Published in final edited form as:
Expert Opin Ther Targets. 2008 August ; 12(8): 1021–1033. doi:10.1517/14728222.12.8.1021.

Grb2 Signaling in Cell Motility and Cancer

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Summary

Metastasis is the primary cause of death in most human cancers, and understanding the molecular mechanisms underpinning this multistep process is fundamental to identifying novel molecular targets and developing more effective therapies. Growth Factor Receptor-bound protein 2 (Grb2) is a key molecule in intracellular signal transduction, linking activated cell surface receptors to downstream targets by binding to specific phosphotyrosine-containing and proline-rich sequence motifs. Grb2 signaling is critical for cell cycle progression and actin-based cell motility, and consequently, more complex processes such as epithelial morphogenesis, angiogenesis and vasculogenesis. These important functions make Grb2 a logical therapeutic target for strategies designed to prevent the spread of solid tumors through local invasion and metastasis. Here we review the role of Grb2 in cancer and specifically in metastasis-related processes, and summarize briefly the development of anti-cancer therapeutics selectively targeting this important adapter protein.

Keywords

Grb2; Cell Migration; Tumor Metastasis

1. Introduction: the metastatic problem

Metastasis is the primary cause of death in most human cancers, and understanding the molecular mechanisms underpinning this multistep process is fundamental to identifying novel molecular targets and developing more effective anti-cancer therapies. Despite progressive advancement in our understanding and in the treatment of cancer over the last decade, the metastatic process remains poorly understood at the molecular level[1]. Many current cancer treatments focus primarily on blocking the proliferation of tumor cells using cytostatic agents and targeted therapies, but these regimens offer limited success, with frequent relapse. Thus, to improve survival rates for most cancers, more effective ways of treating micrometastatic disease are required.

Metastasis is a multistep process[2, 3] in which cells from the primary tumor migrate through the extracellular matrix, enter the circulation through newly formed blood vessels (tumor angiogenesis) and disseminate to distant sites (extravasation), where proliferation begins again. Blocking any stage of this process can potentially be an effective strategy to block the entire process of metastatic disease.

A variety of extracellular signaling molecules, such as growth factors and cytokines, signal through specific binding to membrane receptors endowed with tyrosine kinase activity[4].

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Ligand binding triggers receptor phosphorylation on tyrosine residues within specific structural motifs which constitute docking sites for several signaling molecules, such as SH2 domain containing proteins. Recent findings show that these molecules are dynamic connectors of intracellular networks, able to coordinate spatially and temporally diverse pathways[5]. One such molecule is the adapter protein Growth Factor Receptor bound protein 2 (Grb2), which recruits to receptors a variety of other signaling molecules to form multimeric signaling complexes leading to cellular responses such as proliferation and invasion, two widely recognized hallmarks of cancer[6].

Grb2 was originally characterized for its role in cell proliferation[7]; however, emerging evidence shows that Grb2 contributes to tumorigenesis in several other ways and to other stages of cancer progression. Direct and indirect interactions between Grb2 and several intracellular proteins involved in the metastatic cascade have been the subject of numerous original reports, but unfortunately the role of Grb2 in cell motility and metastasis has not been systematically examined. Here we summarize these findings and offer a perspective on the development of selective inhibitors of this adapter protein as anti-cancer drugs.

2. The adapter protein Grb2

Grb2 is a ubiquitously expressed adapter protein that is essential for a variety of basic cellular functions and acts as a critical downstream intermediary in several oncogenic signaling pathways. The mature 25 kDa Grb2 protein has a modular structure with one Src homology 2 (SH2) domain flanked by two SH3 domains[8]. Originally isolated through screening for epidermal growth factor receptor (EGFR) interacting proteins, Grb2 has been demonstrated to interact with several proteins. In particular through its SH2 domain, which is a conserved sequence of 100 amino acids, Grb2 can interact directly with receptor tyrosine kinases (e.g. hepatocyte growth factor receptor, platelet derived growth factor receptor, etc.) and non-receptor tyrosine kinases, such as focal adhesion kinase (FAK) and Bcr/Abl[9], as well as substrates of tyrosine kinases, via preferential binding to the phosphopeptide motif pYXNX (where N is asparagine and X any residue). The carboxyl and amino-terminal Src homology 3 (SH3) domains, which have a conserved sequence of around 50 amino acids, bind proline-rich regions within interacting proteins.

The canonical model of Grb2 function relies on the widely confirmed observation that Grb2 is constitutively associated with Son of sevenless (Sos), a guanine-nucleotide exchange factor that promotes GDP-GTP exchange on Ras. Upon growth factor receptor activation and tyrosyl phosphorylation, Grb2 brings Sos1 into close proximity of membrane-bound Ras, thereby activating Ras and the downstream mitogen-activated protein kinase (MAPK) cascade[10].

The *grb2* gene is highly conserved among species and Grb2 expression is critical for normal development[11]. Mouse embryos with homozygous deletion of *grb2* die at a very early embryological stage, precluding investigation of these cells later in development. However, using mutations originally identified in *C. elegans*, it has been possible to generate a hypomorphic allele of the mouse *grb2* gene and derive $grb2^{-/hypomorph}$ mice that manifest morphogenic defects in neural crest cell migration into the branchial arches and defects in cardiovascular development[12]. These findings reinforce the idea that Grb2 is critical for epithelial morphogenesis and for processes such as cell motility and vasculogenesis.

3. Grb2 and cancer: an overview

Beside its role as critical downstream intermediary in several oncogenic signaling pathways, Grb2 signaling has also been implicated directly in the pathogenesis of several specific human malignancies. The human *grb2* gene is located in chromosome 17(q22), a region which is known to be duplicated in solid tumors and in leukemias[13]. In chronic myelogenous leukemia

(CML), the chimeric Bcr-Abl tyrosine kinase oncoprotein is able to bind the Grb2 SH2 domain through Y177 in the BCR region, linking the fusion protein to the Ras pathway[14]. Competitive antagonism of the ATP binding site of the Abl tyrosine kinase with small molecules such as Imatinib (STI-571) is currently the main therapy, but inhibition of Grb2-Bcr interaction could become a valid adjuvant therapy or an alternative in patients resistant to this treatment.

Grb2 can also be overexpressed in tumors, such as in breast cancer. Indeed, in addition to its role as a proximal mediator of ErbB2/Neu signaling, Grb2 itself was found to be overexpressed in several breast cancer cell lines and breast cancer tissue samples[15, 16], enhancing signaling through the MAPK pathway. Grb2 is also important in polyomavirus induced mammary carcinoma, and Grb2 gene dosage was rate limiting for the onset and development of experimental mammary carcinomas[12], highlighting its critical role in the transformation process. Grb2 is involved in keratinocyte growth factor (KGF) induced motility in MCF-7 breast cancer cells[17] further suggesting that Grb2 can be a valid therapeutic target for pathological processes such as the spread of solid tumors through local invasion and metastasis. In bladder cancer cells where no EGFR overexpression or H-Ras mutations have been reported, Grb2 has been found overexpressed together with Sos1, as the only observed mechanism of oncogenesis[18]. In the highly metastatic cancer cell line 1-LN, Grb2 was one of the effector proteins significantly induced, together with Sos1, Shc and Raf1, through activation of the [alpha]$_2$-macroglobulin receptor[19].

Consistent with a role in tumor dissemination, several groups have reported specific direct and indirect interactions of Grb2 with molecules involved in cytoskeleton remodeling, motility and other cellular processes recapitulated in the multistep cascade of cancer metastasis. Inhibitors of Grb2 SH2 domain binding have been demonstrated to reduce motility *in vitro* and decrease cancer metastasis in animal models[20–22]. Several studies, summarized below, elucidate the molecular mechanisms by which Grb2 contributes to cell motility and other processes characteristic of cancer metastasis (Figure 1).

4. Grb2 signaling in invasion and metastasis

4.1. Cell adhesion

Early in the metastatic cascade changes occur in the adhesion properties of potentially metastatic cells inside the primary tumor[23]. These cells lose their junctions to other cells and to the extracellular matrix (ECM) and display increased motility. In motile cells, new adhesion sites (focal complexes) located within cell edge protrusions (lamellipodia and filopodia) are transient and small compared to the more stable focal adhesions underlying the cell body and localized at the extremities of actin stress fibers[24]. The interaction of ECM components with the cell surface is mediated mainly by members of the integrin family of transmembrane receptors. Integrins do not have intrinsic catalytic activity but rely on the kinase activity of other nearby intracellular proteins. For example, focal adhesion kinase (FAK) colocalizes with integrin receptors upon engagement of the cell with the ECM and constitutes a nodal point in integrin signaling, and an important regulator of cell migration[25].

FAK overexpression in several types of tumors is associated with increased angiogenesis, metastasis and poor prognosis[26]. FAK function is regulated through tyrosyl phosphorylation at specific sites in its amino acid sequence. Several stimuli, such as those mediated by integrins, can induce FAK autophosphorylation, creating docking sites for proteins containing SH2 domains, including Src. Src can also activate FAK and promote its phosphorylation on other tyrosine residues; one of these residues, Y925, occurs within a consensus sequence (pYXNX) for high affinity binding to the SH2 domain of Grb2[27]. Interestingly, elevated Src activity, as observed during colon cancer progression, specifically promotes phosphorylation on tyrosine Y925, inducing changes in integrin adhesion and deregulation of E-cadherin[28] and leading to

an E-cadherin/N-cadherin switch[29]. This event is part of an important hallmark of cell transformation and metastasis, namely epithelial-mesenchymal transition (EMT). The role of FAK in tumor metastasis has been confirmed in several studies and may explain, at least in part, why Grb2 SH2 domain binding antagonists can inhibit migration *in vitro* and tumor dissemination in animal models[22]. These same binding antagonists were able to suppress N-cadherin expression in primary tumors, suggesting a novel role for Grb2 in EMT[22, 30].

The interaction of Grb2 with FAK, Shc and other proteins also leads to activation of the Ras and extracellular-signal-regulated kinases 2 (ERK2) pathways; integrin engagement of these pathways induces cell spreading through actin cytoskeleton rearrangement. Indeed, one of the key differences between ERK2 pathway activation by growth factor receptors versus by integrins is that the latter require a functional actin cytoskeleton to signal, while growth factor receptors can signal even when the actin microfilaments are disrupted by cytochalasin D, a potent inhibitor of actin polymerization[31, 32].

The discovery of Grb2 mediated adhesion signaling raises questions as to its precise role in this context. In particular, it is important to understand the signaling that permits migrating tumor cells to go through a series of continuous attachments and detachments, and how signaling proteins such as Grb2 direct these transitions and promote cell movement.

4.2. Extracellular matrix remodeling

Once tumor cells have detached from neighbor cells and from the ECM, they must move through the tissue and into blood vessels to reach distant sites. Proteolytic ECM remodeling facilitates this process and involves a wide spectrum of proteinases[33]. Matrix metalloproteinases (MMPs) are a family of secreted and transmembrane proteins capable of degrading virtually every component of the extracellular matrix. They function in physiologic processes such as tissue growth, morphogenesis, tissue repair and angiogenesis, as well as in pathological conditions such as tumor invasion and metastasis[34]. The MMP family includes a class of membrane-anchored metalloproteinases, ADAMs (A Disintegrin And Metalloprotease), involved in proteolytical cleavage and release of membrane-bound growth factors, cytokines and receptors[35]. ADAMs overexpression and dysregulation have been implicated in angiogenesis and metastasis, especially in ErbB ligand cleavage and activation as well as in the processing of other proteins involved in oncogenesis[36]. ADAM12 (Meltrin alpha), upregulated in several cancers, interacts directly with the SH3 domains of Grb2 through prolin-rich sequences in its cytoplasmic tail[37]. Moreover, Grb2 and ADAM12 colocalize at membrane ruffles, structures visible specifically during epithelial cell migration. Another protein in this family, ADAM15 (metargidin) also interacts with Src and Grb2 *in vitro* through proline-rich sequences in the cytoplasmic domain[38]. In addition to weakening the structural integrity of the ECM, these proteolytic activities can liberate matrix-bond chemokines and growth factors, that further enhance to the motility and proliferation of cancer cells. This collection of important oncogenic properties has prompted the development of selective ADAMs inhibitors now entering clinical trials, and reinvigorated hopes placed earlier on MMP inhibitors[39, 40].

4.3. Cytoskeletal plasticity and cell motility

Despite the name “growth factor”, most proteins so named not only stimulate proliferation, but a plethora of other effects. Upon binding to membrane receptors, growth factors initiate signals leading to survival, changes in energy metabolism and other activities such as motility and invasion. Most, if not all, growth factors are also motogens. One of the best characterized growth factors involved in cell motility and invasion is hepatocyte growth factor (HGF), also known as the scatter factor (SF), the ligand for the tyrosine kinase receptor c-Met[41]. Scattering is a spatially and temporally complex response to HGF/c-Met interaction in which a cluster of

grouped cells lose apical/basal polarity and initiate membrane ruffling, then dissociate from the ECM and neighboring cells through disruption of E-cadherin mediated cell-cell adhesion and subsequently migrate[42]. Intracellularly, HGF binding activates the c-Met kinase domain, and through a series of auto- and trans-phosphorylations on specific tyrosine residues, docking sites for effector proteins are created. Grb2 interacts directly with pY1356 in c-Met (Swissprot Database sequence, accession P08581), or indirectly through the adapter protein Gab1 (Grb2 Associated Binder 1). Among the many downstream pathways implicated in this process are activation of the Rho family GTPase Rac and Ras[43, 44]. Activation of the Ras-Rac1/Cdc42-PAK and Gab1-Crk-C3G—Rap1 effector cascades also regulates cytoskeletal and cell adhesion proteins such as Arp2/3, N-Wiskott-Aldrich Syndrome protein (N-WASP), paxillin, integrins, FAK and cadherins.

Grb2 mediated motogenic signaling extends beyond its role as a receptor-proximal adapter protein in growth factor and ECM stimulated cell motility: Grb2 interacts directly with the actin filament machinery. One such interaction is with the cytoskeletal associated protein WASp[45], a regulator of actin cytoskeletal rearrangement. Patients affected by a mutation in the gene encoding this protein show functional defects in platelets, in T and B cell polarization, and in the ability of these cells to migrate in response to external stimuli, resulting in thrombocytopenia, immunodeficiency and a propensity to develop malignancies[46]. The WASp protein contains proline rich sequences that interact with the Grb2-SH3 domains; binding to Grb2 translocates WASp from the cytosol to the plasma membrane, where it can interact with membrane bound proteins such as Rac and Cdc42[47]. Grb2 also links the EGF receptor to WASp protein constitutively and this interaction is enhanced upon EGF stimulation. WASp at the membrane interacts with Nck[48], and together with Grb2, cooperatively stabilizes actin-nucleating complexes. So Grb2 and Nck, both SH2 and SH3 domain containing proteins, link membrane receptors and membrane-bound proteins to intracellular cytoskeletal regulators, increasing their local concentrations at the membrane and facilitating enzymatic reactions and activation. Model organisms, such as *Listeria monocytogenes* and vaccinia virus, have been used to refine our understanding of the role of Grb2 in actin-based motility. These pathogens subvert the actin cytoskeletal machinery to navigate through the host cytoplasm[48, 49]. N-WASp is necessary for the actin-based motility of vaccinia virus, and in mammalian cells regulates actin polymerization through the Arp2/3 complex (the nucleation factor of newly formed actin filaments) and interaction with Cdc42[50]. When N-WASp is not present, Grb2 triggers only a weak activation of Arp2/3 with defective actin polymerization[51]. These findings further define the role of Grb2 in connecting signaling molecules to the actin cytoskeleton and cell motility.

Several motogenic signaling pathways from the cell surface converge on the p21-activating kinases (PAKs), which consequently translocate to the leading edge of the cell and contribute to motility and invasion. Activation of PAKs and translocation to the plasma membrane are processes that involve interaction with adapter proteins such as Nck and Grb2. PAKs are serine/threonine kinases that regulate cancer signaling networks and are considered platforms that amplify and propagate oncogenic signals elicited by extracellular stimuli[52]. PAKs are important regulators of actin cytoskeletal dynamics and the role of PAKs in cancer has been widely reported in literature. Specifically, PAK1, the best characterized member of the PAK family, was discovered in 1994[53] as a target for CDC42 and Rac1, well-known regulators of the actin cytoskeleton and implicated in the formation of fingerlike protrusions (filopodia; CDC42) and sheetlike structures at the cell periphery (lamellipodia; Rac1). Interestingly, PAK can directly and specifically interact with Grb2 through a prolin-rich motif in the PAK sequence[54]. This interaction is independent of EGF stimulation, but it is increased after stimulation of the EGF receptor and EGFR-Grb2-PAK1 interaction is required for EGF induced lamellipodia formation.

The importance of Grb2 in sites of dynamic actin rearrangement is further underscored in the formation of podosomes. Originally identified in cells of mesenchymal origin, podosomes have also emerged as adhesive structures in epithelial cells. These clusters of actin organized in rosette-like structures have components also found in focal contacts, as well as dynamin, cortactin, Arp 2/3, N-WASP and VASP[55]. Grb2, but not Nck, participates in podosome formation, and overexpression of Grb2 interferes with the organization of these structures[56]. Characteristically, these structures disappear when cells become motile and re-form when cells are no longer moving. Podosomes have also a similar structure and composition to the invadopodia found in malignant tumor cells.

The complexity of Grb2 involvement in actin-based cell motility is illustrated by a growing list of interacting cytoskeletal proteins (Table 1). Merlin localizes to the leading edge of cells (membrane ruffles), where it colocalizes with actin and contributes to actin assembly and interaction with the cortical cytoskeleton (actin cortex). Merlin is the product of the Neurofibromatosis 2 (NF2) gene; NF2 mutations give rise to an autosomal dominant syndrome characterized by vestibular schwannomas and meningiomas. The sequence of merlin shows similarities with ERM proteins (Ezrin, Radixin and Moesin) that link membrane proteins to the actin cytoskeleton; merlin has the FERM domain for interaction with F-actin. In a screen for Merlin binding proteins, a novel protein, Magicin (merlin and Grb2 interacting cytoskeletal protein) with a consensus sequence (pYVNG) for the SH2 domain of Grb2, was discovered[57]. Magicin creates a multiprotein complex with merlin, although the main interaction appears to be through the SH3 domain of Grb2. The functional consequences of magicin-Grb2 interaction and how this interaction reflects manifestations of neurofibromatosis syndrome remain unclear.

The filamentous-actin binding protein cortactin (from “cortical actin binding protein”), translocates from the cytoplasm to the cell periphery where it assists the Arp2/3 complex in nucleating actin[58] and promoting the formation of lamellipodia. Grb2 can associate directly with cortactin, linking several receptor tyrosine kinases to the actin cortex. When phosphorylated on serine or threonine by ERK, cortactin assumes a conformation that allows N-WASp and other nucleation-promoting factors to bind to the cortactin-Arp2/3-actin complex[59]. Cortactin is also overexpressed in certain cancers and is associated with invasiveness, formation of invadopodia and secretion of MMPs, favoring the spread of cancer cells through tissue[60, 61]. Moreover, cortactin can bind to another Grb2 interacting protein, caldesmon. When tyrosyl phosphorylated, caldesmon forms a protein complex with Grb2, Nck, Shc, PAK1 and myosin light chain kinase[62], creating an oncogenic platform that signals independently of EGFR ligand activation and distinctly from the Ras mitogenic pathway.

The microtubule network is also affected by Grb2-mediated signaling. For example, dynactin, a multisubunit activator of the microtubule motor protein dynein that contributes to cell polarization during migration, forms a constitutive complex with Grb2 *in vivo*, through its N-terminal SH3 domain, in osteoclasts[63]. Another microtubule associated protein, microtubule associated protein2 (MAP2), a major cytoskeletal protein in neurons[64] that participates in neuronal morphogenesis, also binds Grb2, linking the microtubule network to multimeric signaling complexes in the cytosol. Finally, hungtingtin, whose malfunction is associated with Huntington's Disease, has several proline-rich motifs and interacts specifically with the Grb2 SH3 domains not associated with Sos, linking hungtingtin to the EGF receptor[65]. Hungtingtin is mainly associated with microtubules and appears to function in cytoskeletal anchoring.

4.4. Tumor angiogenesis

In order to grow beyond a relatively small size, primary tumors have to establish a blood supply. Tumor cells secrete several growth factors that induce the formation of new blood vessels (angiogenesis). Angiogenesis is an important step in the transition of the primary tumor to

malignancy. In addition to nourishing the tumor, newly formed vessels provide a means to disseminate metastatic cells. Recent findings describe angiogenesis at the primary tumor as a mosaic of tumor cells and endothelial cells, and this structure facilitates the shedding of cancer cells into systemic circulation.

Many of the pathways in which Grb2 is involved are important in angiogenesis and lymphangiogenesis. Several growth factors such as vascular endothelial growth factor (VEGF), angiopoietin-1, fibroblast growth factor 2 (FGF2) and HGF contribute to the development of new blood vessels in physiologic and pathological conditions, such as tumor angiogenesis. Several other angiogenic signaling pathways, including the ones driven by platelet-derived growth factor (PDGF), ALK and Eph also require Grb2 as a critical downstream effector. VEGF receptor 2 (VEGFR2/KDR) is phosphorylated in response to its ligand, VEGF-A, which is secreted by many tumor cells, leading to the direct recruitment of Grb2, Shc and Nck[66]. Grb2 interacts similarly with VEGF receptor 3 (FLT4L) as well as indirectly through interaction with Shc[67]. Consequently, Grb2 can mediate cell cycle progression via the Sos/Ras pathway and cell motility through activation of the Rac1/Rho pathway. The latter response appears to be sensitive to specific binding antagonists of the SH2 domain of Grb2[68], with reduction of cell invasion and inhibition of more complex processes, such as endothelial cell tubulogenesis and vasculogenesis in the chick chorioallantoic membrane (CAM). Other signaling pathways such as those driven by angiopoietin-1 and FGF-2 also stimulate angiogenesis through Grb2[69, 70]. HGF has been shown to enhance tumor angiogenesis and the interaction between tumor and endothelial cells, mostly by increasing endothelial cell expression of CD44[71] or integrin expression in cancer cells[72]. These events require the activation of multiple signaling for which Grb2 has been demonstrated to be a key intermediate[73, 74].

Grb2 SH2 domain binding to pY925 on FAK can also link ECM-integrin engagement to Ras-ERK pathway activation and increased production of VEGF in tumor cells, stimulating endothelial cell motility and survival[75]. Moreover, previous studies have demonstrated that hypoxia, likely to occur inside tumors, increases phosphorylation of FAK with consequent Grb2 binding[76]. This is in addition to the role of FAK in regulating endothelial cell adhesion and motility during angiogenesis.

4.5. Tumor cell dissemination

After surviving in the systemic circulation, tumor cells adhere to the vessel endothelium, extravasate and seed secondary sites. One of the best characterized models of extravasation is represented by the movement of leukocytes from the circulation to the site of tissue damage or infection, in response to cytokines released by macrophages in the affected tissue. These cytokines induce the expression of a particular class of adhesion molecule, selectins, while specific chemokines act as chemoattractants on circulating leukocytes[77]. Selectins mediate the adhesion of lymphocytes on the endothelial cell luminal surface with a series of low affinity binding sites, slowing down their speed to complete arrest. Studies of the intracellular signaling events upon L-selectin activation have identified Grb2 as an important adapter: Grb2 binds directly to L-selectin and triggers activation of Rac2[78]. In analogy to rolling leukocytes, cancer cells can use the same rolling mechanism to extravasate and lodge in the secondary site[79, 80]. In fact, tumor progression has been associated with increased expression of selectin ligands, and L-selectin can enhance the formation of metastasis to lymph nodes *in vivo*[81]. Endothelial cells in different tissues are not alike and can express different patterns of adhesion molecules; this may explain in part why tumor cells prefer specific organs, skipping organs that may be the most logical target based on vascular drainage from the primary tumor.

Integrins are also important mediators of cancer cell rolling adhesion and subsequent extravasation[77]. Integrins expressed on the surface of circulating tumor cells engage the surface of endothelial cells and this binding triggers association of the integrin cytoplasmic tail to the

intracellular cytoskeleton; consequently, tumor cells can change their shape and form pseudopodia for facilitating passage between endothelial cells. After successful extravasation, tumor cells must again activate ECM degradation, migrate to the metastatic site and proliferate again. Thus metastatic cells must switch from a migratory to a proliferative state, again modulating pathways in which Grb2 is a well known mediator.

5. The development of Grb2 inhibitors as anti-cancer drugs

5.1 Grb2 Silencing

The importance of Grb2 in several oncogenic signaling pathways has prompted significant efforts to selectively disrupt its intracellular interactions. Grb2 gene silencing using RNAi technology has been used to explore the role of this protein in signal transduction[82] and in receptor downregulation[83]. Nuclease-resistant antisense oligonucleotides directed against Grb2 mRNA inhibit the proliferation of Philadelphia-chromosome positive leukemic cells[84] and ErbB2 overexpressing breast cancer cells[85], reinforcing the importance of Grb2 in oncogenesis and enabling gene silencing as a potential anti-cancer strategy for this target.

5.2 Targeting the Grb2 SH2 domain

SH2 domains are well-recognized pharmaceutical targets[86]. The concept that disruption of Grb2 SH2 domain interactions can significantly inhibit Grb2 signaling is supported by the effects of the general transcriptional inhibitor actinomycin D, which has been shown to block Grb2 SH2 domain interaction with Shc and tumor cell cycle progression[87–89]. Natural products and derivatives that block Grb2 SH2 domain binding interactions have also been characterized[90]. Substantial efforts to develop Grb2 SH2 domain binding antagonists have also focused on structure-based approaches, building on the unique features of the Grb2 SH2 domain recognition motif[91, 92].

The Grb2 SH2 domain prefers ligands containing an asparagine residue at the second position carboxy-terminal to the phosphotyrosyl residue (pY+2) and it requires that ligands adopt β-bend configurations[93]. The structural basis for these requirements became clear when the crystal structures of the Src and Grb2 SH2 domains became available for comparison. This showed that while Src family SH2 domains bind phosphopeptides in extended sheet conformations, peptides binding to the Grb2 SH2 domain are forced into a β-bend conformation by the presence of a Trp-121 indole side chain that prevents the extended ligand form.

As a starting point for the synthesis of this class of binding antagonists, short peptides containing pYXN sequences from physiological binding targets of Grb2 have been used. Since the cytosolic environment has a high level of constitutive tyrosine phosphatase activity, a major challenge has been to confer hydrolytic stability to these phosphopeptides *in vivo.* Conferring phosphatase resistance by replacing the pY residue with phosphonomethyl phenylalanine (Pmp), or related structures[94], or by subsequent refinements using non-phosphate containing ligands[95], have been successful strategies (for a detailed review see[96]). Other phosphonate-based mimetics and their corresponding prodrugs have also shown activity in cell models[97]. Recently, Song et al.[98] reported the synthesis of a potent Grb2 SH2 domain inhibitor free of phosphotyrosine or any phosphotyrosyl mimetic, demonstrating that complete replacement of the pY residue can be accomplished without significant loss of binding affinity.

Systematic and stepwise substitution of the pYXN recognition motif and mimicking of the β-turn conformation has led to high affinity synthetic compounds capable of blocking RTK-Grb2 interactions in intact cells[99]. The development of potent peptidomimetic inhibitors of Grb2 SH2 domain binding has been aided by macrocyclization to stabilize the critical β-turn conformation. [For a detailed description of the chemical development of these inhibitors, see[86, 92]]. Non peptidic Grb2 SH2 domain antagonists have also been developed. Building on

the prior structure-based design of peptidomimetic ligands of the Grb2 SH2 domain[100], Caravatti et al. demonstrated that it is possible to design Grb2 SH2 domain antagonists devoid of any peptidic character[101].

Small, synthetic Grb2 SH2 domain-binding antagonists have been shown to potently block HGF-stimulated cell motility, matrix invasion, and branching morphogenesis in epithelial and hematopoietic target cell models[21]. Those compounds did not affect HGF-stimulated mitogenesis, implying that dependence on Grb2 for mitogenic signaling may be cell-type specific. The same compounds also inhibited the basic morphogenetic events required for angiogenesis, such as HGF, VEGF and bFGF-driven endothelial cell migration and invasion in a reconstituted extracellular matrix[68]. Moreover, inhibition of VEGF-stimulated angiogenesis in an *in vitro* human umbilical vein endothelial cell (HUVEC) cord formation assay, and suppression of vasculogenesis *in vivo* in the chick chorioallantoic (CAM) assay, further implicate Grb2 in pro-angiogenic pathways and suggest that its blockade may represent an effective anti-angiogenesis strategy[68].

Because Grb2 SH2 domain binding antagonists were not found to be universal inhibitors of cell proliferation, models of tumor metastasis were favored for further study of these compounds *in vivo*. Using a murine syngeneic melanoma cell line (B16-F1) in an experimental metastasis model (tail vein injection) and a human prostate adenocarcinoma cell line (PC3M) in a spontaneous metastasis model (xenograft transplant), in conjunction with bioluminescence technology, a significant reduction in metastatic burden was achieved using a prototypical Grb2 SH2 domain binding antagonist[22]. These results demonstrated that it is possible to specifically target the spread of solid tumors using small molecules and it implies a critical role for the Grb2 SH2 domain in this process.

Several important steps remain in the preclinical development of Grb2 SH2 domain binding antagonists as anti-cancer drugs. Establishing the selectivity of rationally designed compounds for the Grb2 SH2 domain is critical in avoiding potential toxicity due to antagonism of off-target SH2 domain-mediated interactions. Modification of compounds with chemical tags while maintaining biological activity has been used to create powerful tools for exploring target protein selectivity as well as other basic aspects of Grb2 signaling[102, 103]. The development of pharmacodynamic markers of drug action in intact animals represents another high priority. Global analysis of gene expression in the presence and absence of treatment with selective Grb2 SH2 domain binding antagonists to identify a molecular signature is a promising approach that was recently used to identify N-cadherin as a potential pharmacodynamic marker[22].

5.3 Targeting the Grb2 SH3 domains

SH3 domains are small modules that were identified 20 years ago[104, 105] as one of the first recognized modular protein domains. Unlike SH2 domains, SH3 and SH3-like domains appeared earlier in evolution and are ubiquitous in eukaryotes as well as prokaryotes. The structure of these modules is known in great detail. The ligand binding surface of SH3 domains is relatively flat and hydrophobic and consists of three pockets characterized by conserved aromatic residues. The ligand typically occupies two of these pockets with two hydrophobic prolines, while the third pocket frequently interacts with basic residues, in a so called polyproline-2 (PPII) conformation[106]. The basic residue is important for SH3 domain ligand orientation: when the basic residue is at the amino-terminal end the SH3 domain binds in an amino to carboxyl orientation, while the reverse orientation is preferred when the basic residue is at the ligand carboxyl-terminus. The same conformation is recognized by other proline-recognizing modules such as WW and profilin domains[106], and this similarity has been the subject of several studies to better understand the selectivity of SH3 domains for target proteins. The importance of conformation over sequence for SH3 domain ligand recognition is also gaining acceptance. Although SH3 domains have long been thought to bind preferentially to

proline rich sequences of the form PXXP in target proteins, several exceptions to this rule have been found[107]. The SH3 domains of Grb2 have been found to bind a RXXK core consensus motif[108].

The relatively low binding affinities reported for SH3/target protein interactions, together with the ability of SH3 domains from different proteins to recognize the same target, have raised questions regarding the basis for SH3 domain selectivities observed in intact cells as well as the feasibility of screening peptide libraries using SH3 domains to discover binding antagonists[109]. Nonetheless, progress has been made on both fronts[110]. For Grb2 in particular, SH3 domain target selectivity may be increased through two mechanisms, one intrinsic to the SH3 domains themselves and the other through their context in an SH2 domain containing protein. For example, both Grb2 SH3 domains may interact simultaneously with different sites on a single target molecule, e.g. SOS1, thereby increasing both the apparent affinity and selectivity of Grb2-target interaction[107]. Because the Grb2 SH2 domain restricts Grb2 subcellular localization, the pool of potential SH3 domain binding partners is also likely to be limited, further increasing their apparent selectivity.

The SH3 domains from several proteins have been used to screen short peptide libraries in search of binding antagonists. Peptides binding the SH3 domain containing adaptor protein Mona/Gads with high affinity have been identified and subsequent structural studies using one of these peptides revealed a novel type of peptide-SH3 domain interaction[111]. Similarly, screening for peptides binding the SH3 domains of the *C. elegans* Grb2 homolog Sem-5 yielded a bivalent peptide ligand with nanomolar affinity[112]. Pak1-derived peptides encompassing proline-rich sequences in that protein were found to specifically disrupt Grb2 SH3 domain-Pak1 interactions with relevant impact on growth factor mediated migration and lamellipodia formation[54]. Non-naturalamino acids analogs have been substituted at the proline-requiring site of Grb2 SH3 domain ligands[113]. These have provided peptides with nanomolar affinity and reinforced the concept that proline residues may be dispensable in the design of SH3 domain binding antagonists.

An adaptation of the target peptide screening approach in developing SH3 domain binding antagonists has been to systematically introduce point mutations in target peptide sequences. High affinity peptides capable of blocking the proliferation of primary blast cell cultures derived from patients with chronic myelogenous leukemia (CML) and Bcr/Abl positive cell lines have been developed using this strategy[114]. Subsequent modifications of these peptides to improve their ability to permeate cells yielded agents that more potently disrupted Grb2 signaling complexes in CML-derived cells[115]. These preclinical studies support the concept that Grb2 SH3 domain binding antagonists could provide a therapeutic alternative for CML patients developing resistance to standard treatments[116].

Enhancing the affinity and selectivity of artificial SH3 domain binding antagonists by exploiting the existence of two SH3 domains in Grb2, dimeric peptides with high affinity binding to both SH3 domains of Grb2 have been designed with the goal of disrupting Grb2-SOS1 interactions[117]. These “peptidimers” inhibited cell growth *in vitro* and displayed anti-tumor effects in xenograft models, and thus represent the first examples of *in vivo* activity for this class of compounds[118]. Introducing N-alkylated residues into both monomers of the peptidimer and optimizing the linker improved the affinity for Grb2 to the subnanomolar range[119]. Finally, non-peptidic small molecule inhibitors have also been explored. The first example is the Src signal transduction inhibitor UCS15A that disrupts several SH3 domain mediated interactions, including those of Grb2[120]. Although target selectivity remains to be improved, this and similar chemical structures may provide a platform for the development of small synthetic drugs that potently antagonize specific SH3 domain binding interactions.

Expert Opinion

Virtually all cancers develop as a consequence of abnormal cell signaling, and protein tyrosine phosphorylation controls several important oncogenic signaling pathways. Targeting tyrosine kinases has already demonstrated its utility in the clinical arena. As an alternative and complementary approach, targeting downstream signaling pathways is also logical. Hence, it is not surprising that in the last decade there has been a flourishing interest in better understanding and targeting signal transduction modulators, such as Grb2, as a strategy to develop novel cancer therapeutics. The importance of protein binding modules to complex cell signaling cascades such as those involved in motility and invasion is well known. For example, the SH2 and SH3 domains of the tyrosine kinase c-Src are required for facilitating cell spreading, while its catalytic activity is dispensable for this function[121]. Targeting adapter proteins by developing antagonists of well-characterized protein binding modules builds upon these important basic science advances.

The role of Grb2 as a signal transducer for several oncogenic growth factor receptors and the broad involvement of Grb2 in multiple steps of the metastatic cascade make it an excellent target for anti-tumor therapeutic strategies. Small, synthetic Grb2 SH2 domain binding antagonists have been developed that potently inhibit cell migration, invasion and angiogenesis, supporting their potential as anti-metastatic therapeutics. Innovative approaches, such as the incorporation of fluorophores[122] for tracking cellular localization and the incorporation of biotin[123] for the study of selectivity and the identification of drug-associated proteins, have enabled rapid characterization of the molecular mechanisms of action of these novel compounds in intact cells. Despite improvements in the design and effectiveness of this class of compounds, important questions such as bioavailability and long-term toxicity remain largely unanswered. Considering the widespread expression of Grb2 in tissues, the embryonic lethality of Grb2 gene deletion[11], and the central role of Grb2 in immune receptor signaling[124], detailed analysis of cellular and organ specific toxicity is an important future goal.

The rational design of drugs targeting specific molecules involved in oncogenesis and metastasis is still in its infancy. To address the problem of low efficacy for some single agents therapies, and to address complex processes such as metastasis, combinations of drugs acting at different signaling nodes and processes will probably be needed. For example, targeting multiple tyrosine kinase receptors has a proven advantage over single agent therapy and can overcome resistance to treatment and biological heterogeneity[125]. Hence, Grb2 SH2 and SH3 domain antagonists may become part of individualized combination of targeted therapies built on the molecular profiles of specific cancers. Further studies are needed to define the tumor-specific biology of Grb2 in diverse human cancers in order to determine where and when these inhibitors may have the best chance to be of benefit. Considering the fundamental role of adapters such as Grb2 in cell signaling and in the development of human cancer, we anticipate substantial growth in efforts to develop drugs that selectively target adapter proteins as a class. Such efforts are likely to be rewarded with improved cancer treatments and a deeper understanding of adapter protein function.

Acknowledgments

We would like to thank Georgia Shaw for her help in creating the artwork in figure 1. This research was supported by the Intramural Research Program of the NIH, National Cancer Institute, Center for Cancer Research.

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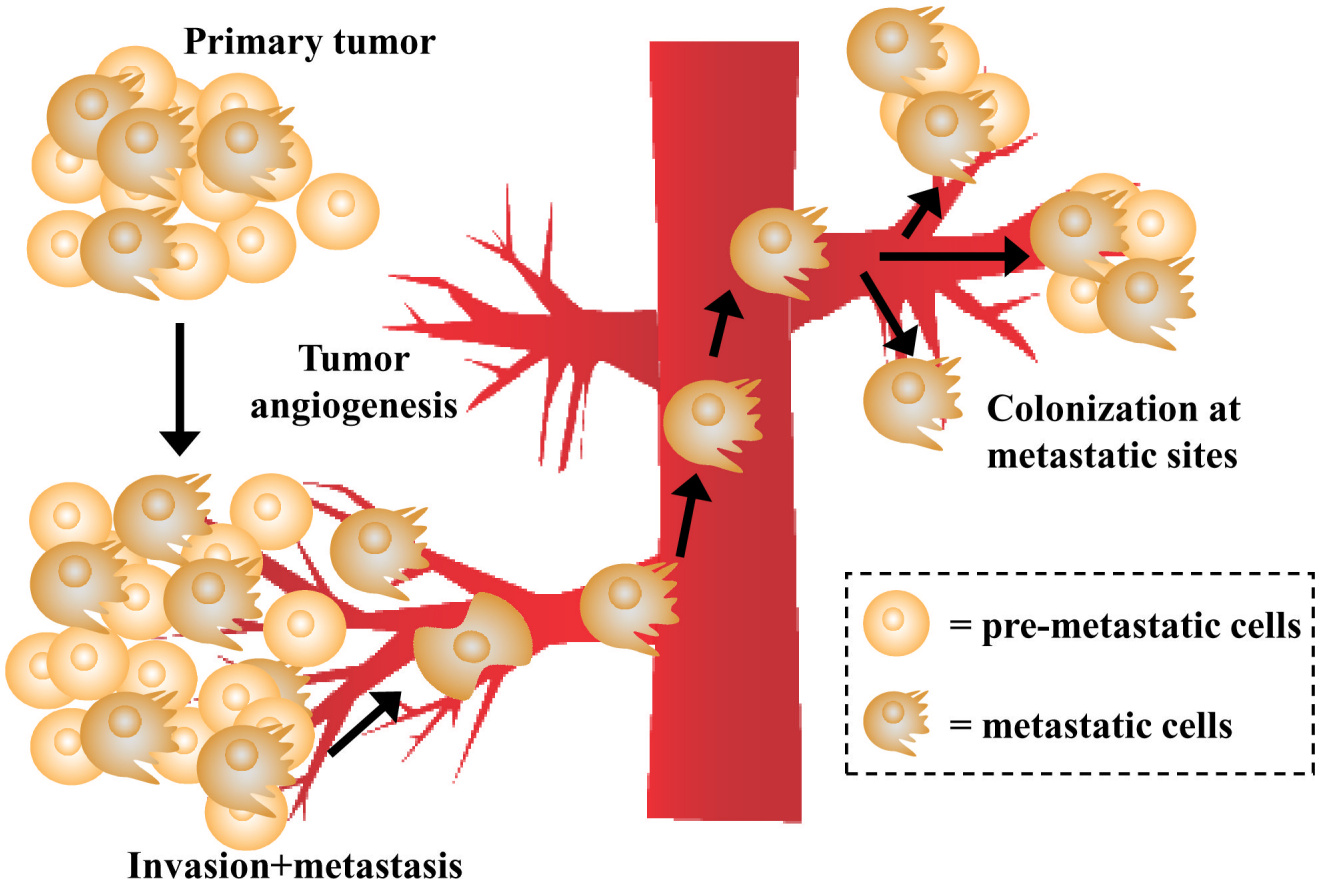

Figure 1. The role of Grb2 in the metastatic process

When primary tumors progress and establish a new blood supply, cells within the primary site detach from neighboring cells as an effect of the Integrin-FAK-Grb2-ERK2 signaling cascade. Invasion through surrounding tissue is preceded by abnormal activation of matrix metalloproteinases. Subsequently, tumor cells reorganize their cytoskeleton, extending lamellipodia and filopodia, and migrate; Grb2 interactions with several cytoskeletal proteins is crucial in this process. Several angiogenic signals rely on Grb2 in order to develop new blood vessels, either through growth factor receptor or integrin signaling. Tumor cells, after infiltrating and surviving in the systemic circulation, adhere to the endothelial cell luminal surface through cell membrane Selectins and extravasate. In the new environment, tumor cells can rapidly proliferate, or remain dormant until new signals stimulate their growth. Proliferation and invasiveness at the metastatic site is also supported by the Grb2-Sos-Ras-MAPK cascade.

Table 1

Reported interactions of Grb2 with cytoskeletal proteins.

Grb2 interacting protein	**Function**
ACTIN cytoskeleton	
Focal Adhesion Kinase (FAK)	Focal Adhesion formation
WASp and N-WASp	Transducers of signals from surface receptors to the actin cytoskeleton
p21-activated kinases (PAK)	Regulators of cell cycle progression and cell shape change
Magicin	Cytoskeletal organization and transcriptional repressor
Cortactin	Cytoskeletal and adherens-junction organization
Caldesmon	Regulates actin and calmodulin interaction
MICROTUBULE cytoskeleton	
Dynactin1	Dynein activator
MAP2	Stabilizes microtubule elongation
Huntingtin	Cytoskeletal anchoring to tubulin