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REGULATION AND FUNCTIONAL ROLES OF GRB14

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1. ABSTRACT

Grb14 is the last described member of the Grb7 family of adaptors, containing Grb7, Grb10 and Grb14. These proteins share a series of conserved domains involved in protein-protein and protein-lipid interactions: an amino terminal proline-rich region, a C-terminal SH2 domain, and a central GM region containing a RA, a PH domain, and a newly described PIR (BPS) region. As shown for the other members of the Grb7/10/14 family, Grb14 binds to various receptor tyrosine kinases (RTKs) under ligand induction. This interaction involves the SH2 and PIR domains, and the respective participation of these domains is likely to be a determinant in the specificity of action of Grb14. At the present time, a role for this Grb14-RTK interaction was established only for insulin (IR) and FGF receptors (FGFR). Grb14, through its PIR, is an inhibitor of IR tyrosine kinase activity and thus of insulin effects. Grb14 also decreases FGF signaling, but more probably by interfering with cellular effectors downstream from the receptor. Only a few cytosolic partners of Grb14 are identified. One of them, the adaptor ZIP, allows phosphorylation of Grb14, and regulation of its inhibitory action on IR signaling. The identification of further proteins interacting with Grb14 is required to elucidate the biological role of this protein.

2. INTRODUCTION

The reception of extracellular signals by cellular plasma membrane receptors activate intracellular networks leading to the appropriate cell response. A particular class of proteins, the molecular adaptors, play a pivotal role in the assembly of proteins into intracellular biochemical pathways. Molecular adaptors are formed by the juxtaposition of various protein-protein interacting domains, implicated in protein activation cascades, and formation of signaling complexes. Recently, a new family of cytosolic adaptors has emerged, the Grb7/10/14 family of proteins, comprising of Grb7, Grb10 and Grb14. These adaptors were originally identified as binding partners of several activated receptor tyrosine kinases (RTKs) (1-4). Grb7/10/14 family members and a *Caenorhabditis elegans* gene product, Mig-10, share significant sequence homology. Grb7/10/14 have a highly conserved Src-homology 2 (SH2) domain at the C-terminus, and a central GM (Grb/Mig) region (1), that includes a pleckstrin homology (PH) domain, a putative RA (Ras-associating) domain amino-terminal to the PH domain (5), and a more recently described region known as the PIR (phosphorylated insulin receptor interacting region) or BPS (between the PH and SH2) domain (6, 7) (Figure 1). The N-terminus is the less conserved domain among the different isoforms, but it contains a highly conserved proline-rich sequence (PS/AIPNPFPEL), which is a putative binding region for Src-homology 3 (SH3) domain containing proteins (8). The presence of these multiple interacting domains within the Grb7/10/14 family members indicates that they have the potential to bind to a variety of proteins and phospholipid components of cellular signaling pathways. Currently, a number of potential downstream binding partners of the Grb7/10/14 family of proteins have

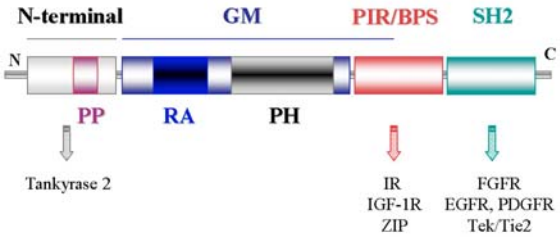

Figure 1. Schematic representation of Grb14 primary structure. Identified proteins binding to different domains of Grb14 are indicated. GM region, for Grb and Mig; PP: proline-rich conserved motif: PS/AIPNPFPEL; RA: homology to Ras-associating domains; PH: plextrin-homology domain; SH2: Src-homology-2 domain; PIR/BPS: phosphorylated insulin receptor interacting region, also known as between PH and SH2 domain; IR: insulin receptor; FGFR: fibroblast growth factor receptor; EGFR: epidermal growth factor receptor; PDGFR: platelet-derived growth factor receptor; ZIP: protein kinase C zeta interacting protein.

been identified, but the significance of the majority of these interactions is unknown. In addition, the physiological function of the Grb7/10/14 proteins is not fully elucidated. Grb7 has been found to regulate cell migration and tumor progression (9-11), whereas various isoforms of Grb10 have been shown to regulate growth factor-induced cell proliferation positively (12-14) or negatively (15-17). In this review, we will focus on the last identified member of the Grb7/10/14 family, Grb14. We will discuss its interactions with other proteins and its biological action, especially in insulin signaling.

3. GRB14 EXPRESSION

According to the newly adopted nomenclature (2), there are three variants of Grb14 which differ from their species of origin: hGrb14 (18), rGrb14 (7), and mGrb14 (19). Alignment of the amino acid sequences of each variant reveals 83% of sequence identity between human and rat or mouse, and 93% between rat and mouse. The most conserved region is in the C-terminus of the protein. To date, only one splice variant of Grb14 has been identified. This contrasts with Grb7 and Grb10 which are expressed as 2 or 4 splice variants.

In rat, Northern and Western blot analysis revealed that Grb14 is expressed in the liver, skeletal muscle, heart, white adipose tissue, pancreas and brain (7). Grb14 expression pattern in humans is slightly different with an additional expression in kidney, gonads and at lower levels in placenta, small intestine and colon, but Grb14 was not detected in the lung, spleen, thymus and prostate (18). It is noteworthy that Grb14 seems to be preferentially expressed in insulin target tissues (liver, skeletal muscle and white adipose tissue), and also in more recently described insulin-sensitive organs such as pancreas and brain. In the 3T3-F442A adipose cell line, Grb14 expression increases during adipocyte differentiation, whereas Grb7 does not vary in the same cells, suggesting that Grb14 can be considered as a marker of adipose cell differentiation (7).

Interestingly, genes encoding the Grb7/10/14 family of proteins map on chromosomes close to members of the epidermal growth factor receptor (EGFR) family, suggesting that they cosegregated during evolution. Grb7 gene colocalizes with ErbB2 within a region of mouse chromosome 11 (20) or with its human homolog HER2 to a syntenic locus on human chromosome 17q (21). Grb10 is localized in a region of mouse chromosome 11 (22) and human chromosome 7 (23) close to EGFR. Fluorescence in situ hybridisation localized the human Grb14 gene to a region of human chromosome 2q22-q24 close to ErbB4 (26). An additional gene is also proposed at 12q13, close to ErbB3 (26). Grb7 is overexpressed in human breast, gastric and esophageal cancer cell lines in concert with HER2 (20, 24, 25). Immunoprecipitation studies demonstrated that a large fraction of autophosphorylated HER2 is bound in a complex with Grb7 in cell lines (20). However, no data has been yet published concerning Grb10 co-amplification with EGFR in human cancers. Grb14 is expressed in human breast epithelial strains and in various human cancer cell lines (18). However, to date, there are no reported cases of ErbB4 co-amplification in such cancer cell lines, as described for Grb7 and HER2. Taken together, these data suggest that aberrant expression of Grb7/10/14 family adaptor proteins in human cancer cells may contribute to tumorigenesis by modulating RTK signaling.

4. GRB14 INTERACTION WITH RECEPTOR TYROSINE KINASES

4.1. Cloning of Grb14

Grb14 was originally cloned as a growth factor receptor-binding protein by interaction with EGFR, using the CORT technique (cloning of receptor targets) (18). However, association between EGFR and Grb14 could not be detected in intact mammalian cells (HEK 293), even upon transient co-overexpression. Similarly, *in vitro* interaction observed between Grb14 and platelet-derived growth factor receptors (PDGFR) could not be confirmed in transfected cell lines. This suggests that Grb14 does not play a major role in EGFR or PDGFR signal transduction. Grb14 was thereafter cloned in yeast two-hybrid screens by interaction with different RTKs, like insulin receptor (IR), fibroblast growth factor receptor (FGFR) and Tek/Tie2 (7, 19, 27). Grb14 binding to the insulin and FGF receptors was confirmed in cell lines overexpressing the proteins. Furthermore, this association is dependent on ligand stimulation of the receptors, and no interaction is observed with kinase inactive receptor mutants.

In vivo insulin stimulation induces the association between endogenous Grb14 and IR in rat liver, and in 3T3-F442A adipocytes (7, AKJ & AFB unpublished results). Kinetics studies of Grb14 binding to the IR performed in CHO-IR/Grb14 cells indicate that the interaction is induced by insulin within 1 min and reaches a maximum after 1-2 min (28, 29). This binding remains detectable throughout the 90 min of insulin stimulation and even after the withdrawal of the hormone (29). In rat liver, Grb14/IR interaction can be detected as early as 30 seconds after insulin stimulation (B. Desbuquois and AFB unpublished data). Taken together, these studies demonstrate that Grb14

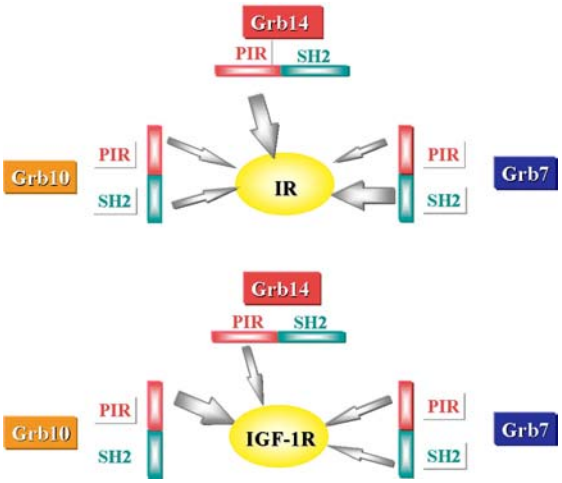

Figure 2. Role of the PIR and SH2 domains in Grb7/10/14 binding to IR and IGF-1R. The relative participation of the PIR and SH2 domains in the interaction of Grb7/10/14 with insulin receptors (IR) and insulin-like growth factor receptor (IGF-1R) is indicated by arrows. The size of the arrows linking two proteins is proportional to the interaction measured in the two-hybrid system and *in vitro* in GST pull-down studies (6, 7, 29, 30).

is a physiological interacting partner of the IR. In overexpressing systems, Grb7 and Grb10 also bind to the IR after insulin stimulation (15, 30), and this interaction occurs rapidly (17). However, further studies are needed to establish if these interactions also occurs with endogenous proteins in physiological insulin targets.

4.2. Mapping studies of Grb14-RTK interactions

The observation that the binding of Grb14 to RTKs requires an intact receptor tyrosine kinase activity led to the hypothesis that the SH2 domain of Grb14 may preferentially interact with a phosphotyrosine residue of the receptor. Actually, Grb14 binding to EGFR, PDGFR, FGFR and Tek is mediated by the SH2 domain, and potential phosphotyrosine targets on FGFR and Tek have been mapped (18, 19, 27). In contrast, deletion of the SH2 domain does not impair Grb14 recruitment by the IR, as this interaction is mediated by the PIR (BPS) (7). PIR is a new protein-protein binding domain conserved only in the Grb7/10/14 family of proteins, which, in concert with the SH2 domain, is implicated in the binding of activated RTKs. The relative importance of these two domains varies, depending on the individual the RTK and the Grb protein. Strikingly, the PIR plays the predominant role in Grb14 binding to the IR (7). Interestingly, it is also the domain responsible for the interaction between Grb14 and the insulin-like growth factor-1 receptor (IGF-1R), which is structurally similar to the IR (29). In contrast, the SH2 domain is the main binding domain in the Grb7/IR interaction (30), whereas both the PIR and the SH2 domains are equally implicated in the Grb10/IR interaction (6). The PIR is also the main Grb10 binding domain to IGF-1R (6). These differences in binding between the PIR and SH2 domains of Grb7/10/14 family members should then be important for the specificity of interactions between the various RTKs and these adaptors (Figure 2). The N-terminal domain of Grb14 can bind to FGFR, regardless of its activation state, but the conformation of the full-length protein prevents its binding to non-phosphorylated receptor (19). Thus, other domains of Grb14 may also contribute to the binding specificity.

On the other hand, association of Grb14 with IR involves the tyrosine kinase regulatory loop of the receptor. Mutation of Tyr^{1150} or Tyr^{1151}, which does not impair IR tyrosine kinase activity, almost suppresses Grb14 binding (7). The juxtamembrane domain (Tyr^{960}) and the C-terminal domain (Tyr^{1316} and Tyr^{1322}) of the IR are not implicated in the association with Grb14. These results are partly similar to those observed for Grb10 and Grb7 (6, 12, 30, 31), since some studies indicated that SH2 domain of Grb10 can also bind to the juxtamembrane site and to the C-terminus of the IR (32, 33). Nevertheless, when analysed in the context of full-length proteins, these results indicate that the members of the Grb7/10/14 family display similar binding specificities and can specifically interact with the phosphorylated kinase loop of the IR. Concerning the interaction between FGFR and Grb14, similar studies indicated that the C-tail, and to a lesser extent, the juxtamembrane region of FGFR, are involved in mediating association with Grb14 (19).

4.3. Structural basis of Grb14-RTK interaction

These biochemical characteristics may be explained, at least in part, by the structural basis for the interaction of the Grb7/10/14 family of adaptors with RTKs. The SH2 domains of Grb7/10/14 share 68-73% amino acid identity, and despite their high conservation, differences in the phosphopeptide-binding properties have been reported (7, 27, 29, 30, 34). The SH2 domains can be placed into four groups on the basis of the amino acid at the bB5 position, which is the most influential residue in determining phosphopeptide specificity (35). The Grb7/10/14 SH2 domains are classified into the group I due to the presence of a Tyr or a Phe at the betaD5 position. Most members of this group exhibit a preference for the target amino acid sequence: pTyr- hydrophilic residue - hydrophilic residue- hydrophobic residue, where the selectivity at the +2 position following the phosphotyrosine is lower or similar to that at +1 and +3 position (35, 36). The SH2 domain of Grb14 requires the presence of a large hydrophobic residue at the +3 position as it was described in the interaction with phosphopeptides designed from the Tek receptor (pY^{814}PVL and pY^{1106}EKF) (34), and the interaction with pY^{766}LDL in the FGFR (19). Compared to the Grb14 SH2 domain, the SH2 domain of Grb7 presents an overlap in binding specificity with Grb2 which bind preferentially to motif with an asparagine at the +2 position. Like Grb2, the Grb7 SH2 domain binds strongly to pTyr1139 (pYVNQ) in HER2, whereas it is not the case for Grb14 (34). The substitution of individual amino acids in the Grb14 SH2 domain by the corresponding residues from Grb7 demonstrate that the bD6 amino acid (which is a leucine in Grb7 and a glutamine in Grb14) plays a determinant role for SH2 domain specificity within the Grb7 family.

Although specific interactions vary between SH2 domains, the overall architecture of SH2 is remarkably

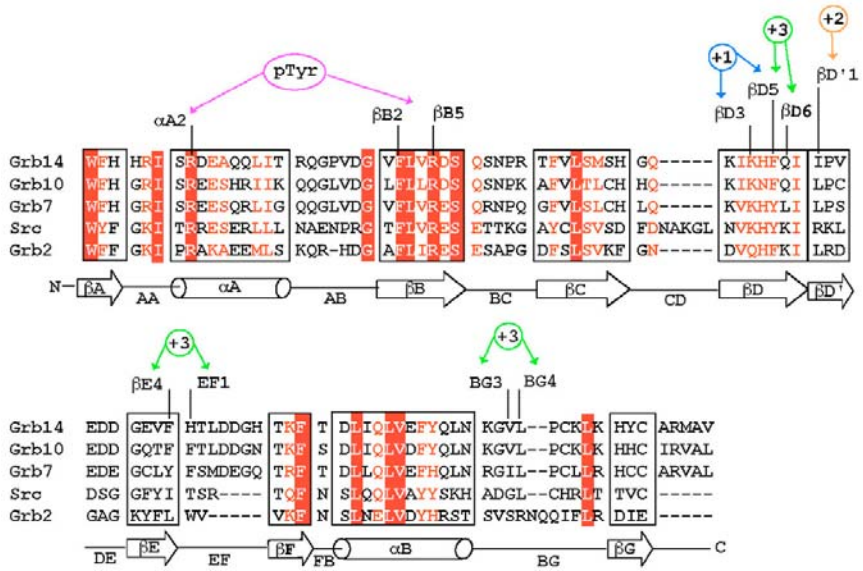

Figure 3. Sequences alignments of the SH2 domain of Grb7/10/14, Src and Grb2. Boxes indicate the canonical secondary structural elements which are shown below the sequences (87). Red shading indicates residues strictly conserved. In red are the homologous residues. The arrows indicate residues from SH2 domain that are predicted to make contact with the side chains of the associated peptide: pTyr - +1 - +2 - +3. For example, betaB2 (Phe in Grb14) refers to the second residue of the betaB strand, and BG3 (Val in Grb14) is the third residue of the BG loop.

conserved. In most SH2-ligand structures, peptide ligands bind in an extended conformation. Phosphotyrosine recognition elements are provided by αA, βB, bD and the BC loop. The binding site for residues C-terminal to the phosphotyrosine is formed by the EF and BG loop (37). The recent crystallographic study of the Grb10 SH2 domain provides information on structural features that is predicted to discriminate against binding of canonical extended phosphotyrosine sequences (38). By homology sequence, this crystal structure of Grb10 SH2 domain suggests that the SH2 domains of Grb10/14 are partially impaired in their ability to bind phosphotyrosine-containing ligands. Indeed, the binding pocket for the P+3 residue of the phosphopeptide in the Grb10 SH2 domain is absent because of the position of Val522 (conserved in Grb14) at BG3 rather than a glycine which is found in other SH2 domains. The presence of a side chain at this position masks the P+3 binding pocket. In addition, the non-glycyl residue at BC5 in Grb7/10/14 (a lysine in Grb10/14 and a glutamine in Grb7) is likely to impair phosphate coordination by the BC loop. However, the SH2 domains of the Grb7/10/14 family are characterized by an insertion of 4-5 residues in the EF loop relative to all others SH2 domains described (Figure 3). Recent assignments of resonances of hGrb14 and hGrb7 SH2 domains in complex with the phoshorylated peptides pY^{766}LDL and pY^{1139}VNQ respectively, show significant chemical shifts in the extended EF loop, suggesting a predominant role for this insertion as an SH2 domain specificity determinant (39, 40). Crystallographic or solution NMR determination of the structures of SH2 domains in complex with phosphorylated peptide ligand will be necessary to provide insights into the mechanism of peptide recognition and to elucidate the role of the EF loop insertion in ligand specificity.

Concerning the PIR domain, the structural basis for its interaction with RTKs, particularly IR, is still unknown. The PIR domain is a structurally uncharacterized region of approximately 50 residues that is unique to this family of adaptor proteins. Actually, it is still unclear if the PIR binds directly to a phosphorylated tyrosyl residue or if the binding depends on a new epitope unmasked after spatial rearrangement of the kinase loop occurring after activation of the IR (41). In agreement with the latter hypothesis, a recent study using peptide competition experiments demonstrated that the PIR of Grb10 does not bind directly to phosphotyrosine residues in the IR kinase loop (42).

5. DOWNSTREAM PARTNERS OF GRB14

Grb14, as well as Grb7 and Grb10, were isolated as binding partners of RTKs, and, as discussed above, they interact with these receptors through their C-terminal region, containing the PIR and SH2 domains. Nevertheless, the presence of other conserved interacting regions in their N-terminal part, indicates that they also have the potential to bind to a variety of protein or phospholipid components implicated in downstream signaling pathways (Figure 1). Using different techniques, a number of intracellular proteins were identified as binding partners of Grb7 and Grb10. Grb10 was shown to interact mainly with various kinases, like Tec, BCR-ABL, Jak2, MEK1, Raf1 and Akt (14, 43-47). An interaction with the ubiquitin protein ligase Nedd4 was also reported (48). Although Grb10 is not ubiquitinated, it allows the recruitment of Nedd4 to IGF-1R, leading to ubiquitination and destabilization of receptors (49). On the other hand, Grb7 was shown to bind to a wide variety of proteins: the tyrosine phosphatase SHPTP2 (50), the adaptor Shc (20, 51), the kinase FAK (9),

the GTPase Rnd1 (52), caveolin-1 (53), or to phosphoinositides (54). These interactions, and their functional role in Grb10 and Grb7 functions are discussed in the related reviews in the same issue.

In contrast, currently only two proteins were identified, using the yeast two-hybrid system, as binding partners of Grb14: tankyrase 2 and PKC-zeta interacting protein ZIP (55, 56). Tankyrase is a human telomeric poly(ADP-ribose)polymerase (PARP), positively implicated in telomere elongation (57 , 58). Telomere maintenance is required for long-term proliferation of eucaryotic cells, like immortalized cells and cancer cells. Tankyrase 2 is a novel human tankyrase, which was cloned by interaction with either the telomere-binding protein TRF1 (59), the insulin-responsive amino-peptidase IRAP (60) and Grb14 (55). Tankyrase 2 lacks the N-terminal histidine/proline/serine-rich (HPS) region of tankyrase 1, but contains 24 consecutive ankyrin-type repeats, a sterile alpha motif module, and a C-terminal region with homology to the poly (ADP-ribose) polymerase (PARP) catalytic domain. The PARP domain of tankyrase 2 is catalytically active, and in a manner similar to tankyrase 1, tankyrase 2 is supposed to be important in telomere elongation (58). The N-terminal 110 amino acids of Grb14, containing the poly-proline P(S/A)IPNPFPEL conserved motif of the Grb7/10/14 family of adaptors, interacts with a region of the protein corresponding to ankyrin repeats 10-19 (55). It is noteworthy that this region does not contain an SH3 domain that target specific proline-rich sequences. Subcellular fractionation studies indicated that both Grb14 and tankyrase 2 associate with the low density microsome fraction which is enriched in Golgi vesicles and endosomes. One hypothesis is that tankyrase 2 may be involved in regulating the subcellular localization of Grb14, and of activated RTKs bound to Grb14. Interestingly, in the same yeast two-hybrid screen, Lyons *et al* also reported that the SH2 domain of Grb14 binds *in vitro* to the ubiquitin protein ligase Nedd4 in a phosphotyrosine independent manner (55), as previously described for the Grb10-Nedd4 interaction (49). It should be interesting to determine if, as for Grb10, this interaction can target other proteins for ubiquitination.

Recently, our laboratory has shown that ZIP (also known as p62) interacts with Grb14 (56). ZIP is a molecular adaptor, characterized by the succession of several conserved protein domains: an N-terminal atypical PKC-interacting domain (AID), a cysteine-rich sequence called ZZ zinc finger domain, two central PEST domains, which are likely to be implicated in protein stability, and a C-terminal ubiquitin-associated domain potentially involved in the ubiquitination protein degradation (for review, see (61)). Mapping studies indicate that the PIR of Grb14 interacts with the ZZ zinc finger domain of ZIP (56). Previous studies have demonstrated that ZIP interacts with PKC zeta and behaves as a link between PKC zeta and other proteins, such as RIP (62), TRAF6 (63), or Kvbeta2 (64) in ternary complexes. In addition, the PKC zeta binding domain of ZIP is located in N terminal of the ZZ zinc finger domain, raising the possibility that ZIP can bind simultaneously Grb14 and PKC zeta through two distinct but proximal modules. Using coimmunoprecipitation experiments *in vivo* using lysates of rat liver, we confirmed that endogenous Grb14, ZIP, and PKC zeta form a heterotrimer under physiological conditions (56).

It was reported that proteins of the Grb7/10/14 family can oligomerize. Grb10 can be found as oligomers in cell extracts, and tetramers are formed by the interaction of the N-terminal region with the PH and the BPS (PIR)-SH2 domains (65). Furthermore, Grb10 binds to Grb7 in a yeast two-hybrid assay (unpublished data from J. Cooper in (65)). However, as tested in the two-hybrid system, we could not detect an oligomerization for Grb14 (DP and AFB, unpublished results).

Other downstream partners of Grb14 should be identified in the near future. A likely candidate is a protein of approximately 125 kDa that is phosphorylated on tyrosine residues and coprecipitates with Grb14 *in vitro* (7). After insulin stimulation, this protein either dissociates or is dephosphorylated. A similar protein was also observed to interact with Grb10 (12, 32). As noticed for the Grb10-associated protein, this Grb14 binding protein does not immunoreact with antibodies against the focal adhesion kinase (V.B. unpublished data) and its identity is still unknown. Thus, in contrast to Grb7, Grb10 and Grb14 do not bind to FAK, and this absence of interaction with FAK is in agreement with the lack of effect of Grb14 and Grb10 on cell migration, as opposed to Grb7 (10).

6. PHOSPHORYLATION OF GRB14

All members of the Grb7/10/14 family of adaptors bind to a number of activated RTKs after ligand stimulation, suggesting that they may be substrates of these receptors. As expected, Grb7 is phosphorylated on tyrosine residues by the EGFR (66), Tek (27), EphB1 (67), or by the non-receptor tyrosine kinase FAK (10). Grb10 is also a substrate for non-receptor tyrosine kinases like Tec (43) and Src (68, 69). However, although it is phosphorylated on tyrosine residues after insulin or vascular endothelial growth factor (VEGF) stimulation, Grb10 is unlikely to be a direct substrate of the IR or of the VEGFR (32, 68, 69). In contrast, Grb14 is not phosphorylated on tyrosine residues in response to either insulin (7) or FGF-2 (19).

In the basal state, Grb14 is phosphorylated on serine and threonine residues (18, 19). Furthermore, *in vivo* metabolic labeling experiments and two-dimensional phosphoaminoacid analysis demonstrated that serine phosphorylation of Grb14 is increased after stimulation with PDGF (18) or FGF-2 (19). The kinase(s) implicated in this effect and the role/s of this phosphorylation are not yet identified. More recently, our laboratory showed that Grb14 undergoes insulin-stimulated phosphorylation on serine/threonine residues (56). The serine/threonine kinases involved in Grb7 protein phosphorylation do not appear to be either cyclic AMP- or calcium-stimulated kinases or classical or novel PKC isozymes. In this context, the identification of ZIP as a binding partner of both Grb14 and PKC zeta led to the hypothesis that Grb14 could be a substrate for this kinase. Previous studies reported that ZIP

allows PKC zeta to be associated with specific effectors in several signaling complexes and to phosphorylate them (62-64). In agreement with this hypothesis, PKC zeta is able to phosphorylate Grb14 both *in vitro* and in intact cells (56). In addition, insulin-stimulated Grb14 phosphorylation is decreased by a PKC zeta pseudo-substrate and increased by the overexpression of the kinase, suggesting that PKC zeta is a potential candidate for *in vivo* insulin-induced Grb14 phosphorylation (56). However, a participation of other kinases cannot be excluded, and further experiments are needed to clarify this point.

7. FUNCTIONAL ROLES OF GRB14

The functional role of the Grb7/10/14 family of adaptors is not yet understood. Since all members of this family bind to activated RTKs, it can be hypothetized that they are implicated in growth factors actions, and thus in the regulation of cell proliferation. However, to date there is no report of transgenic models allowing to investigate the function of these proteins in a whole organism. The functional role of these proteins was then essentially analysed on the basis of the nature of their interacting partners, and using *in vitro* approaches or overexpression studies.

7.1. ROLE ON INSULIN ACTION

7.1.1. Inhibition of insulin receptor catalytic activity

Grb14 tissue distribution, and insulin stimulation of Grb14 binding on the IR in rat liver strongly argued in favor of a role for Grb14 in insulin signaling. Furthermore, the observation that Grb14 specifically binds to the IR tyrosine kinase loop led to the hypothesis that this interaction may alter the catalytic activity of the receptors. In vitro tyrosine kinase assays demonstrate that Grb14 directly inhibit IR catalytic activity towards the synthetic substrate poly (Glu :Tyr), without significantly altering receptor autophosphorylation (29). This is consistent, considering that tyrosine phosphorylation of the IR is necessary for Grb14 association. The Km for ATP is not altered, indicating that Grb14 acts as a non-competitive inhibitor for ATP. Grb14, which decreases the Vmax by 70-80%, but only slightly reduces the Km for the synthetic substrate, behaves like an uncompetitive inhibitor (29). Crystallographic studies have shown that the IR regulatory kinase loop adopts an open conformation after autophosphorylation, allowing ATP and substrate access to their respective sites (41, 70). The kinetics data indicate that Grb14 reduces IR-catalyzed reactions without blocking the access to the ATP and substrate binding sites. Thus, a plausible explanation could be that the binding of Grb14 maintains the kinase under an inactive form and potentially blocks the phosphoryl transfer from ATP to the substrate. Similar experiments performed with the various domains of Grb14 revealed that inhibition of IR catalytic activity was due to the PIR of Grb14, whereas the SH2 domain alone had no effect. Interestingly, the domain responsible for the inhibition of IR tyrosine kinase activity is also the PIR for both Grb10 and Grb7 (29, 42). This supports a direct link between the binding ability and the inhibitory action of the PIR, and underlines its pivotal role in the regulation of IR catalytic activity. Moreover, PIR is the domain of Grb14 which is preferentially phosphorylated *in vitro* by PKC zeta. This phosphorylation is likely to be physiologically relevant since it increases the inhibitory effect of Grb14 on IR tyrosine kinase activity (56). However, the phosphorylated residues and the molecular mechanism whereby Grb14 phosphorylation increases its inhibitory effect are still unknown. This increased activity may reflect an increased binding affinity of the phosphorylated PIR for the IR kinase regulatory loop. A definitive answer should be given by crystallographic studies of the PIR domain bound to the activated IR. In this context, it is noteworthy that the adaptor SH2-B, which also binds to the activation loop of the IR, does not modify IR catalytic activity (29, 71, 72). This implies that a protein bound to the activated IR regulatory loop does not necessarily inhibit its kinase activity.

Grb10 and Grb7 also inhibit IR catalytic activity in *in vitro* experiments, but are less effective than Grb14 (29, 42). Grb7 induces a maximal inhibition of 40% whatever the amounts of GST-Grb7 tested. In the case of Grb10, a maximal inhibition of 80% can be obtained, but the IR is less sensitive to the inhibitory effect of Grb10 than of Grb14 (29). Recently, a study proposed an alternative mechanism for Grb10-mediated inhibition of insulin signaling which results from a physical disruption of insulin receptor substrates (IRS) association with phosphorylated residues of the IR kinase domain and/or the NPXY motif surrounding tyrosine 972 (17). This physical disruption was only assessed in yeast, and must be confirmed in mammalian cells. Furthermore, this study does not exclude a role of Grb10 on IR catalytic activity to decrease IRS phosphorylation.

IGF-1 receptors are structurally and functionally closely related to IR (73), and members of the Grb7/10/14 family of adapters are able to bind to these receptors (6, 16, 29, 31). Comparison of the effect of Grb14 on tyrosine kinase activity of IGF-1R and IR revealed that IGF-1R catalytic activity is less sensitive than IR to the inhibitory effect of Grb14 (29). Taken together these results are in favor of a specific inhibitory effect of Grb14 on IR catalytic activity.

7.1.2. Role on cellular insulin actions

After insulin binding on its receptor, the first step of intracellular insulin action is the induction of IR autophosphorylation on multiple tyrosine residues. Then, these residues serve as docking sites for specific intracellular effectors, such as the IRS family or Shc, leading to the transmission of the insulin signal (for review see (74)). In CHO-IR cells, the overexpression of Grb14 does not impair insulin-induced IR autophosphorylation (29). One can argue that these findings do not validate an inhibitory effect of Grb14 on IR tyrosine kinase activity in cells. Nevertheless, the tyrosine phosphorylation of the IR is necessary for Grb14 association and represents the first signaling event. Thereafter, the binding of Grb14 may maintain the regulatory kinase loop in an inactive

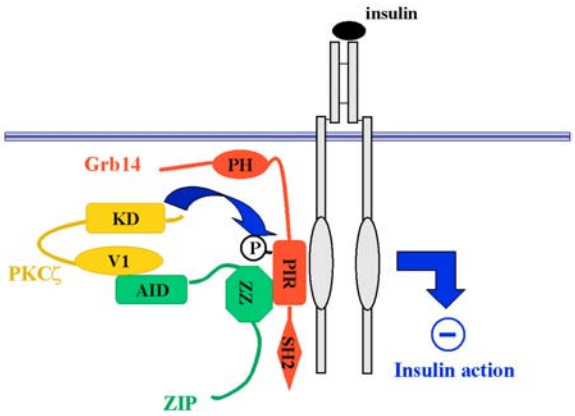

Figure 4. Model for the role of ZIP in Grb14 mediated insulin signaling inhibition, adapted from (56). ZIP connects PKÇzeta to Grb14 through respectively its AID and ZZ domains. Phosphorylation of Grb14 PIR by PKÇzeta enhances the inhibitory action of Grb14 on the insulin receptor catalytic activity and thus on insulin action. AID: atypical PKC-interacting domain; ZZ: ZZ zinc finger domain; V1: first variable domain; KD: kinase domain; P: phosphorylated Ser/Thr residue.

conformation, decreasing subsequent substrate phosphorylation. In support of this hypothesis, insulin-stimulated tyrosine phosphorylation of IRS-1, Shc and Dok is decreased in CHO-IR/Grb14 cells compared to CHO-IR (7, 28). In addition, the activation of ERK1 and ERK2 was both decreased and delayed in the presence of Grb14 (29). While the overexpression of Grb14 also delayed by 5-10 min the phosphorylation of Akt, it does not affect the maximal insulin-induced Akt phosphorylation (29). This discrepancy may be explained by the fact that in this study the activation state of Akt was estimated by using anti-phospho-Ser 473. Indeed, a similar study which focused on Grb10γ indicated that its overexpression in CHO/IR cells preferentially reduced Akt^{T308} versus Akt^{S473} phosphorylation (17). In addition, Ser-473 phosphorylation of Akt does not always correlate with its activity (75). Thus, the direct inhibitory effect of Grb14 on IR catalytic activity is likely to be responsible for the alterations in the early steps of insulin signaling.

On the other hand, overexpression of Grb14 in CHO-IR cells led to a sustained IR phosphorylation which remains at its maximal level up to 90 min (29). One explanation is that the binding of Grb14 on the activated IR sterically hinders the action of tyrosine phosphatases such as PTP1B, therefore maintaining the IR under a phosphorylated state. These results suggest that Grb14 and PTP1B decrease the catalytic activity of the IR through two different mechanisms: PTP1B inactivate the receptor by dephosphorylation (76), whereas the binding of Grb14 on the phosphorylated tyrosine kinase loop directly inhibits its catalytic activity. Nevertheless, additional studies in models with physiological levels of Grb14, PTP1B and specific substrates are needed to determine the kinetic of their respective binding and thus their subsequent action on IR signaling.

The decrease in IR substrates phosphorylation and effector activation in presence of Grb14 is accompanied by an inhibition of both DNA and glycogen synthesis in CHO-IR/Grb14 cells (7). Taken together, these results are consistent with a negative role for Grb14 on mitogenic and metabolic effects of insulin. Using the *Xenopus laevis* oocyte system, our laboratory has shown recently that microinjection of Grb14 specifically blocks insulin-induced reinitiation of meiosis (56). Interestingly, the co-injection of ZIP is able to potentiate Grb14 inhibitory action. This effect requires the recruitment of PKC zeta and also can be mimicked by previous *in vitro* Grb14 phosphorylation. Thus, a new negative feedback pathway of insulin signaling emerges whereby ZIP acts a scaffolding protein which specifically targets the PKC zeta activity to Grb14 (Figure 4).

7.1.3. Comparison with the role of the APS/SH2-B family of adapters on insulin signaling

Another family of adaptors, comprising of APS (for Adaptor protein with a Pleckstrin homology (PH) and Src homology 2 domain (SH2)), SH2-B and Lnk (a lymph node adaptor protein), exhibits structural and functional analogies with the Grb7/10/14 family (77). The primary structure of these proteins is also composed of an N-terminal proline-rich domain, a central PH domain, and a C-terminal SH2 domain, and they are likely to be involved in RTK signaling. SH2-B is expressed as four alternative splice variants, which are differently involved in growth factor induced mitogenesis (78). Both APS and SH2-B bind to the phosphorylated tyrosine kinase loop of the IR through their SH2 domain (77, 79-81). However, the interaction of SH2-B with the receptor does not alter its catalytic activity (29). APS is a good substrate of the IR tyrosine kinase, whereas SH2-B is not, or only poorly, phosphorylated on tyrosine residues in response to insulin (71, 80-82). Overexpression studies provided evidence that APS and SH2-B enhance IR autophosphorylation, insulin stimulation of ERK and PI-3 kinase pathways, and insulin stimulated mitogenesis (82, 83). In addition, APS promotes tyrosine phosphorylation of the proto-oncogene Cbl by the IR (84, 85). Cbl is then involved in insulin stimulation of GLUT4 glucose transporter translocation, and in glucose transport (86). APS and SH2-B are thus positive effectors of insulin signaling. On the other hand, recruitment of Cbl by APS after insulin stimulation leads to ubiquitination of the receptor and initiation of internalisation and degradation, and thus resulting in down-regulation of insulin signal transduction (85). Taken together, these studies clearly show that these molecular adapters can act through various mechanisms to regulate, either positively or negatively, different signaling pathways.

7.2. Role of Grb14 on FGF signaling

FGFR is, after the IR, the second RTK whose interaction with Grb14 was reported in intact cells. As shown for insulin, overexpression of Grb14 inhibits FGF-induced cellular proliferation (19). Interestingly, expression of a Grb14 SH2-mutant unable to bind to FGFR potentiates the effect of FGF, suggesting that Grb14 interact with unidentified downstream effectors. No effect on FGFR catalytic activity was reported. Thus, the mechanisms

implicated in the inhibition by Grb14 of insulin and FGF action on cellular proliferation are likely to be different.

It is noteworthy that Grb14 mRNA is highly expressed in a number of human breast and prostate cancer cells (18). This could suggest a positive role of Grb14 in cellular proliferation of this cell lines. However, from the current data, it is not clear if the level of Grb14 protein expression is also increased, and if it is a wild type or a mutant form of Grb14. Thus, the role of Grb14 in regulating cellular proliferation requires further clarification.

8. PERSPECTIVES

Grb14 is the last identified member of Grb7/10/14 family of adaptors, and therefore there is a limited amount of data published on the subject. Nevertheless, all the accumulated studies provide evidence for an inhibitory action of Grb14 on RTKs signaling. This contrasts with studies on Grb10 which are controversial, depending on which isoform is analysed. One of the future prospects in this field will be to understand the molecular mechanisms by which Grb14 decreases the catalytic activity of RTKs, especially the IR. Structural analysis of the interaction between the PIR and the activated tyrosine kinase loop is needed to elucidate this question. To further assess the putative role of Grb14 in regulating insulin action, functional studies in insulin-sensitive cells are needed. One can suppose that overexpression of Grb14 in target-tissues of insulin may lead to insulin-resistance, and subsequently to type 2 diabetes *in vivo*. In this context, Grb14 may also appear as a new target for anti-diabetic drugs which could disrupt its association with the IR. Another area of future interest will be to identify new downstream partners of Grb14. Given its role as a molecular adaptor, it is likely that additional downstream effectors will interact with its other conserved domains like the PH domain or the proline-rich region. The identification of these Grb14 binding-partners will be of considerable interest to fully understand the function of this protein.

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Abbreviations: Grb: growth receptor binding protein; BPS: between PH and SH2; CHO, Chinese hamster ovary; MAPK: mitogen activated kinase; IGF-I: insulin-like growth factor I; IR: insulin receptor; IGF-IR: IGF-I receptor; EGF: epidermal growth factor; PDGF: platelet-derived growth factor; PKC, protein kinase C; PH: pleckstrin homology; PIR, phosphorylated insulin receptor interacting region; RTK, receptor tyrosine kinase; SH2: Src homology 2; ZIP: PKC zeta interacting protein

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