T3 library [8], was found to be structurally similar to the previously identified Grb7 and the *Caenorhabditis elegans* protein F10E9.6/Mig-10, both of which contain a central region of

similarity [9]. Soon afterwards, an additional protein with homology with this central region, Grb14, was identified in a screen of a breast epithelial cDNA library [10]. Thus a new family of proteins with a common multidomain structure emerged (Figure 1). The central region of similarity, shared with Mig-10 and hence referred to as the GM (Grbs and Mig) region [11], is almost 60% identical between Grb7, Grb10 and Grb14. Within the GM region are RA (Ras-association)-like and PH (pleckstrin homology) domains. An SH2 (Src homology 2) domain located towards the C-terminus is conserved among mammalian family members, although the Mig-10 protein lacks an SH2 domain. The so-called BPS (between the PH and SH2 domains) region [12] is unique among this family of proteins. All the Grb7/10/14 family, as well as Mig-10, possess a proline-rich region towards their N-termini. Apart from Mig-10, neither *C. elegans* nor *Drosophila melanogaster* possess orthologues of Grb7/10/14. The acquisition of Grb7/10/14 structure and function therefore appears to have been relatively late, in evolutionary terms.

The Grb7/10/14 proteins are superficially similar to those of the APS (adapter protein with PH and SH2 domains)/SH2-B family [13], with regard to the presence and disposition of PH and SH2

Abbreviations used: EGF(R), epidermal growth factor (receptor); Erk, extracellular-signal-regulated kinase; FAK, focal adhesion kinase; FGF(R), fibroblast growth factor receptor; GIGYF protein, Grb10 interacting GYF protein; GK, Goto-Kakizaki; Grb, growth factor receptor-bound protein; GM, Grbs and Mig; GST, glutathione S-transferase; IGF-I, type I insulin-like growth factor; IGFR, IGF-I receptor; IR, insulin receptor; IRS, IR substrate; IUP, intrinsically unstructured protein; MAPK, mitogen-activated protein kinase; Meg, maternally expressed gene; MEK1, MAP kinase/Erk kinase; Nedd4, neuronal precursor cell-expressed developmentally down-regulated; PDGF(-BB), platelet-derived growth factor(-BB); PDK-1, 3'-phosphoinositide-dependent kinase 1; PH, pleckstrin homology; SH2, Src homology 2; APS, adapter protein with PH and SH2 domains; BPS, between the PH and SH2 (domains); PI3K, phosphoinositide 3-kinase; PKC ζ , protein kinase C ζ ; PTP, protein tyrosine phosphatase; RA, Ras-association; Shc, Src homology collagen; siRNA, small interfering RNA; SRS, Silver-Russell syndrome; TZD, thiazolidinedione; (m)UPD7, (maternal) uniparental disomy of chromosome 7; UTR, untranslated region; VEGF(R2), vascular endothelial growth factor (receptor 2); ZIP, PKC ζ -interacting protein.

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Figure 1 A schematic representation of the Grb7/10/14 family of proteins, including Mig-10

The Grb10 isoform shown is hGrb10 γ , which is most similar in size and architecture to Grb7 and Grb14. These proteins share a common central GM region of homology, containing an RA-like domain and a PH domain. They also have a proline-rich region (P) near their N-termini. Mig-10 has an additional proline-rich region near its C-terminus, whereas the Grb7/10/14 proteins possess a C-terminal SH2 domain. The BPS (between PH and SH2) domain is apparently unique to the Grb7/10/14 family. The domain structure of APS is also shown for comparative purposes. Near the C-terminus, Tyr⁶¹⁸ is phosphorylated by the insulin receptor [119]. The domains are drawn to scale, and the amino acid (aa) lengths of the proteins are indicated.

domains and their interaction with the autophosphorylated IR (Figure 1). However, the structural similarity seems more likely to be a result of convergent, rather than divergent, evolution. There is no significant sequence similarity between the PH domains of Grb7/10/14 and those of APS/SH2-B, and the sequence similarity between SH2 domains of the two protein families is only 25–30%.

STRUCTURE–FUNCTION RELATIONSHIPS IN THE GRB7/10/14 FAMILY

Grb10 is the most studied member of the protein family, in terms of both structure and function. Multiple isoforms of Grb10 have been described, which differ in their N-terminal regions and arise from alternative splicing or translation initiation. Of these isoforms, human Grb10 γ corresponds most closely to Grb7 and Grb14. Gel filtration and sedimentation experiments show that Grb10 exists in solution in oligomeric form [14,15]. The first such study concluded that Grb10 was most likely a tetramer, and that oligomerization involved interactions between the N-terminal region and both PH and BPS–SH2 domains [14]. However, a subsequent study, supported by both crystallographic and mutational analysis, proposed that Grb10 most likely forms an extended dimer, mediated by self-association of SH2 domains [15].

SH2 domain

The SH2 domain is the most highly conserved region among members of the Grb7/10/14 family. Compared with Grb10, the SH2 domains of Grb14 and Grb7 have 72 and 67% amino acid sequence

identity respectively. This phosphotyrosine-binding module is important for interactions with receptor tyrosine kinases and other intracellular signalling proteins. However, different binding preferences are exhibited by Grb10, Grb14 and Grb7. Full-length Grb10 interacts particularly well with the IR and IGFR, and in yeast two-hybrid screens with these activated receptors, the SH2 domain of Grb10 represented the greatest number of independent clones relative to other binding proteins [16,17]. Similarly, the isolated SH2 domain of Grb14 is able to bind the activated IR in a yeast two-hybrid screen, although a GST (glutathione S-transferase) pull-down assay is not sufficient to support this interaction [18].

The isolated SH2 domain of Grb10 has been crystallized and its structure determined at 1.65 Å (1 Å = 0.1 nm) resolution (Figure 2) [15]. Although SH2 domains are typically monomeric, the Grb10 SH2 domain crystallizes as a dimer. The dimer interface is made up of residues within and flanking the SH2 C-terminal α -helix, which are conserved within the Grb7/10/14 family, but not in other SH2 domains. On the basis of the structural analysis and previous biochemical studies, it was concluded that Grb10 and Grb14 have a partially impaired ability to bind phosphotyrosine-containing ligands due to a non-glycyl residue at the end of the BC loop, and the lack of a P+3 binding pocket [15]. The solution structure of the Grb14 SH2 domain, as determined by NMR spectroscopy, is indeed very similar to the crystal structure of the Grb10 SH2 domain, apart from some differences in the relative orientation of the C-terminal helices [19]. The Grb7 SH2 domain, for which a solution structure has also been determined by NMR, may bind phosphopeptides with higher affinity, and especially the pYVNQ sequence in HER2/ErbB2 (where pY represents phosphotyrosine), due to some variations in its sequence relative to Grb10 and Grb14 [20]. The structural features of the Grb10/14 SH2 domains should favour binding of turn-containing phosphotyrosine sequences, such as those found in the phosphorylated activation loops of the IR and IGFR, rather than an extended conformation, as usually bound to other SH2 domains. Binding to IR and IGFR might additionally be favoured by their dimeric nature and by interactions involving the BPS domain of Grb10, as discussed below.

The crystal structure of the SH2 domain of APS has been solved in complex with the phosphorylated tyrosine kinase domain of the IR [21]. This also reveals a dimeric arrangement of SH2 domains, in this case associated via an extended and non-canonical C-terminal α -helix. The APS–SH2 dimer engages two IR kinase molecules, with pTyr¹¹⁵⁸ of the activation loop bound to the canonical phosphotyrosine-binding pocket of the SH2 domain, whereas pTyr¹¹⁶², together with aspartate residues at positions 1156 and 1161, also interact. Thus dimerization may be a particular feature of adapter proteins involved in signalling from receptors such as the IR and IGFR, which themselves exist as pre-formed covalent dimers.

BPS domain

The BPS domain was first identified in Grb10, and was named after its location between PH and SH2 domains [12]. In the same year, it was identified in Grb14, where it was referred to as the PIR (for phosphorylated IR-interacting region) [18]. This region, consisting of approx. 80 amino acids, shows 56–64% sequence identity among the Grb7/10/14 family members, but is not homologous with any other known protein interaction domain. The BPS region appears to be intrinsically unstructured, and has been categorized into the expanding family of IUPs (intrinsically unstructured proteins) [22]. However, the isolated domain does retain physiological activity: it interacts with the activated IR and IGFR [12], it can inhibit IR catalytic activity [23,24] and it can

Figure 2 Crystal structure of the Grb10 SH2 dimer

A ribbon diagram of the Grb10 SH2 dimer. Individual SH2 domains are coloured gold and purple. The N- and C-termini are indicated. The dimer interface consists of residues in the C-terminal α -helices, as well as some flanking residues. Figure modified from [15] with permission. © (2003) American Society for Biochemistry and Molecular Biology.

prevent insulin-induced germinal vesicle breakdown in *Xenopus laevis* oocytes [22]. In addition, heat treatment of the BPS domain does not alter its physiological activity, characteristic of the IUP family [22]. A more recent study provides evidence for a transiently structured short stretch of the Grb14 BPS domain, encompassing residues 399–407, which may be significant in receptor binding [25]. Indeed, the BPS domain is an important receptor binding determinant which, in spite of its sequence conservation, is thought to impart specificity within the Grb7/10/14 family, as will be discussed in greater detail below.

PH domain

The PH domain is also well conserved amongst the Grb7/10/14 family, with approx. 60% sequence identity between family members. It is generally assumed that PH domains facilitate localization to membranes through interaction with phospholipids [26]. The binding specificities of the Grb10 and Grb14 PH domains have not been determined, although the Grb7 PH domain has been shown to bind specific phosphoinositides [27]. The highest affinity binding was observed for PtdIns3P and PtdIns5P, and there was weak association with PtdIns(3,4)P₂ and PtdIns(3,4,5)P₃. The interaction of Grb7 with phospholipids has been implicated in promoting its phosphorylation by FAK (focal adhesion kinase) and subsequent enhancement of cell motility. An arginine residue in the second β -sheet of the Grb7 PH domain is critical for phosphoinositide binding, as for the PH domains of other proteins. This arginine is not conserved in Grb10 or Grb14, although they do have a lysine at this position, and may therefore have a similar capacity to bind phospholipids.

Proline-rich region

In the N-terminal regions of Grb7/10/14 is a conserved proline-rich sequence, P(S/A)IPNPFPEL, as well as other PXXP motifs that are the minimal consensus for classical SH3 binding. The SH3 domain of c-Abl binds Grb10 *in vitro*, and the interaction is abolished in the presence of a peptide corresponding to the conserved proline-rich sequence [28]. Recently, two novel Grb10-interacting GYF proteins (GIGYFs 1 and 2) were discovered by

yeast two-hybrid screening [29]. These proteins bind to tandem proline-rich regions in the N-terminus of Grb10 via GYF motifs rather than SH3 domains. Stimulation of IGFR-overexpressing fibroblasts with IGF-I (insulin-like growth factor type I) increased binding of GIGYF1 to Grb10, and induced transient association of both these proteins with the IGFR. Whether these interactions are physiologically important remains to be determined. The N-terminal region of Grb14 interacts with tankyrase 2, although the exact binding site has not been characterized [30]. Tankyrase 2 contains a poly(ADP-ribose) polymerase homology domain and an ankyrin repeat region, of which repeats 10–19 interact with Grb14. Grb14 and tankyrase 2 associate *in vivo* in a low-density microsome fraction, but the functional significance of the interaction is unclear. No interactions have been reported thus far for the N-terminal region of Grb7.

RA domain

An RA-like domain was identified within the central GM region of the Grb7/10/14 family using a bioinformatics approach based on sequence analysis and fold recognition [31]. The RA domain is found in a variety of signalling proteins, several of which are known to be RasGTP effectors [32]. It is very similar in structure to the Ras-binding region of c-Raf, although it has little sequence identity [33]. It has been speculated that the RA-like domain of the Grb7/10/14 family may allow it to bind to proteins of the Ras superfamily. Indeed, recent evidence has demonstrated an interaction between Grb7 and the Ras family members N-Ras, K-Ras and R-Ras3 [34].

GRB7/10/14 FAMILY: GENES AND ISOFORMS

Grb10

Cloning of mouse and human Grb10 revealed several isoforms of this protein, differing between species (Figure 3). Partial clones have also been obtained for rat Grb10. In each case, the isoforms are generated from a single gene by alternative splicing. Further heterogeneity of mouse isoforms, both endogenous and ectopically expressed from cloned cDNA [8,17], apparently reflects

Figure 3 A schematic representation of the mouse and human isoforms of Grb10

All the mouse and human isoforms of Grb10 have a proline-rich region (P) near the N-terminus and a highly conserved SH2 domain at the C-terminus. They also share a central region consisting of RA-like, PH and BPS domains, although the hGrb10 β isoform has a truncated PH domain. The mGrb10 α and mGrb10 δ isoforms have additional mouse-specific regions (M) between the proline-rich and RA-like domains. The hGrb10 β , hGrb10 ζ and hGrb10 ϵ isoforms have a common extended N-terminal region with additional unique segments at the extreme N-terminus. Amino acid (aa) lengths and predicted molecular masses are indicated.

the use of alternative translation initiation sites. The human (h)Grb10 γ isoform is the prototypical member of the family, being most similar to Grb7 and Grb14, whereas other human isoforms have extended N-terminal sequences. The mouse (m) forms of Grb10 possess additional 'mouse-specific' regions of 80 (mGrb10 α) or 55 (mGrb10 δ) amino acids between the proline-rich and RA-like domains. The discovery and naming of Grb10 isoforms by different laboratories initially created a confused nomenclature. However, a system for consistent nomenclature has been established at <http://cbr-rbc.nrc-cnrc.gc.ca/thomaslab/grb7.html>, and this will be followed in this review.

The mouse Grb10 gene is located on chromosome 11 and spans approx. 110 kb [8,35]. The gene consists of 18 exons, with the translational start site(s) in exon 3 and the termination site in exon 18. mGrb10 α includes coding from exon 5, whereas mGrb10 δ does not. The existence of two promoters, in exon 1 and exon 1a, can also give rise to alternative transcripts of ≈ 5.5 kb [35]. In mice that have disruption of exons 2–4, Northern blot analysis with a probe to exon 18 reveals an additional transcript of 1.5 kb, believed to encode another isoform referred to as mGrb10 ι . This transcript is not detected by a probe to exons 11–16, which encode the BPS, and part of the PH and SH2 domains of the mGrb10 proteins. By Western blotting of lysates from e12.5 embryos with a C-terminal antibody to Grb10, small immunoreactive fragments were detected, which could correspond to translation of mGrb10 ι [35]. Further work is required to determine the functional significance of these short Grb10-related peptides.

The human Grb10 gene is located on chromosome 7, spans greater than 190 kb, and consists of at least 22 exons [36–38]. While the hGrb10 γ protein is most similar to Grb7 and Grb14, the other isoforms of hGrb10 are distinguished by their N-terminal extensions, which are created by alternative splicing involving use of different exons. A common extension of 41 amino acids is shared between hGrb10 β , hGrb10 ζ and hGrb10 ϵ . Unique regions of extension are also found in these isoforms (17 amino acids for both hGrb10 β and hGrb10 ζ ; 11 amino acids for hGrb10 ϵ). Whether these extensions convey distinct functional properties remains to be examined. hGrb10 β and hGrb10 ζ differ only in their PH regions, with hGrb10 β lacking a 46-amino-acid segment in this domain that would be expected to abrogate its function, at least in terms of phospholipid binding. The functional significance of this deletion remains unclear, although it has been suggested it might be related to the ability of Grb10 to protect against apoptosis [39]. Apart from the differences in coding sequence, a number of 5'-untranslated exons are also subject to alternative splicing [38]. The significance of this is again unclear, although the structure of 5'UTRs (untranslated regions) might be expected to influence the efficiency of mRNA translation.

Grb14

In the case of Grb14, the mouse, rat and human orthologues exhibit close similarity. Only one isoform has been reported for each species, although recent evidence supports the existence of a variant of hGrb14, called hGrb14 β , with a truncated SH2 domain (R. Kairouz and R. J. Daly, personal communication).

Grb7

For Grb7, the mouse, rat and human orthologues also have a high degree of identity. A variant of hGrb7 has been reported, which lacks a C-terminal portion of the SH2 domain [40]. However, unlike the putative truncated hGrb14 β , hGrb7V expresses a short hydrophobic region in place of the truncation, resulting from a frame-shift. The expression of Grb7V has been associated with invasive oesophageal carcinoma.

TISSUE DISTRIBUTION AND RELATIVE EXPRESSION OF GRB7/10/14

Murine expression profile

Murine Grb10 mRNA is highly expressed in insulin target tissues, such as skeletal muscle and adipose tissue, as well as in heart and kidney, as determined by Northern blot analysis [8,17]. It is also detectable, at a lower level, in brain, lung and liver. In mouse embryos, analysis of mRNA levels by *in situ* hybridization showed high levels of expression in a variety of muscle types, and in liver, brain and cartilage [35]. Additionally, a study of rat liver by reverse transcriptase (RT)-PCR found that Grb10 mRNA was highly expressed in fetal tissue, but was not detectable in adult liver [41]. Rodent Grb14 transcripts are also found at relatively high levels in the major insulin-responsive tissues (liver, white adipose and skeletal muscle), as well as in the heart [18,42]. Similarly, the protein is readily detected in these tissues [42], although expression in murine liver is approx. 5-fold higher than in adipose tissue and 20-fold higher than in skeletal muscle [43]. Unlike Grb10 and Grb14, Grb7 transcripts are not detected in murine skeletal muscle, although they are present in liver. Indeed, expression is highest in the liver and kidney, and is relatively restricted to these two tissues [44].

Human expression profile

The reported distribution of mRNA and protein for human Grb7/10/14 generally parallels that of murine tissues, but with

a few exceptions. Expression of hGrb10 transcripts is high in skeletal muscle, as for mGrb10. But, unlike mGrb10, expression of hGrb10 is also high in the pancreas [28,45,46]. Both skeletal muscle and pancreas have several RNA transcripts, consistent with the presence of more than one splice variant in these tissues, although this could also reflect variation in non-coding sequence. Expression of hGrb10 is intermediate in cardiac muscle and brain, whereas low-level expression is observed in a variety of other tissues (placenta, lung, liver, kidney, spleen, prostate, testis, ovary, small intestine and colon). Although there is no published information on the abundance of Grb10 in human adipose tissue, two mRNA transcripts were detected in rhesus monkey adipose tissue [46]. In the case of hGrb14, transcripts are highly expressed in liver and heart, as for rodent tissues. Transcripts are also detected in human skeletal muscle, pancreas, kidney and gonads, with relatively lower levels in the brain and placenta [10].

Overall, Grb10 and Grb14 exhibit similar, but by no means identical, patterns of expression across a broad range of tissues, with insulin target tissues prominent among those with highest levels of expression. Available data suggest that Grb14 is more abundant than Grb10 in adult liver, whereas Grb10 may be more abundant than Grb14 in skeletal muscle, and perhaps also in adipose tissue. However, no direct quantitative comparison of the expression levels of Grb10 and Grb14 within a given tissue has been made. In contrast, the distribution of Grb7 is more limited, consistent with a different function for this protein. Expression of hGrb7 has been reported in the pancreas, prostate, liver and small intestine, whereas expression in the mouse is apparently restricted to the liver and kidney [28].

THE ROLES OF GRB10 AND GRB14 IN INSULIN AND IGF-I ACTION

Receptor binding of Grb10 and Grb14

Although all the members of the Grb7/10/14 family were initially discovered through their binding to autophosphorylated EGFRs, it was rapidly realized that, as with other SH2-domain-mediated interactions, these adapter proteins could bind to numerous other receptors and tyrosine kinases. The interactions of Grb10 and Grb14 with IR, and to a lesser extent with IGFR, have been well studied, but interactions, particularly involving Grb7, have also been reported with c-kit/SCFR (stem cell factor receptor), EphB1, ErbB2/3/4, FGFR (fibroblast growth factor receptor), PDGF β R (platelet-derived growth factor β -receptor), Ret and Tek (tunica endothelial kinase)/Tie2 (see Table 1 for a comprehensive list). Interactions have been demonstrated by a variety of approaches, often involving yeast two-hybrid screens, overexpression systems or pull-down assays, which do not provide information on relative affinity of different receptors for a given adapter or of different adapters for a given receptor, and systematic studies of a comparative nature have not been carried out. However, it is recognized, for instance, that Grb10 binds relatively weakly to the EGFR compared with the IR [12]. It is difficult to know which interactions have the potential to be physiologically significant, bearing in mind also the levels of expression of adapters and receptors in different tissues. In the absence of quantitative experimental data, significance is perhaps best inferred from information about the binding mechanisms of the adapters. In this context, the specificity of the SH2 domains and the additional contribution to binding from the BPS domains must be taken into account.

Receptor regions involved in Grb7/10/14 binding

Grb7 has a broad binding profile [3,4], consistent with its SH2 domain specificity for pYXN motifs which are present in a variety of receptor tyrosine kinases, and in tyrosine-phosphorylated pro-

Table 1 Interactions of the Grb7/10/14 family members with cell surface receptors

Binding of the Grb7/10/14 family is indicated by $\checkmark$ for numerous cell surface receptors. Where known, the binding preference is also indicated. GHR, growth hormone receptor; n.s., not studied; x, no binding detected.

Receptor	Grb7	Grb10	Grb14	Preference	References
c-kit/SCFR	$\checkmark$	$\checkmark$	n.s.		[63,107]
EGFR	$\checkmark$	$\checkmark$	$\checkmark$	Grb7 > Grb14	[8,10,12,28,40,44]
Eph family	$\checkmark$	$\checkmark$	n.s.		[108,109]
ErbB2	$\checkmark$	x	x		[10,110,111]
ErbB3	$\checkmark$	n.s.	n.s.		[112]
ErbB4	$\checkmark$	n.s.	n.s.		[112]
FGFR	$\checkmark$	$\checkmark$	$\checkmark$	Grb7/14 $\gg$ Grb10	[50,52,53]
G6f	$\checkmark$	n.s.	n.s.		[113]
GHR	n.s.	$\checkmark$	n.s.		[114]
IGFR	n.s.	$\checkmark$	n.s.		[12,24,46,54,115]
IR	$\checkmark$	$\checkmark$	$\checkmark$		[12,16,18,28,45,46,49,51]
PDGF β R	$\checkmark$	$\checkmark$	$\checkmark$	Grb7 = Grb14	[10,28,47,50,52]
Ret	$\checkmark$	$\checkmark$	n.s.	Grb7 > Grb10	[116,117]
Tek/Tie2	$\checkmark$	n.s.	$\checkmark$		[118]
VEGFR	n.s.	$\checkmark$	n.s.		[72]

teins such as Shc (Src homology collagen) [9,47]. Grb7 appears to interact less well than Grb10/14 with IR/IGFR. The SH2 domains of Grb10 evidently prefer multiply phosphorylated motifs in β -turns, as in the autophosphorylated activation loops of the IR and IGFR [15]. It might be expected therefore that Grb10 and Grb14, like APS [48], would also bind Trk family members which have a similar activation loop motif, although this has not been reported. However, it is recognized that Grb10 and Grb14 interactions are also mediated substantially by their BPS domains, which particularly confer high-affinity binding to the IR.

Association of Grb10 with the IR is dependent on receptor autophosphorylation. In cells, association is observed only following insulin binding [16,45], and mutation of the critical lysine residue within the ATP-binding site of the receptor tyrosine kinase abrogates binding [46]. Additionally, the replacement of two IR activation-loop tyrosine residues (Tyr¹¹⁶²/Tyr¹¹⁶³) with phenylalanine results in reduced binding of full-length Grb10 in yeast two-hybrid assays [46,49], although mutation of Tyr¹¹⁵⁸ has little effect on binding [12,49]. Binding of full-length Grb10 protein does not require either the C-terminal (Tyr¹³²⁸/Tyr¹³³⁴) or juxta-membrane (Tyr⁹⁶⁵/Tyr⁹⁷²) tyrosine residues of the IR [28,46,49]. The reported inability of a C-terminally truncated IR to bind the isolated Grb10 SH2 domain [16], and the apparent localization of the SH2 domain binding site to the C-terminal Tyr¹³³⁴ of IR [50], are at odds with the studies clearly identifying the activation-loop tyrosine residues as the site of SH2 domain interaction. Grb7 and Grb14 are very similar to Grb10 in their requirements for IR binding [18,51].

Grb7/10/14 domains involved in receptor binding

Several studies have assessed the relative contributions of different regions of the Grb7/10/14 proteins to IR and IGFR binding. In the case of interaction with the IR, many have supported an important role for the SH2 domain. However, full-length Grb10 or Grb14, mutated within the SH2 FLVRES motif, both retain IR binding [12,18]. These findings suggested the involvement of a second receptor-binding region, identified as the BPS domain. The individual BPS and SH2 domains of Grb10 are able to interact with the phosphorylated IR, and binding of a FLVRES-mutated SH2 domain is restored when it is coupled with the BPS domain [12]. This BPS-SH2 (mutant) fusion interacts well with the IR

kinase domain *in vitro*, indicating that the BPS domain binds within the tyrosine kinase region of the IR. However, it is thought that the BPS domain does not bind directly to phosphorylated tyrosine residues of the activation loop, but rather that the conformational change of the IR following autophosphorylation exposes a binding epitope for the BPS domain [15,23]. The presence of two different receptor-interacting domains is thought to confer relative specificity for IR binding of Grb10 and Grb14 in particular, although Grb14, but not Grb10, also binds effectively to the FGFR [52,53]. It has been suggested that the BPS and SH2 domains of Grb10 contribute equally to IR binding [12]. However, a direct comparison with Grb7 and Grb14 revealed different specificities of each domain for the activated IR [18,51]. The isolated BPS region of Grb14 bound IR better than the BPS regions of Grb7 and Grb10. In contrast, the SH2 domain of Grb7, but not Grb14, bound efficiently to IR. Thus, although Grb7 can also bind to the IR, it may do so with lower affinity than Grb10/14, and with less specificity for this receptor over many others. In summary, both SH2 and BPS domains may contribute to specificity of Grb10 and Grb14 for IR over other tyrosine kinases, albeit with differing importance in terms of overall binding. No studies have yet been reported that compare directly the affinities of full-length Grb7, Grb10 and Grb14 for IR. In intact cells, the PH domain additionally plays a role in the interaction of Grb10 with the IR [49].

Preferential binding to IR versus IGFR?

Owing to the very high homology between IR and IGFR tyrosine kinase domains, it might be expected that members of the Grb7/10/14 family would bind equally well to these two receptors. Indeed, in yeast two-hybrid screens Grb10 was readily identified as a binding partner for the IGFR [17,54] as well as the IR [16,45,46]. However, there is some evidence that Grb10 binds preferentially to the IR compared with the IGFR. It has been reported that Grb10 is more effectively co-precipitated with activated IR than IGFR [17]. It has also been reported that, although both the BPS and SH2 domains contribute substantially to the interaction of Grb10 with the IR, this is not the case for the IGFR, which interacts well only with the BPS domain of Grb10 [12]. However, it is difficult to reconcile the reported lack of binding of the Grb10 SH2 domain to the IGFR with current understanding of the mechanism of SH2 domain binding to the IR and the conservation of relevant residues between the IR and IGFR. Indirect evidence for the converse specificity was provided by a report that overexpression of Grb10 inhibited IGF- but not insulin-stimulated cell growth [55]. No studies of Grb14 or Grb7 binding to IGFR have been reported, although the IGFR was sensitive to inhibition of tyrosine kinase activity by Grb14 *in vitro*, albeit less so than the IR [24]. It remains to be clearly established whether some or all members of the Grb7/10/14 family exhibit differential interaction with the IR and IGFR that could result in selective regulation of one receptor compared to the other *in vivo*.

Effects of Grb10 and Grb14 on receptor tyrosine kinase activity

Binding of the SH2 and/or BPS domains of Grb10 and Grb14 to the IR/IGFR tyrosine kinase domains might well be expected to affect kinase activity, and this has indeed been shown to be the case. Studies performed *in vitro* have shown that both full-length and BPS-containing fragments of Grb7/10/14 inhibit IR and, in some cases, IGFR catalytic activities towards peptide substrates [23,24]. The inhibition occurs by an uncompetitive mechanism, and with a potency ranking for the IR of Grb14 > Grb10 > Grb7. Overexpression of Grb10 or Grb14 in intact cells similarly inhibits insulin-stimulated phosphorylation of endogenous substrates, including IRS-1 (IR substrate 1), IRS-2, Shc and p62dok ([18,45,

Figure 4 A model of the mechanisms by which Grb10 and Grb14 may regulate insulin action, at the level of the IR

The binding of insulin to its receptor triggers receptor autophosphorylation on multiple tyrosine residues. This allows recruitment of IRSs, which, in turn, triggers activation of signalling cascades to induce metabolic and mitogenic responses. Grb10 and Grb14 can act to inhibit the catalytic activity of the IR, and may also block access of substrates to the activated receptor. In addition, Grb10 and Grb14 may act to regulate access of PTPs to the IR, or may recruit negative regulators via their adapter function. TK, tyrosine kinase.

56–59], and L. J. Holt, D. L. Cope, J. K. Sethi and K. Siddle, unpublished work). In a yeast tri-hybrid assay, both IRS-1 and IRS-2 binding to the IR were disrupted by a BPS–SH2 fragment of Grb10, suggesting that the inhibitory effect of Grb10 on IRS phosphorylation is due, at least in part, to steric hindrance [58]. Thus the inhibitory effects of Grb10 and Grb14 may be explained by a combination of decreased activity of the receptor kinase itself and restricted access of substrates to the receptor (Figure 4).

Effects of Grb10 and Grb14 on metabolic and mitogenic signalling

Consistent with the observed inhibitory effects on insulin-induced phosphorylation of IR substrates, overexpression of Grb10 or Grb14 has been shown to inhibit the activation of downstream insulin-signalling cascades involving PI3K (phosphoinositide 3-kinase) and protein kinase B/Akt, as well as Erk/MAPK (extra-cellular-signal-regulated kinase/mitogen-activated protein kinase) ([18,24,45,56,58,59], and L. J. Holt, D. L. Cope, J. K. Sethi and K. Siddle, unpublished work). A number of studies have also investigated metabolic endpoints, and concluded again that overexpression of Grb10 or Grb14 in cultured cells inhibits insulin-induced glycogen synthesis [18,60], phosphorylation of Elk1 transcription factor [59], DNA synthesis [18] and cell cycle progression [55,61]. Similarly, microinjection of a GST fusion protein containing the BPS and SH2 domains of Grb10 inhibited mitogenesis induced by insulin (or IGF-I) in fibroblasts [46], although interpretation of this experiment is ambiguous, since it could be argued that the microinjected construct is acting as a dominant-negative, rather than a mimic, of endogenous Grb10. Overall, however, a number of studies from different laboratories, involving several Grb10/14 isoforms and a variety of cell lines (including 3T3-L1 adipocytes and primary rat hepatocytes as well as CHO and HeLa cells), all support the notion that both Grb10 and Grb14

Figure 5 Intracellular binding partners for the Grb7/10/14 family

A diagram to show downstream binding partners for Grb7/10/14, and the potential cellular functions resulting from these interactions. NIK, nuclear factor κ B-inducing kinase.

have the potential to exert general inhibitory effects on insulin signalling (and, at least in the case of Grb10, on IGF signalling). These effects are not dependent on cell context, but stem from the inhibition of receptor kinase activity when Grb10 or Grb14 binds to the autophosphorylated IR. One exception to this generalization is the report that overexpression of Grb10, as mediated by recombinant adenovirus in primary hepatocytes, inhibited insulin-stimulated glycogen synthesis without affecting IRS phosphorylation or the activities of PI3K, Akt, glycogen synthase kinase-3 or Erk/MAPK [60]. This study concluded that Grb10 acted by inhibiting a novel and unidentified pathway leading to glycogen synthase activation in liver. However, it is perhaps more likely that the results were in some way artefactual, and certainly the observed effects of insulin on glycogen synthesis were very small, irrespective of whether Grb10 was overexpressed.

Despite the numerous studies that have detailed inhibitory effects of Grb10 on insulin receptor kinase and signalling pathways, other work, from one laboratory in particular, has suggested that Grb10 may play a positive role in modulating the actions of insulin and other growth factors. Overexpression of mGrb10 α in either NIH-3T3 or BHK fibroblasts was reported to enhance DNA synthesis and cell proliferation induced by insulin, IGF-I or PDGF-BB, but not EGF [50]. Consistent with these observations, introduction into cells of putative dominant-negative Grb10 fragments (SH2 domain or proline-rich region), by microinjection or as cell-permeable fusion peptides, inhibited mitogenic responses induced by insulin, IGF-I or PDGF-BB. Further studies by the same laboratory suggested that Grb10 also enhances metabolic responses to insulin [62]. Overexpression of Grb10 in L6 myocytes or 3T3-L1 adipocytes enhanced insulin-induced glucose transport, glycogen synthesis and lipogenesis, whereas expression of dominant-negative constructs, particularly the Grb10 SH2 domain, inhibited the insulin response [62]. The potentiation of metabolic responses was accompanied by enhanced activation of Akt, but not p70S6K or p38MAPK. Evidence was provided that selective activation of the Akt pathway by Grb10 resulted from its ability to bind the p85 regulatory subunit of PI3K (Figure 5),

and thus act as a functional link between the IR and PI3K activity independently of IRS-1 [62]. The activity of Grb14 was not examined in these studies.

Two further studies have suggested additional mechanisms whereby Grb10/14 might act as positive mediators of insulin signalling. It has been reported that Grb10 associates constitutively with Akt (Figure 5) when both proteins are overexpressed in COS-1 cells [63]. The interaction was thought most probably to involve the N-terminal region of Grb10, and was not dependent on its SH2 or PH domains. Importantly, interaction with Grb10 apparently increased Akt activity independently of the PI3K pathway, and it was suggested that recruitment of the Grb10–Akt complex to activated c-kit receptors could play an important role in c-kit signalling. To date, these intriguing results have not been replicated with other cell lines or receptor systems. On the contrary, association of Grb10 with Akt was not detected in CHO-IR cells, where overexpression of Grb10 was instead shown to inhibit insulin-induced Akt activation [58]. Very recently, a proteomics-based screen identified Grb14 as a binding partner for PDK-1 (3'-phosphoinositide-dependent kinase) (Figure 5) [64]. When overexpressed in HEK-293 cells, Grb14 and PDK-1 were shown to associate constitutively via a specific PDK-1 binding motif located between the RA and PH domains of Grb14 (and conserved in Grb10). It was suggested that Grb14 could act as a scaffold in the recruitment of PDK-1 to the activated IR, thus promoting Akt phosphorylation and transduction of the insulin signal. Again, these intriguing data await confirmation.

It is difficult to reconcile the data from different laboratories suggesting that Grb10, and perhaps Grb14, have the potential to act as both negative and positive influences on signalling by the IR. Opposite effects of Grb10 overexpression on insulin action have been reported in separate studies in the same 3T3-L1 cell line [58,62], so that dependence of Grb10 function on cell context does not appear to be a plausible explanation. It remains possible that apparently conflicting results might reflect the use of different isoforms of Grb10, or the effect of different levels of expression, but the key issue is whether any of the data provide insight into the physiological role of Grb10.

A potential criticism of all these studies discussed so far is that they involve overexpression of Grb10/14 in cell lines which, with a few exceptions, are not representative of classical insulin target tissues. A number of recent studies have addressed this problem by using gene targeting techniques to examine the consequences of knocking down expression of endogenous Grb10/14. When endogenous expression of Grb10 in HeLa cells overexpressing the IR was almost completely ablated by using siRNA (small interfering RNA), insulin-induced phosphorylation of Akt, Shc and Erk/MAPK was significantly enhanced compared to wild-type HeLa/IR cells [59]. A surprising feature of this study was that, although the relative expression of Grb10 and the IR was not determined, endogenous Grb10 was apparently sufficient to substantially inhibit IR function even though the IR was overexpressed.

The most compelling evidence for the physiological role of Grb10 and Grb14 has come from gene knockout studies in mice. Disruption of the Grb10 gene by a gene-trap insertion has substantial phenotypic effects with both metabolic and growth-related components [35]. Mice lacking Grb10 exhibited overgrowth of both the embryo and placenta, such that mutant mice were 30% larger than normal at birth. The overgrowth was not proportionate, the liver being particularly affected, while the brain was spared, and in adult animals adipose tissue actually decreased (A. Ward, personal communication). Moreover, overgrowth of the liver was accompanied by enhanced glycogen deposition. These observations establish that Grb10 is a potent tissue-selective growth

inhibitor, particularly in the embryonic phase of growth, but do not in themselves shed light on its mechanism of action. It was shown that absence of Grb10 caused proportionately similar growth effects in IGF-2-deficient mice [35] or IGFR-deficient mice (A. Ward, personal communication), arguing against a site of action downstream of the IGFR. Although the increase in hepatic glycogen in Grb10-deficient mice is consistent with enhanced insulin signalling in the liver (fetal mouse liver normally expresses relatively high levels of Grb10), further studies will be required to establish whether insulin action is indeed potentiated in this or other tissues by ablation of Grb10. Given the data obtained in the Grb14 knockout mouse, it would certainly be expected that ablation of Grb10 should increase insulin sensitivity in at least some tissues, and thus affect glucose homeostasis. In any case, it will be important to determine whether any improvement in whole-body glucose tolerance in the Grb10 knockout mouse is a direct result of enhanced insulin signalling or a consequence of changes in tissue growth and body composition.

Disruption of the Grb14 gene in mice gives rise to a quite different phenotype [42]. Grb14^{-/-} mice exhibit slightly decreased body mass (5–10% overall) compared with wild-type littermates, although the weight loss is not uniform in all tissues. Liver is among the tissues most affected, with a 16% reduction, whereas heart mass is increased by 18%. In metabolic terms, Grb14-deficient mice display improved glucose tolerance in spite of lower circulating insulin levels, indicating enhanced insulin sensitivity. Moreover, following insulin stimulation *in vivo*, liver and muscle of Grb14^{-/-} mice exhibited increased incorporation of glucose into glycogen and increased activation of insulin signalling pathways, as reflected by IRS-1 tyrosine phosphorylation, IRS-1 association with the p85 subunit of PI3K and Akt Ser⁴⁷³ phosphorylation. However, no such potentiation of insulin signalling was detected in adipose tissue of Grb14^{-/-} mice. Consistent with these observations, in *ex vivo* studies insulin-stimulated glucose uptake was modestly enhanced in soleus muscle, but not adipose tissue, from Grb14^{-/-} mice. These observations indicate that Grb14 is a significant tissue-specific inhibitor of insulin signalling. The manifestly different roles of Grb10 and Grb14 at the whole-body level, as revealed by the phenotypes of the respective gene knockouts, might in part reflect differences in expression of the two proteins between tissues and at different stages of development in normal animals. It remains to be determined whether the proteins also have distinct functions at a molecular level, in terms of the specificity of their interactions with receptors or other proteins and the consequent effects on intracellular signalling pathways. Simultaneous targeting of both Grb10 and Grb14 (either with siRNA in cell culture systems or by gene knockouts in mice) should in future provide information about functional redundancies between these proteins and thus further delineate their specific actions.

Available evidence suggests that Grb10 and Grb14 would compete in binding to the IR, not only with each other, but also with APS and SH2-B, in those tissues where the proteins are co-expressed. The possibility exists that at least some aspects of the phenotypes of individual gene knockouts could reflect not simply a loss of function of the targeted protein, but also a gain of function of other proteins that potentially compete for binding at the same sites on receptors. Mice deficient in APS exhibit increased insulin sensitivity and hypoinsulinaemia [65] that is superficially similar to the phenotype of Grb14-deficient mice. However, when individual tissues of APS^{-/-} mice were examined *ex vivo*, it was observed that insulin-stimulated glucose uptake was enhanced in adipose tissue, but not skeletal muscle, in contrast with what was seen in tissues of Grb14^{-/-} mice. The molecular basis of this tissue specificity is unclear, given that both APS and

Grb14 are reportedly expressed at higher levels in adipose tissue than in skeletal muscle. Mice deficient in the APS-related adapter SH2-B exhibit a very different phenotype of age-dependent hyperglycaemia, hyperinsulinaemia and insulin resistance, with evidence of impaired insulin receptor activation and signalling in liver, skeletal muscle and fat [66]. Other studies suggest that SH2-B acts as a positive mediator of signalling by diverse receptors [48,67–69]. It will obviously be interesting in future to study the phenotype of compound knockouts involving different members of the Grb10/14 and APS/SH2-B protein families.

OTHER POTENTIAL ACTIONS OF GRB10 AND GRB14

Regulation of receptor phosphorylation

Association of Grb10 with the IR is dependent on autophosphorylation of tyrosine residues in the IR kinase activation loop. It might be expected that binding of Grb10 would protect these sites from dephosphorylation, while potentially inhibiting phosphorylation of other domains (juxtamembrane and C-terminal; Figure 4). The net effect of Grb10 on overall receptor phosphorylation is therefore difficult to predict, and studies involving overexpression of Grb10 have produced apparently conflicting data. In primary cultured hepatocytes, expression of Grb10 substantially decreased the level of insulin-stimulated IR phosphorylation, as quantified by blotting with a generic anti-phosphotyrosine antibody [60]. However, in CHO-IR cells, overexpression of Grb10 did not decrease receptor phosphorylation as quantified by blotting with phosphorylation site-specific antibodies [58]. Indeed, in similar experiments we have observed that Grb10 overexpression substantially enhances insulin-stimulated phosphorylation of the IR kinase activation loop as detected particularly by anti-pTyr^{1162/1163} antibody (L. J. Holt, D. L. Cope, J. K. Sethi and K. Siddle, unpublished work). It seems likely that the various sites of receptor phosphorylation are affected differently by Grb10, and that this explains the results obtained with different anti-phosphotyrosine antibodies (and certainly it cannot be assumed that generic anti-phosphotyrosine antibodies react equally with all autophosphorylation sites). However, the levels of Grb10 expression in different studies, and other cell-specific factors, may also have a bearing on the results.

Ablation of the Grb14 gene in mice markedly decreased IR phosphorylation (as detected by anti-phosphotyrosine or site-specific anti-pTyr^{1162/1163} antibody) in liver but not in skeletal muscle, although IRS-1 phosphorylation was enhanced in both tissues [42]. In that study, treatment with the PTP (protein tyrosine phosphatase) inhibitor pervanadate normalized IR phosphorylation between wild-type and knock-out hepatocytes, suggesting that the decreased IR phosphorylation in cells lacking Grb14 is due to enhanced PTP-mediated dephosphorylation. This interpretation is consistent with data showing that IR dephosphorylation by PTP1b *in vitro* was reduced in the presence of Grb14 [24]. Thus, although binding of Grb10 or Grb14 to autophosphorylated IR inhibits kinase activity towards intracellular substrates, it may also have the effect of protecting key sites from dephosphorylation, thus maintaining the IR in a potentially active state (Figure 4). The fraction of IR affected in either sense will clearly depend on the level of expression of Grb10/14, which may vary considerably between tissues.

Regulation of receptor degradation

Among the Grb10- and Grb14-interacting proteins discovered in yeast two-hybrid studies is the protein Nedd4 (neuronal precursor cell expressed developmentally down-regulated), an E3 ubiquitin protein ligase (Figure 5) [30,70]. Grb10 and Nedd4 were

co-immunoprecipitated from mammalian cells, whereas the Grb14–Nedd4 interaction was not observed in this context. Grb10 was not itself ubiquitinated by Nedd4 [70]. However, overexpression of Grb10 in mouse embryo fibroblasts resulted in a strong ligand-dependent increase in ubiquitination of the IGFR, associated with increased internalization and a shortened half-life of the receptor [71]. It was suggested that Grb10 functions *in vivo* to mediate the ubiquitination and degradation of the IGFR. However, ablation of the Grb10 gene in mice apparently does not affect the level of IGFR expression (A. Ward, personal communication), casting doubt on whether the Grb10/Nedd4 pathway makes a significant contribution to IGFR turnover in normal tissues. There is no evidence that Grb10 or Grb14 influences degradation of the IR. Overexpression of Grb10 in a number of different cell types does not affect the level of IR expression [58,60,72], and levels of IR appear to be normal in both Grb10 and Grb14 knockout mice [42], and A. Ward, personal communication). In endothelial cells, Grb10 associates with and is phosphorylated by the VEGFR2 (vascular endothelial growth factor receptor-2) [72]. Overexpression of Grb10 in these cells actually increases VEGFR2 expression, apparently by inhibiting Nedd4-mediated receptor degradation [73]. Thus there is at present no evidence to support a physiologically important role of Grb10 or Grb14 in promoting the degradation of receptors with which they interact.

The adapter protein APS has, however, been implicated in mediating degradation of the IR. APS is recruited to and phosphorylated by the activated IR and, in turn, binds c-Cbl, a multifunctional protein with E3 ubiquitin protein ligase activity [74]. The c-Cbl protein promotes the ubiquitination and down-regulation of several tyrosine kinases with which it interacts directly [75], and it has been suggested that it might act similarly on the IR when recruited by APS [74]. More recently, it was shown that APS interacts also with Asb6, a SOCS box protein that can in turn bind the elongin B–C-ubiquitin ligase complex [76]. This suggests a second pathway whereby APS might regulate IR expression and function. Consistent with a negative role of APS in insulin signalling, ablation of the APS gene in mice increased insulin sensitivity, although neither the lack of APS nor its overexpression affected the level of IR expression [65]. The role of APS is potentially complex, and several studies in a variety of cell types have indicated that it may enhance signalling by the IR and other receptors [48,77–79]. It is notable that the SH2 domains of APS and Grb10/14 bind to similar sites in the IR-activation loop, and there may be an element of competition in binding of these adapters to activated receptors. Any manipulation that changes the expression of Grb10/14 might influence IR function indirectly through effects on APS-dependent pathways.

Regulation of apoptosis

Grb10 has been suggested to play a role in the regulation of apoptosis that is independent of direct interaction with receptors. The SH2 domain of Grb10 has been reported to bind to Raf-1 in a constitutive manner, and to MEK1 (MAP kinase/Erk kinase) following insulin treatment (Figure 5) [80]. Expression of SH2-mutated forms of Grb10, which cannot interact with Raf-1 or MEK1, induced apoptosis, while co-expression of wild-type Grb10 reversed the apoptotic effect [80]. It is unclear how this anti-apoptotic action of Grb10 might be mediated, although a fraction of Grb10 appeared to co-localize with Raf-1 to mitochondria [39]. However, the PH-deleted hGrb10 β was found only in the cytosol, implying that functional differences may exist between the Grb10 isoforms in their abilities to protect against apoptosis. In general, Grb10 has been found to be largely cytosolic in unstimulated

cells, and in part to translocate to the plasma membrane following insulin stimulation [28,36].

Regulation of cell migration

The Grb7/10/14 family of proteins share a region of sequence homology, the GM domain, with the *C. elegans* gene, Mig-10. This gene is required for the long-range migration of neuronal cells in embryonic development [81], thus suggesting a possible role for the Grb7/10/14 family in regulating the migration of mammalian cells. Cell migration is important for diverse biological processes, including embryonic development, the inflammatory immune response, wound healing and tumour metastasis. FAK is a key player in mediating these responses, following its activation by integrins. FAK is localized at focal adhesions, and Grb7 is also found in focal adhesions (as well as in the cytoplasm), where it binds to tyrosine-phosphorylated FAK via its SH2 domain (Figure 5) [82,122]. A number of studies have implicated Grb7 in the process of growth factor-induced cell migration [3,4]. A comparative study involving transient expression of Grb7, Grb10 and Grb14 in CHO cells indicated that only Grb7 could stimulate cell migration under the conditions tested [82]. However, there have been very few studies on the effects of Grb10 and Grb14 in this context, and the extent of functional divergence between Grb7/10/14 family members remains to be fully explored.

REGULATION OF GRB10 AND GRB14 ACTION

It seems inherently unlikely that the Grb7/10/14 family of adapters would be present in cells simply to act as tonic inhibitors of receptor tyrosine kinases. It is, of course, possible that their effects extend beyond simple inhibition of kinase activity and downstream signalling. While both Grb10 and Grb14 can clearly act as modulators of insulin action, the question arises as to whether they are themselves regulated. Regulation of adapter function might in principle be exerted by post-translational modification or by modulating levels of expression.

Regulation by phosphorylation

The Grb7/10/14 family can be phosphorylated on serine/threonine and tyrosine residues *in vivo*, although the functional significance of these modifications is unclear. In intact cells, both Grb10 and Grb14 are basally phosphorylated on serine residues [8,10]. Serine phosphorylation of Grb10 was increased following stimulation with a variety of growth factors, including EGF, PDGF, FGF and insulin, and this was accompanied by a shift in protein mobility on SDS/PAGE [8,36]. Inhibitor studies suggested that basal and insulin-stimulated phosphorylation is mediated, at least in part, by the PI3K and MAPK signalling pathways [36]. Moreover, Grb10 has been reported to associate directly with Raf-1 and MEK1 kinases [80], and to be phosphorylated by MAPK *in vitro* [83]. It is possible that phosphorylation of Grb10 by these insulin-regulated kinases acts as a feedback mechanism to modulate its actions on insulin signalling. However, as yet, the sites of phosphorylation and the effects of phosphorylation on Grb10 function are unknown.

Serine phosphorylation of Grb14 was increased upon stimulation of cells with PDGF and FGF, but not with EGF [10,52]. Atypical protein kinase C ζ (PKC ζ) phosphorylates Grb14 *in vitro*, primarily within the BPS domain, and appears to mediate stimulation of Grb14 phosphorylation by insulin in CHO-IR cells [84]. Significantly, phosphorylation of Grb14 increased its potency as an inhibitor of the IR tyrosine kinase *in vitro*. In cells, PKC ζ interacts indirectly with Grb14 in a ternary complex with the

molecular adapter ZIP (PKC ζ -interacting protein), which binds the BPS domain of Grb14. Surprisingly, ZIP did not affect the phosphorylation of Grb14 by PKC ζ *in vitro*, but overexpression of ZIP in oocytes potentiated the inhibitory effect of Grb14 on insulin-induced germinal vesicle breakdown. Thus the phosphorylation of Grb14 by PKC ζ may allow the enhancement of its function as a negative regulator of insulin action (Figure 4), although it remains to be determined whether the pool of Grb14 associated with ZIP and PKC ζ can also associate with the IR. Grb10, but not Grb7, can also bind ZIP *in vitro* (Figure 5) [84], and given the sequence homology between Grb10 and Grb14, it would seem likely that both proteins might be similarly phosphorylated by PKC ζ , but this remains to be demonstrated.

Tyrosine phosphorylation of Grb7/10/14 family members is even more enigmatic than serine/threonine phosphorylation. Although Grb10 and Grb14 bind well to the IR, it seems clear that they are not direct substrates of its tyrosine kinase activity [18,85]. Transient, weak phosphorylation of a 65 kDa protein, presumed to be Grb10, has been observed following insulin stimulation of HIRc cells [28], and the use of tyrosine phosphatase inhibitors also revealed the potential of insulin to induce tyrosine phosphorylation of Grb10 [85]. However, in this latter case at least, it was proposed that Grb10 phosphorylation was mediated by intracellular tyrosine kinases of the Src/Fyn family, rather than the IR itself. Tyr⁶⁷ was identified as the major site of phosphorylation, a residue that is not conserved in Grb7 or Grb14, and mutation of this residue to glycine increased the affinity of Grb10 for the IR. Phosphorylation of Grb10 in endothelial cells in response to VEGF was also shown to be mediated in part through activation of Src [72]. Additionally, it has been reported that Grb10 interacts with and is phosphorylated by the intracellular tyrosine kinase Tec (Figure 5) [86]. Thus tyrosine phosphorylation of Grb10 might conceivably be involved in terminating Grb10 action at the receptor level, or might play a role in signalling by non-receptor tyrosine kinases. Tyrosine phosphorylation of Grb14 has not so far been described.

Regulation by changes in protein expression

Another mechanism by which the actions of Grb10 and Grb14 might in principle be controlled is by regulation of the levels of these proteins within the cell. The complexities of Grb10 expression, involving tissue- and isoform-specific imprinting (as discussed below), multiple promoter elements and alternative splicing of long 5'UTRs within the mRNA [38], certainly suggest a high potential for developmental and hormonal regulation at both transcriptional and translational levels. Although effects of insulin on Grb10 levels have not been reported, VEGF can stimulate Grb10 expression [72]. In this context, Grb10 evidently participates in a positive feedback loop, causing an increase in the number of VEGF receptors (VEGFR2/KDR) on endothelial cells. In fact, VEGF may act synergistically with hepatocyte growth factor to up-regulate the expression of both Grb10 and Grb14 in endothelial cells [87]. Whether Grb10 levels are regulated by insulin or IGF-I remains to be examined. However, Grb14 has been studied in this regard. Insulin increased Grb14 expression by approx. 2-fold in cultured 3T3-F442A adipocytes, although the insulin-sensitizing drugs known as TZDs (thiazolidinediones) reduced the expression of Grb14 [43]. Insulin also enhanced Grb14 expression in MCF-7 cells incubated under serum-free conditions, whereas oestradiol both down-regulated basal expression and repressed the effect of insulin [61]. Given the role of Grb14 as an inhibitor of insulin signalling, the action of insulin on Grb14 expression would appear to be a negative feedback, in contrast with the action of VEGF on Grb10 expression.

INVOLVEMENT OF GRB10 AND GRB14 IN DISEASE

Grb10 is a candidate gene for the growth retardation of SRS (Silver–Russell syndrome)

GRB10 is an imprinted gene in both mice and humans, being expressed preferentially, if not predominantly, from the maternally inherited allele in most tissues. In contrast, *GRB7* and *GRB14* are not imprinted. In a systematic screen for maternally expressed genes (*Megs*) in mice, it was found that one such imprinted gene, *Meg1*, was identical with *mGrb10* [88], which had previously been mapped to the proximal region of mouse chromosome 11, near the *EGFR* gene [8]. Maternal or paternal duplication of murine proximal chromosome 11 is associated with prenatal growth retardation or promotion respectively [89,90], and because of the proposed involvement of Grb10 in insulin- and IGF-I-mediated growth, it was considered a good candidate for the imprinted effects on growth in mice. It was also suggested that *GRB10* was a candidate gene for the human SRS (MIM180860), a disorder involving pre- and post-natal growth retardation and morphological abnormalities [88]. This hypothesis gained credence when it was shown that the human *GRB10* gene, which is located on chromosome 7, is also imprinted [38].

The majority of cases of SRS are sporadic, although familial associations have been described, consistent with both autosomal dominant and recessive patterns of inheritance, suggesting that this is a genetically heterogeneous disorder. Approx. 10% of cases are associated with mUPD7 (maternal uniparental disomy of chromosome 7, i.e. the inheritance of both chromosomes from only the mother) [91–93]. In contrast, in humans paternal UPD7 has no effect on growth [94]. These observations led to speculation that one or more imprinted genes on chromosome 7 play a role in SRS, reflecting either lack of a paternally expressed growth-promoting gene, or excess of a maternally expressed growth-suppressing gene. Human *GRB10* has been mapped to chromosome 7p11.2–p12 [36,95], and several studies have supported a role for *GRB10* in SRS, at least in a subset of patients. Thus SRS has been associated with duplication of the 7p11–p13 segment of chromosome 7 that includes the *GRB10* gene [96–98]. However, mUPD7-associated SRS has also been reported in a patient with duplication of a segment at 7q31, and normal biparental inheritance of *GRB10* [99]. Screens for *GRB10* mutations in SRS have produced similarly mixed results. One study of 58 patients identified two unrelated subjects with maternal inheritance of a variant form of *GRB10*, having a replacement of Pro⁵⁹ → Ser, which was not present in 100 controls [100]. The affected residue lies in the proline-rich N-terminal region of Grb10, but the effect of the substitution on protein function was not investigated. Three further studies involving over 200 patients did not find any sequence variants that were specifically associated with SRS [38,101,102]. It is difficult to predict what sort of mutation within the coding sequence of *GRB10* might give rise to an 'activated' form of the protein. Regulatory mutations that affect either the *GRB10* promoter or its imprinting, for instance by switching on the paternal allele, are perhaps more likely, and such loss-of-imprinting defects have been reported in other imprinted disorders. Some workers have sought to rule out *GRB10* as an SRS candidate gene, on the basis of the observation that it is not imprinted in growth-plate cartilage, the tissue most directly involved in linear growth [103].

However, the imprinting status of *GRB10* is far from simple, differing among species, tissues and isoforms, and it seems that conclusions may also depend on the methods used to study expression [100,104]. Even in the mouse, it has become apparent that imprinting is more complex than originally proposed. Although expression is predominantly, if not entirely, from the maternal

allele in many tissues, and especially in skeletal muscle [35,38, 88,105], there is evidence of paternal expression, albeit at lower levels than for the maternally transmitted allele and possibly involving distinct promoters and transcripts, in a subset of tissues including brain [105,106] and cartilage [35]. However, notwithstanding the potential for some expression from the paternal allele, disruption of the maternal allele alone is sufficient to elicit a dramatic effect on overall body size [35]. Humans express a more diverse set of Grb10 splice variants than mouse, and these largely involve differential use of 5' exons associated with distinct promoters. Maternal mono-allelic expression of human *GRB10* is highly specific for the $\gamma 1$ isoform in skeletal muscle, whereas in the brain, expression of most transcripts is confined to the paternal-derived allele, and in other tissues expression is bi-allelic [38,101,106].

Although expression of the human *GRB10* gene may not be so dependent on imprinting as in the mouse, a case can still be argued for the involvement of *GRB10* in SRS. The possibility cannot be excluded that there are alterations in regulatory elements or epigenotype. It is also conceivable that submicroscopic duplication of either the maternal or paternal *GRB10* alleles, which are bi-allelically expressed in most tissues, could result in a 'double-dose' of these Grb10 proteins in the majority of tissues. Alternatively, mUPD7 could generate a double-dose of hGrb10 $\gamma 1$, which is maternally expressed in skeletal muscle. Thus a contribution of Grb10 (through aberrant expression or mutation) to the pathogenesis of SRS cannot yet be completely ruled out, although it now seems likely that any involvement must be confined to only a small subset of cases.

Grb14 expression is elevated in models of insulin resistance

While aberrant expression of Grb10 may influence growth and development, elevated expression of Grb14 may contribute to states of insulin resistance. In two rodent models of Type II diabetes, the *ob/ob* mouse and the non-obese diabetic GK (Goto-Kakizaki) rat, expression of Grb14 mRNA and protein were found to be increased by 75–100% in adipose tissue, but not in liver [43]. Expression of the adapter protein ZIP, a known binding partner for Grb14, was similarly elevated in the adipose tissue of *ob/ob* mice, but not in GK rats. In human Type II diabetics, expression of Grb14 mRNA was increased by 43% in subcutaneous adipose tissue, but was not significantly altered in skeletal muscle, compared with healthy controls, whereas expression of ZIP mRNA was increased by 150% and 50% in adipose tissue and muscle respectively. In cultured 3T3-F442A adipocytes, insulin increased Grb14 expression, whereas TZD decreased expression, and tumour necrosis factor α (TNF α) and high glucose were without effect. In *ob/ob* mice, prolonged (72h) fasting or treatment with insulin-sensitizing agents (metformin or TZD) ameliorated the metabolic manifestations of insulin resistance and induced modest (30–45%) decreases in Grb14 expression in peri-epididymal adipose tissue [43]. Thus, in several situations, the expression of Grb14 was inversely related to insulin sensitivity. Taken together with the increased insulin sensitivity of Grb14 knockout mice, these results are consistent with a pathophysiological role of Grb14 in the regulation of insulin sensitivity and the development of insulin resistance.

CONCLUDING REMARKS AND FUTURE DIRECTIONS

Many aspects of the function of the Grb10 and Grb14 adapter proteins remain enigmatic. Studies on cells *in vitro* have variously reported both positive and negative roles for Grb10 in signalling by both insulin and IGFR. A smaller amount of work on Grb14 has focused particularly on its inhibitory role in insulin signalling, but

where direct comparisons have been made its mechanism of action appears similar to that of Grb10. Studies on mice lacking Grb14 have clearly established that this adapter protein plays a significant role in glucose homeostasis and strongly suggest that its predominant action on IR signalling is inhibitory. A physiological role for Grb10 in the regulation of insulin signalling and glucose homeostasis is very likely, but remains to be clearly defined. Notwithstanding this potential overlap, the phenotypes of Grb10- and Grb14-knockout animals are very distinct, implicating Grb10, but not Grb14, as a tissue-selective regulator of embryonic growth and development. Moreover, the knockout studies appear to exclude a major role of either adapter in IGF signalling. Grb14 knockout mice are, if anything, slightly smaller than wild-type, while the marked overgrowth of Grb10 knockout animals is independent of IGFR function. Although much previous work has focused on the regulation of insulin signalling, it is likely that the IR is not the only, and perhaps not even the major, target of Grb10. It seems likely that the distinct phenotypes resulting from ablation of Grb10 and Grb14 in part reflect differences in the normal levels of expression and tissue distribution of these proteins. However, it is probable that the adapters are also functionally distinct at the molecular level, in terms of their interactions with receptors or other binding partners. Both adapters, and especially Grb10, appear promiscuous in interacting with multiple receptor and non-receptor tyrosine kinases and other signalling proteins *in vitro*, but the physiological relevance of many of these interactions remains unclear. Defining the major targets of regulation by Grb10 and Grb14, and the basis of adapter specificity, will be important goals of future studies.

Grb10 and Grb14 differ in other important respects. Expression of the *GRB10* but not the *GRB14* gene is imprinted, whereas the Grb10 but probably not the Grb14 protein is expressed as multiple isoforms generated by alternative mRNA splicing. However, there are differences in both imprinting and alternative splicing of *Grb10/GRB10* between mouse and human, suggesting that some aspects of function might be species-specific. It remains to be determined whether the splice variants of Grb10 are functionally distinct, and studies to date have emphasized similarities rather than differences. The BPS and SH2 domains implicated in receptor binding are common to all splice variants, and show a high degree of sequence conservation between Grb10 and Grb14, but the variable N-terminal regions might be expected to contribute specificity for other potential interactions. This is another area in which further work is merited.

The demonstration through knockout studies that Grb10 and Grb14 are physiologically important regulators of growth and metabolism raises the question of whether these adapters are themselves regulated, either in terms of acute activity or concentration. There is evidence that Grb10 can be phosphorylated on both serine/threonine and tyrosine residues, but the effects of such post-translational modifications on function are unknown. Regulation of Grb10 and Grb14 expression has received little attention, although one recent study showed that levels of Grb14 mRNA and protein are increased in human Type II diabetes and animal models of insulin resistance. Specific factors influencing the transcription, translation or phosphorylation of Grb10 or Grb14 have not been identified, and this should be yet another fruitful area for future studies. Overall, much remains to be done to evaluate the contribution of aberrant adapter function to metabolic disease or developmental disorders, and the potential for adapter-targeted therapeutic intervention.

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