Grb10 Interacts Differentially with the Insulin Receptor, Insulin-like Growth Factor I Receptor, and Epidermal Growth Factor Receptor via the Grb10 Src Homology 2 (SH2) Domain and a Second Novel Domain Located between the Pleckstrin Homology and SH2 Domains*

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The Grb10 protein appears to be an adapter protein of unknown function that has been implicated in insulin receptor (IR) signaling. The interaction of this protein with the IR has been shown to be mediated in part by the Src homology 2 (SH2) domain of Grb10. Here we demonstrate the existence of a second novel domain within Grb10 that interacts with the IR and insulin-like growth factor receptor in a kinase-dependent manner. This domain was localized to a region of approximately 50 amino acids, and we term it the BPS domain to denote its location between the PH and SH2 domains. The BPS domain does not bear any obvious resemblance to other known protein interaction domains but is highly conserved among the Grb10-related proteins Grb7 and Grb14. We show that the BPS domain interaction is dependent upon receptor tyrosine kinase activity. Furthermore, interaction of the BPS domain requires the kinase domain of the IR, since mutation of the paired tyrosine residues (Y1150F/Y1151F) within the IR activation loop dramatically reduced the interaction. Last, our data suggest that the presence of two distinct protein interaction domains may help to determine the specificity by which Grb10 interacts with different receptors. Specifically, the IR, which appears to interact most strongly with Grb10, interacts well with both the SH2 and BPS domains. Conversely, the insulin-like growth factor receptor and EGFR, which interact less avidly with Grb10, interact well only with the BPS domain or the SH2 domain, respectively. In summary, our findings demonstrate the existence of a previously unidentified tyrosine kinase activity-dependent binding domain located between the Pleckstrin homology and SH2 domains of Grb10.

The binding of hormones to their receptor tyrosine kinases triggers receptor tyrosine autophosphorylation (1). In the case of the insulin receptor (IR) and insulin-like growth factor I receptor (IGFIR), phosphorylation of multiple tyrosine residues leads to activation of receptor kinase activity (2) and, in addition, creates docking sites for downstream adaptor proteins such as SHC, insulin receptor substrate-1 (IRS-1), and insulin receptor substrate-2 (IRS-2) (3–5). Phosphorylation of IRS-1 upon multiple tyrosines results in its association with Src homology 2 (SH2) domain-containing proteins including Grb2, Sy, NCK, and the p85 subunit of phosphatidylinositol 3-kinase, thus activating various signaling cascades (6–10). Tyrosyl phosphorylation of SHC also leads to its interaction with Grb2 and mediates the activation of the guanine nucleotide exchange factor SOS and subsequent activation of the Ras signaling pathway (11–14).

SHC, IRS-1, and IRS-2 proteins have been shown to interact with the IR or the related IGFIR through phosphotyrosine binding domains, which recognize the phosphorylated NPXY motif in the juxtamembrane domains of receptors (3, 5, 15). In addition, a second poorly understood receptor binding domain has also been identified within the central domain of IRS-2 (5, 16). Both SHC and IRS-1 have been implicated in insulin-stimulated mitogen signaling transduction (14, 17–19), and IRS-1 has also been proposed to mediate some of the metabolic effects of the IR, including regulation of glucose transport partly through the activation of phosphatidylinositol 3-kinase (20–22). Whether these proteins are sufficient to explain the diverse effects of insulin receptor signaling is a matter of intense investigation. In this regard, the recent discovery of the related proteins that interact with the IR termed Grb-IR, Grb10/IR-SV1, or GrbIR/Grb10 by several groups is of interest (23–25). These three Grb10 variants are derived from the same gene via alternative splicing and have recently been renamed Grb10α for Grb-IR and Grb10β for Grb10/IR-SV1 and Grb-IRβ/Grb10, the latter two being identical cDNAs. Grb10α contains a distinct amino terminus compared with Grb10β, and in addition, Grb10β contains an intact PH domain, which is partially deleted due to a splicing event in the Grb10α mRNA. Since the Grb10 domains that we are addressing in this paper are found in both the α and β forms of Grb10, for simplicity we will refer to the protein as Grb10 in the remainder of the paper. Grb10 (26) is a member of a family of proteins that also

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1 The abbreviations used are: IR, insulin receptor; IRS, insulin receptor substrate; SH2, Src homology 2; IGF1, insulin-like growth factor I; IGFIR, IGF1 receptor; PH, pleckstrin homology; GST, glutathione S-transferase; EGFR, epidermal growth factor receptor; CHO, Chinese hamster ovary; BrdUrd, 3-bromo-5′-deoxyuridine.
includes Grb7 (27, 28) and Grb14 (29). These proteins share highly homologous amino acid sequences and common domains, including a C-terminal SH2 domain, a conserved proline-rich region within the N terminus, and a ~300-amino acid conserved central domain that includes the PH domain and other conserved regions of unknown structure or function located both upstream and downstream of the PH domain (26, 28, 29). The Grb7/Grb10/Grb14 proteins were originally cloned by screening cDNA expression libraries with a labeled tyrosyl-phosphorylated carboxyl-terminal fragment of the EGFR (26, 28, 29). However, neither Grb10 nor Grb14 have been demonstrated to interact efficiently with the EGFR in cells (26, 29). Grb10 has also been identified using yeast two-hybrid methodologies using the cytoplasmic domains of the IR or IGFIR as “bait” (23, 24, 30, 31), and it has recently been proposed that Grb10 associates preferentially with the IR as compared with the IGFIR in mouse fibroblasts (30). The role that Grb10 plays in cellular signaling mediated by any of these receptor tyrosine kinases remains unclear, as do the roles of Grb7 and Grb14.

The multidomain structure of these proteins suggests that they act as adapter molecules, but the signaling pathways that are affected and the nature of this effect remain to be identified.

It has previously been shown that the SH2 domain located at the extreme C terminus of Grb10 is important for interaction with the IR (23, 25, 31, 32). These data are consistent with the previous demonstrations that (a) Grb10 interaction depends upon IR kinase activity in the two-hybrid assay, (b) in vitro interaction of the IR with Grb10-GST fusion proteins is insulin-dependent, and (c) coimmunoprecipitation of Grb10 with the IR is insulin-dependent (23–25, 30–32). However, some recent evidence has suggested that the SH2 domain of Grb10 may not fully explain the interaction of Grb10 with the IR. Specifically, Frantz et al. showed that a full-length Grb10-GST fusion protein was able to interact more efficiently with the IR in vitro than the SH2 domain alone (25). These authors suggested that the PH domain might play a role in mediating the Grb10 interaction, since a construct containing both the PH and SH2 domains showed more efficient interaction. To address this issue, we used the yeast two-hybrid system and assays of in vitro protein interaction to demonstrate that Grb10 does indeed contain a second domain capable of independently interacting with the IR in an insulin-dependent manner. We demonstrate that this novel domain is relatively small (~50 amino acids) and is located between the PH and SH2 domains. We therefore term it the BPS domain to denote this location. We present data to suggest that the two binding domains within Grb10 may play a role in determining the specificity of receptor interaction with Grb10.

MATERIALS AND METHODS

Yeast Strains and Plasmids—S. cerevisiae EGY48 (a-trp1, ura3–52, his 3, leu2) and all yeast expression plasmids were provided by the laboratory of Roger Brent and have been previously described (3, 4, 33–35). All procedures for routine growth and maintenance of yeast strains were described previously (36). Plasmid transformation of yeast strains was performed as described (4, 40), and the units of β-galactosidase activity were calculated by the method of Miller (41). The values shown present averages of more than three assays ± S.E. (each assay representing an independent colony).

Cell Lines—CHOIR cells overexpressing human IRs (42) were grown in F-12 nutrient mixture (Ham’s) containing 10% fetal bovine serum and antibiotics. 5HR11 cells (43), which overexpress human EGFRs were grown in Dulbecco’s modified Eagle’s medium containing 10% fetal bovine serum, antibiotics, and G418 (400 ng/ml). NWTb3 cells (a gift from Derek LeBooth) overexpressing the human IGF1 receptors (44) have been grown in Dulbecco’s modified Eagle’s medium plus 10% fetal bovine serum, antibiotics, and G418 (400 ng/ml).

In Vitro Interaction Studies—GST fusion proteins were generated by introducing Grb10 cDNA fragments corresponding to the PH domain (amino acids 216–358), the SH2 domain (amino acids 435–536), BPS plus SH2 domains (amino acids 358–536), and various other fragments surrounding the BPS region (amino acids 434–396, 396–412, 396–402, 364–402, and 369–402) into the pGEX5X plasmid (Amersham Pharmacia Biotech). After transformation of Escherichia coli strain DH5α, induction of fusion protein expression with isopropylthio-β-D-galactoside, and cell collection and lysis by sonication, the proteins were purified using immobilized glutathione-agarose beads (38). Cultured CHOIR cells, NWTb4 cells, or 5HR11 cells were washed with phosphate-buffered saline (140 mM NaCl, 3 mM KCl, 6 mM NaH2PO4, 1 mM KH2PO4, pH 7.4) warmed to 37 °C, stimulated with or without ligands for 10 min at 37 °C, washed with ice-cold phosphate-buffered saline, and solubilized with lysis buffer (50 mM HEPES, 150 mM NaCl, 1 mM EGTA, 20 mM Na2HPO4, 10 mM NaF, 1 mM Na3VO4). The lysates were clarified by centrifugation at 15,000 × g for 15 min at 4 °C and incubated overnight (~500 μg of total protein/reaction) at 4 °C with (~3–4 μg) immobilized GST fusion proteins. After extensive washing with 50 mM HEPES (pH 7.6), 150 mM NaCl, and 0.1% Triton X-100, the proteins that coprecipitated with the Grb10 or control GST proteins were separated by SDS-polyacrylamide gel electrophoresis, transferred to nitrocellulose membranes (Schleicher and Schuell), and immunoblotted with antibodies against the IR (C-19, Santa Cruz Biotechnology).

Microinjection Studies—NWTb3 cells, which overexpress the IGFIR, were maintained as described previously (19) and grown on glass coverslips for microinjection experiments. The cells were rendered quiescent by serum deprivation overnight. Fusion proteins were expressed in DH5α cells and purified with glutathione-agarose beads by standard techniques (38). The fusion proteins were microinjected at a concentration of 3.5 mg/ml, and preimmune sheep IgG was added to the protein for detection purposes. Parallel injections of sheep IgG alone were also performed. One hour after injection, the cells were stimulated as indicated with 100 ng/ml IGF1. A solution of 3-bromo-5-deoxyuridine (BrdUrd) (Amersham Pharmacia Biotech) was also added to the medium for 16 h to label newly synthesized DNA. Following the labeling period, the cells were fixed and processed for immunofluorescence as described by Xiao et al. (9). Briefly, the cells were stained with a rat monoclonal anti-BrdUrd antibody (Accurate Scientific), followed by a tetramethylrhodamine B isothiocyanate-conjugated donkey anti-rabbit IgG antibody and a fluorescein isothiocyanate-conjugated donkey anti-sheep IgG antibody to detect the injected cells. This technique results in red nuclear staining in cells that have progressed to S phase of the cell cycle and green cytoplasmic staining in the injected cells. Results were analyzed and quantitated using an epifluorescence photomicroscope (Carl Zeiss, Inc.).

RESULTS AND DISCUSSION

Grb10 Interacts with the IR via the C-terminal SH2 Domain and a Novel Domain Located between the PH and SH2 Domains—Grb10 has been shown to interact directly with the IR and IGFIR (23–25, 30–32). We had previously reported that the IR interacts strongly with a region of Grb10 that spans amino acids 358–536, a region that includes the SH2 domain. Since this interaction was kinase-dependent in the two-hybrid assay and because in vitro interaction of Grb10-GST fusions with cell-derived receptors was insulin-dependent (25), we concluded that the interaction was mediated via the SH2 domain. In fact, it has been demonstrated that the SH2 domain of Grb10 does mediate an interaction with the IR (24, 25, 30–32).

However, in subsequent experiments we found that mutagenesis of the critical arginine (to lysine) within the SH2 FLVRES motif, which would be predicted to disrupt phosphotyrosine binding, had only minor effects upon full-length Grb10 interaction with the IR despite the demonstration that the
acts Independently with the IR—As shown schematically in Fig. 2, the known members of the Grb7/Grb10/Grb14 protein family show a high degree of identity within the SH2 domain and the conserved 300-amino acid central domain (26, 29), which includes the PH domain, and two conserved blocks of ~100 amino acids each located both N-terminal and C-terminal to the PH domain. We term the domain between the PH and SH2 domains BPS. To further examine the receptor binding activity of the BPS region, we generated a series of hybrids from this region and assayed their interaction with the IR in the two-hybrid system. As shown in Fig. 2, interaction of BPS domain amino acids 358–434 resulted in high activity, which was approximately equivalent to that observed with the SH2 domain. Further truncation of the C terminus of the BPS region to amino acid 412 or 402 showed no apparent loss of activity. Truncation of the N-terminal residues to 364 showed no significant loss of activity, whereas further truncation to residue 369 showed consistently reduced activity. The kinase-inactive IR (K1018A) hybrid showed no interaction with any BPS domain hybrid proteins (data not shown). We conclude that a distinct domain exists between the PH and SH2 domains, which is capable of binding to the IR in a manner independent of the SH2 domain. Furthermore, the interaction depends upon IR kinase activity. A BLAST search of the data bases using the amino acid sequences between the PH and SH2 domains showed that only the Grb7/10/14 proteins gave significant scores (p = 1.2e−16 or lower; data not shown), suggesting that this domain is not found in other known proteins.

Both the SH2 and BPS Domains of Grb10 Interact with the IR in Vitro—To further examine the SH2 and BPS domain interactions, we produced nine GST-Grb10 fusion proteins corresponding to the PH, SH2, and BPS domains (Fig. 3A). Immobilized GST fusions were incubated with lysates derived from CHO-IR cells that were or were not insulin-stimulated. After extensive washing, the precipitated proteins were resolved by SDS-polyacrylamide gel electrophoresis and subsequently immunoblotted with either anti-phosphotyrosine or anti-IR antibodies. Neither the GST protein alone nor the GST-PH fusion showed any in vitro interaction with the IR (Fig. 3B, GST and lane 3). As we observed previously, the Grb10 hybrid containing both the BPS and SH2 domains interacted strongly with the IR (23). Consistent with our two-hybrid data, mutation of R462K within the Grb10 SH2 FLVRES motif had no significant effect on interaction of the BPS plus SH2-containing fusion (compare lanes 1 and 2). The SH2 domain alone interacted efficiently with the IR (lane 4), and as expected, the R462K mutation eliminated all SH2 binding (lane 5). Several GST fusions containing various BPS domain residues (amino acids 358–434, 364–412, and 369–412) were also found to interact efficiently with the IR to an extent roughly similar to that of the SH2 domain (lanes 6–8), whereas the BPS amino acids 369–402 bound only very weakly to the IR (lane 9). The majority of these results are consistent with our findings from the yeast two-hybrid assay. However, the 369–402 fusion showed less in vitro binding activity (Fig. 3B, lane 9) than was expected from the two-hybrid assay. Consistent with this finding, two additional GST constructs, 358–402 and 364–402, both showed poor interaction in vitro (data not shown), suggesting that amino acids between 402 and 412 are important for in vitro interaction but are not necessary for the more sensitive two-hybrid assay. Alternatively, these hybrid proteins may not fold properly in bacteria as compared with yeast. To prove that the SH2 and BPS domains interact specifically with the activated IR and not the unstimulated IR, we immunoblotted the precipitates with an antibody against the IR (Fig. 3C). These fusion proteins only showed interaction with the IR derived

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**Fig. 1.** Grb10 interacts specifically with the IR via the SH2 domain and a second region located between the PH and SH2 domains. Various regions of Grb10 were assayed for interaction with the wild-type IR “bait” hybrid in the yeast two-hybrid assay. Transformants were assayed for β-galactosidase activity by the solution assay as described under “Materials and Methods.” β-Galactosidase (β-Gal) activity is reported in Miller units, and the data represent an average from a minimum of three independent colonies. + + +, a dark blue color in the colony color assay; −, white colonies.
from insulin-stimulated CHO/IR cells, showing that both the SH2 and BPS bind in an insulin-dependent manner.

The Grb10 BPS Domain Interacts More Strongly with the IGFIR than the SH2 Domain—The IGFIR shares a high sequence homology with the IR (45). Nevertheless, coimmunoprecipitation studies of the Grb10 protein with either the IR or IGFIR have suggested that Grb10 associates preferentially with the IR (30). Thus, we investigated the Grb10 interaction with the IGFIR to see if these results might be attributed to differential Grb10 domain interaction. We therefore examined the interaction of Grb10 with the IGFIR using GST pull-down assays. As shown in Fig. 4, the control GST and PH domain fusions did not precipitate any IGFIR. Surprisingly, although the Grb10 SH2 domain fusion showed significant activity with the IGFIR in the yeast two-hybrid assay (80–100 units, data not shown), the GST-SH2 hybrid showed virtually no interaction with IGFIR in vitro. In comparison, the GST-BPS fusion containing amino acids 364–412 interacted efficiently with the IGFIR in vitro. Interestingly, despite the apparent lack of interaction of the SH2 domain and the IGFIR in vitro, the BPS plus SH2 fusion protein consistently coprecipitated more IGFIR than the BPS domain alone. In addition, the R462K mutation introduced into the BPS plus SH2 construct reduced the interaction by about 50%, yet this mutant protein still showed a relatively stronger interaction with the IGFIR than the BPS domain alone. These data suggest that the SH2 and BPS domains may bind to the IR and IGFIR in a cooperative manner and that even the mutant SH2 domain may stabilize and strengthen the BPS domain interaction as long as the BPS domain is present to direct the SH2 domain to the receptor. Alternatively, the SH2 domain may act to stabilize the structure of the BPS domain such that it binds more efficiently to the IR. These data with the mutated SH2 domain are in agreement with results from the yeast two-hybrid assay, since the 358–536 (BPS plus SH2) showed similar activities whether or not they contained the SH2 mutation (Fig. 1).

The BPS Domain Does Not Interact with the EGFR—Although the murine Grb10 protein was initially discovered by screening an expression library with a 32P-labeled cytoplasmic domain of the EGFR, in vivo interaction has not been easily demonstrated (26). Furthermore, it has been shown that GST-Grb10 fusion proteins interact with the EGFR with less avidity than with the IR and platelet-derived growth factor receptor (24, 25). We reasoned that the weak interaction with the EGFR might be due to differential association of the receptor with the two domains of Grb10. To address this question, we examined EGFR coprecipitation with the previously described Grb10 GST fusions. As shown in Fig. 4, only the SH2 domain was able to bind efficiently to the EGFR in vitro. The BPS domain (364–412) showed no binding to the EGFR. In support of this, the substitution mutation (R462K) within the Grb10 SH2 domain totally abolished this interaction with both the SH2 and the BPS plus SH2 hybrids.

IR Domains Required for Interaction with the BPS and SH2 Domains of the Grb10—Several tyrosine residues in the cytoplasmic domain of the IR and IGFIR become phosphorylated during receptor autophosphorylation, and these phosphotyrosines either activate the kinase activity of the receptor (2) or act as docking sites for substrates of receptors. Specifically, the three tyrosines (positions 1146, 1150, and 1151) within the kinase domain activation loop play a role in activation of the IR catalytic activity (46). In addition, two juxtamen-
brane tyrosines become phosphorylated (953 and 960), the latter of which is located within an NPXY motif (where pY represents phosphorylated tyrosine), which is required for binding to the phosphotyrosine binding domains within IRS-1, IRS-2, and SHC (3–5). In addition, two tyrosines within the C terminus become phosphorylated (1316 and 1322), and although 1322 has been shown to interact with p85 (47), the role of interaction of the IR with Grb10 may be due to elimination of a specific interaction site for Grb10 within the IR rather than being due to a global loss of catalytic activity by this IR mutant. We next examined the independent interaction of either the SH2 domain or the BPS domain with these same IR mutants. As shown in Fig. 5, B and C, both the SH2 and BPS domains showed markedly reduced interaction with the Y1150P/Y1151F double substitution mutant, showing that this receptor region is essential for interaction with both domains. It is of note that the p85 SH2 domain, which has been shown to interact with the C-terminal tyrosine 1322 (47), retains an interaction with the C-terminally deleted IR. This appears to be due to interaction with another region of the IR, which may include the tyrosines within the activation loop (data not shown). We conclude from these studies that the paired tyrosines (Tyr1150 and Tyr1151) within the activation loop are essential for binding to both the SH2 and BPS domains of Grb10.

To further examine whether the SH2 and BPS domains within Grb10 can interact in vitro with the kinase domain of the IR, we tested the ability of the GST fusions to interact with a purified IR kinase domain produced in an insect cell (Sf9) expression system. We utilized a purified preparation of baculovirally expressed IR kinase domain (a gift of Steve Hubbard), which had been activated in vitro and consisted primarily of a form of the kinase domain that was triply phosphorylated within the activation loop (data not shown). As shown in Fig. 5E, the BPS plus SH2-GST fusion interacted well with the IR kinase domain whether or not the SH2 domain contained an Arg or Lys within the FLVRES box. The GST control and PH fusion showed no interaction with the IR kinase domain. Individually, both the SH2 and BPS domains showed interaction with the IRK3P, but at lower levels as compared with the BPS plus SH2 constructs (data not shown). These data are consistent with our two-hybrid results and clearly show that Grb10 can interact with the kinase domain in the absence of both the juxtamembrane and C-terminal IR domains. In addition, these data show conclusively that the interaction between these proteins is direct, since both the IR and Grb10 proteins are highly purified.

Microinjection of the Grb10 Region Containing the SH2 and BPS Domains Inhibits Mitogenesis Induced by IGFI, while the
Individual Domains Have No Effect—As a means of examining the effects of these Grb10 domains on IGFI signaling in a cellular system, we produced GST fusion proteins, which contained the Grb10 PH, SH2, and BPS domains. We have previously used this approach to examine the effect of a Grb10 fragment (amino acids 358–536) upon insulin, IGFI, epidermal growth factor, and serum-stimulated mitogenesis (23). Injection of this fusion protein inhibited insulin- and IGFI-stimulated progression to S phase but had no effect upon epidermal growth factor- or serum-stimulated cell cycle progression. Since this Grb10 fusion protein contains both the SH2 and BPS domain (which we were previously unaware of), it was necessary to examine the effects of injecting each domain independently to examine its effect upon cell cycle progression. These new fusion proteins were microinjected into fibroblasts that overexpress IGFI in a cooperative manner and that the mutated SH2 domain may be able to interact with the IR but only if the BPS domain is also present. Alternatively, the mutated SH2 domain may act sterically to more effectively block interactions with endogenous signaling proteins.

Potential Roles of Multiple Receptor Interaction Domains within Grb10—Our results clearly demonstrate the existence of a second domain within Grb10 that is capable of mediating a kinase-dependent interaction with the IR and IGFI. This region, which we have termed BPS, is highly conserved within the related proteins Grb7 and Grb14, and it is likely that these domains may play a similar role within these proteins. Our data show that the BPS domain binding to the IR requires receptor kinase activity and more specifically appears to require the paired tyrosines within the activation loop within the kinase domain. What remains unclear is whether the BPS domain interacts directly with the activation loop tyrosines or alternatively whether autophosphorylation of these tyrosines causes a conformational change within the kinase domain, which exposes a binding site for the BPS domain. Since this Grb10 fusion protein does not contain the SH2 and BPS domain (which we were previously unaware of), it was necessary to examine the effects of injecting each domain independently to examine its effect upon cell cycle progression. These new fusion proteins were microinjected into fibroblasts that overexpress the IGFIIR, and the injected cells were monitored for mitogen-stimulated BrdUrd incorporation using previously described methods (19). In this assay, all microinjection samples contained preimmune IgG to allow the detection of injected cells, which were then scored as positive or negative for cell cycle progression based upon the presence or absence of nuclear rhodamine staining. As shown in Fig. 6, treatment of cells with IGFI resulted in an increase in the percentage of BrdUrd-positive cells from a basal level of 16.3% to an average of 56.1%. Microinjection of the PH domain had no effect upon BrdUrd incorporation (52.8%). Injection of the individual domains encoding the SH2 (49.5%), SH2-R/K (43.9%), or BPS (48.6%) domain also showed no significant inhibition of cell-cycle progression. Injection of either the BPS plus SH2 or the BPS plus SH2-R/K constructs did show significant inhibition of IGFI-stimulated mitogenesis of ~50% (32.2 and 33.1%, respectively) as compared with IgG-injected, IGFI-treated cells. These data suggest that efficient interaction of Grb10 with the IGFIIR requires both the SH2 and BPS domains. Interestingly, as we had previously observed in the two-hybrid and GST pull-down assays, a BPS plus SH2 construct with a mutated SH2 FLVRES motif retained the ability to inhibit IGFIIR mitogenic signaling, suggesting that the BPS and SH2 domains may bind in a cooperative manner and that the mutated SH2 domain may be able to interact with the IR but only if the BPS domain is also present. Alternatively, the mutated SH2 domain may act sterically to more effectively block interactions with endogenous signaling proteins.
and IGFR in the activation loop, it is surprising that the SH2 domain appears to interact preferentially with the IR in vitro (Fig. 4) and perhaps in vivo (30). It remains possible that these two receptors may have distinct structures in the active state that confer differential binding profiles to receptor substrates.

Our demonstration of two independent interaction domains within a single signaling protein such as Grb10 is interesting for a number of reasons. First, as we have discussed, this may be a mechanism that helps to determine the specificity by which signaling proteins such as Grb10 interact with different receptor tyrosine kinases or other phosphoproteins. As shown schematically in Fig. 7, our data suggest that Grb10 may interact strongly with the IR by virtue of its interaction with both the SH2 and BPS domains. Conversely, Grb10 may interact less efficiently with the IGFR and EGFR due to preferential interactions with either the BPS or the SH2 domain, respectively. In this regard, it is becoming increasingly clear that signaling proteins that contain multiple SH2 domains show a much higher affinity of interaction with their bis-phosphorylated peptide binding partners if both SH2 domains are expressed together (49, 50). In other words, two binding domains within a molecule have been shown to lead to greatly increased affinity, which has been shown to be increased by many orders of magnitude as compared with the individual binding domains. Thus, it is possible that the reason that Grb10 can be coimmunoprecipitated efficiently with the IR but not with the IGFR or EGFR may be explained by these findings (25, 26, 30). Thus far, we have only considered the possibility that Grb10 may interact within or near the activation loop within the IR kinase domain, it seems possible that Grb10 may physically block access of substrates or ATP to the catalytic pocket, which is located very close to the activation loop. However, we cannot rule out the idea that Grb10 might also serve to increase signaling by the IR perhaps by stabilizing the phosphorylated activation loop in the activated configuration or by protection of these phosphotyrosines from tyrosine phosphatases. Further experiments will be required to address these issues. A real understanding of the function of Grb10 in receptor tyrosine kinase signaling is likely to become clear only when we begin to identify components that lie downstream of Grb10.

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**FIG. 7. Model of Grb10 interaction with the IR, the IGFR, and the EGFR.** Our data suggest that both the SH2 domain and the BPS domain interact strongly with the IR. The BPS domain interacts well with the IGFR, whereas the SH2 domain binds weakly as compared with the IR. The SH2 domain interacts well with the EGFR, whereas the BPS domain shows no interaction with the EGFR. This differential interaction may result in relatively high affinity binding of Grb10 to the IR as compared with the IGFR and EGFR.
Dual Receptor Interaction Domains within Grb10

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