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Grb7 – A Newly Emerging Target in Pancreatic Cancer

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1. Introduction

Growth factor receptors are transmembrane glycoproteins involved in many aspects of cell biology ranging from protein and nucleic acid synthesis, cell growth, differentiation and migration to ultimate death of cells (Kiel et al., 2010; Lemmon et al., 2010). On binding by growth factors, the receptors undergo dimerization and autophosphorylation (Burz et al., 2009; Prenzel et al., 2001). The receptor phosphorylation is in turn responsible for recruiting intracellular molecules so as to form a network of signalling complexes critical for the transfer of the signal to downstream events. One class of cytoplasmic proteins recruited in such a way is the growth factor receptor binding (Grb) proteins. As the name implies, Grb proteins were originally identified because of their ability to associate with growth factor receptors (Margolis et al., 1994). Characteristically, Grb proteins form supramolecular complexes with growth factor receptors essential for growth factor mediated signal transduction (Songyang et al., 1993, 1994), though interactions with non-growth factor receptors is also well documented (Margolis et al., 1994; Songyang et al., 1994). Currently 14 Grb proteins are identified, with several implicated in the genesis and development of human cancers (Margolis et al., 1994).

Growth factor receptor bound protein 7 (Grb7) belongs to a subfamily of Grb proteins comprising Grb7, growth factor receptor bound protein 10 (Grb10) (Frantz et al., 1997; Lim et al., 2004) and growth factor receptor bound protein 14 (Grb14) (Cariou et al., 2004; Holt et al 2005). The Grb7 family of adaptor proteins share high sequence and functional homology (Songyang et al., 1993; Holt et al 2005). The group was discovered using a technique dubbed CORT (cloning of receptor targets), an expression/cloning system that uses a tyrosine phosphorylated receptor as a probe to screen protein libraries (Margolis et al., 1992). Specifically, Grb7 was identified using CORT screening of a mouse cDNA expression library with tyrosine phosphorylated C-terminus of the epidermal growth factor receptor (Skolnik et al., 1991; Lowenstein et al.,1992). In common with other adaptor proteins, Grb7 facilitates the coupling of multiple transmembrane and cytoplasmic receptors to downstream effectors molecules (Margolis et al., 1994; Yokote et al., 1994). Grb7 has attracted particular attention since it was noticed to be massively overexpressed, along with EGFR2, in a number of cancers including pancreatic cancer (Stein et al., 1994; Tanaka et al., 1997).

1.1 Expression of Grb7

The human Grb7 gene is located on the positive strand of chromosome 17. Cytogenetic analysis shows that the gene is mapped to the 17q12-q21 loci, as documented by the National Centre for Biotechnology Information(NCBI) (http://www.ncbi.nlm.nih.gov/mapview/). The human Grb7 gene is 9, 352 nucleotides in length and known to encode a primary Grb7 RNA transcript of 4,596 nucleotides in size with the mature Grb7 mRNA known to be composed of 14 exons comprising 1,599 nucleotides. The chromosomal location of Grb7 is found within the erbB2 amplicon (Lucas-Fernández et al.,2008; Kauraniemi et al., 2007), a region known to comprise genes frequently over amplified in cancers (Mano et al.,2006). As will be described later, the localization of Grb7 on this amplicon appears to explain the occurrence of Grb7 over-expression. Fig. 1 shows the overall organization of Grb7 gene and its products.

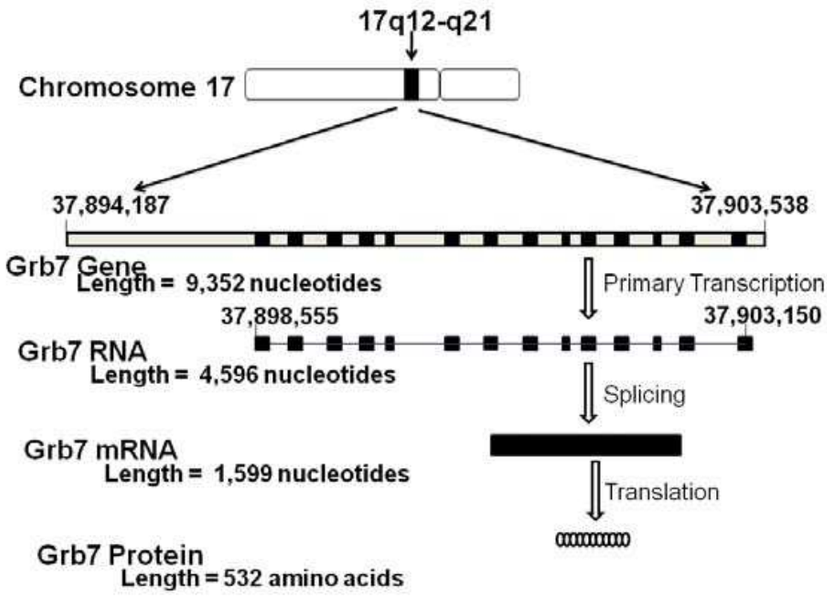

Fig. 1. The overall organization of Grb7 and its products. Grb7 is localized on the long arm of chromosome 17, at 17q12-q21. It is comprised of 14 exons, indicated by filled boxes. In Grb7 mRNA all the exons are merged and represented by single full filled rectangle. The numbers on top of each structure represent the start and end of a given nucleotide sequence. Grb7 amino acids are represented by a string of ellipses.

Grb7 protein displays a distinct expression profile across species, tissues and organs (Margolis et al., 1992). Under normal conditions it is expressed in human tissues including placenta, intestine, brain, lung, kidney, esophagus, mouth, prostate, mammary gland, uterus, ovary, cervix, liver, pancreas, testis, embryonic tissue, lymph node, trachea, larynx, bladder, thymus, skin, eye, ascites, stomach, pharynx and connective tissue [Unigene, www.ncbi.org]. Grb7 is an intracellular protein primarily found in the cytosol though it is localised to focal contacts, mitochondria and cell membrane under certain circumstances (Shen et al., 2004). In addition, Grb7 is found to be localized as an integral component of stress granules (Tsai et al., 2008). It is found to be conserved amongst mammals as the gene and its protein product are found in a number of mammalian species with high sequence

homology. Nonetheless, it is the over expression of Grb7 that is associated with a number of human maladies such as pancreatic and other cancers.

1.2 Grb7 as a mediator of multiple signalling pathways

Grb7 was initially identified as a binding partner of growth factor receptor (Margolis et al., 1992; Han et al., 2001). It has been shown in numerous studies to interact with a diverse spectrum of biomolecules since its initial identification. These include the various growth factor receptors, transmembrane receptor tyrosine kinases, cytoplasmic protein kinases (in particular focal adhesion kinase (FAK)), phosphatases, GTPases, ligases, adaptor proteins, caveolins, phosphoinositides and other biomolecules (Shen et al., 2004; Han et al., 2001; Daly et al., 1998; Holt et al., 2005). These binding partners are known to participate in a myriad of biochemical signalling in their own right. Noteably, while a large number of binding partners functioning upstream of Grb7 have been identified, the precise downstream events leading to Grb7 effects are not yet elucidated. The most recent data suggest that Grb7 is able to recruit RasGTPases leading to phosphorylation of ERK1/2 and cell proliferation (Chu *et al*., 2010). Another set of studies have identified interactions between Grb7, RNA and the RNA-binding protein HuR leading to the proposal that Grb7 can act at the level of translational regulation (Tsai *et al*., 2007, 2008).

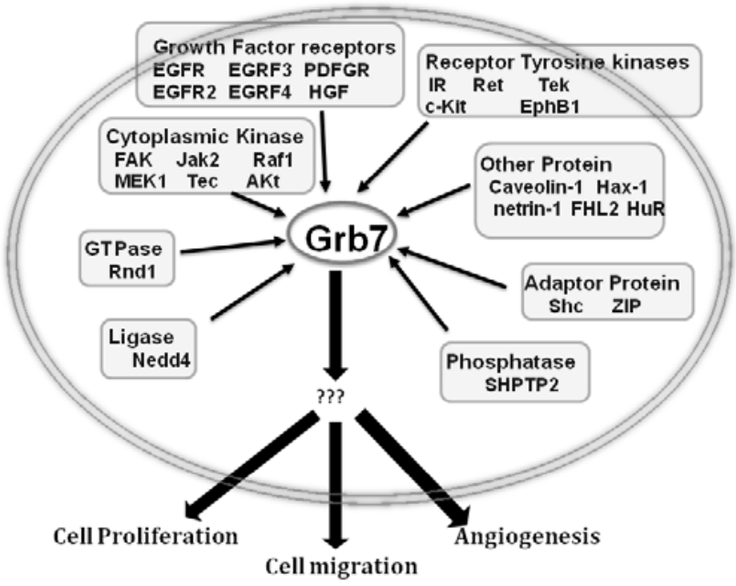

Fig. 2. Grb7 as a mediator of several signalling pathways.

Fig. 2 displays various identified binding partners of Grb7. Importantly, many upstream binding partners of Grb7 are connected with cancer cell properties (Pero et al., 2003; Golubovskaya et al., 2009). For example, the integrin pathway via FAK is important for cell migration (Golubovskaya et al., 2009 ; Zhao et al., 2009). In addition, various growth factor receptors are frequently implicated in growth and proliferation of cancer cells (Witsch et al,. 2010). Indeed there are clinically available anticancer drugs in use that act on EGFR2 such as Trastuzumab (Herceptin®)(Roy et al., 2009), Erlotinib(Tarceva®)(Kim et al., 2002), and Gefitinib(Iressa®)(Velcheti et al., 2010). Trastuzumab is a humanized monoclonal antibody

that binds to the extracellular domain of EGFR2 whereas Gefitinib and Erlotinib are a small molecule drugs binding to the ATP binding site of the intracellular kinase domain of the receptor. Apart from the above shown interaction partners of Grb7, a number of membrane bound macromolecules such as phosphoinositides are reported as important partners of Grb7 mediated signalling (Reiske et al., 2000).

2. Grb7 in pancreatic cancer

Grb7's overexpression in cancer cells has prompted investigation of its role in different properties of cancer cells such as migration, proliferation, invasion and metastasis. Other disease states related with cell development have also been at the centre of investigation. Pancreatic cancer is among the most aggressive and leading causes of cancer deaths worldwide (Bardeesy et al., 2002). The clinical relevance of Grb7 expression in pancreatic cancer was studied by Tanaka and co-workers with the application of immunohistochemical analysis (Tanaka et al., 2006). Through a comparative study of Grb7 overexpression in normal vs maliginant cells, they showed that Grb7 was expressed in 61% of pancreatic cancer cell lines as compared to non-cancerous samples. Furthermore, the study reported Grb7 and erbB2 genes co-amplification as high as 59% in the pancreatic tumour cells that overexpress Grb7 but not in those cells that did not over express Grb7. Furthermore, upregulation of Grb7 has also been separately reported in pancreatic cancer cell lines (Jonson et al., 2003).

Interestingly, Grb7 overexpression was noted to contribute to the migratory, proliferative, metastatic and invasive properties of pancreatic cancer cells. For example, in a study of patients with lymph node metastasis, Grb7 overexpression was noted in 67% of the studied cases, indicating a relationship between Grb7 level and metastatic potential of pancreatic tumours (Tanaka et al., 2006). In a cell motility assay, Grb7 over-expression was shown to directly correlate with the migratory potential of NIH 3T3 cell lines, particularly when it is phosphorylated by FAK (Han et al., 1999). In addition, the association of Grb7 with FAK is reportedly an important factor in the regulation of cell proliferation, and cancer cell growth indicating the crucial role of Grb7 in tumourigenesis. To further evaluate the role of Grb7 in mediating tumourigenesis, BrdU incorporation assay was conducted on A431 carcinoma cells to find out that the knockdown of Grb7 resulted in an evident inhibition of cell proliferation (Chu et al., 2009). A similar conclusion was reached with the use of siRNA to knockout Grb7 and assess its impact on pancreatic cancer cell migration by Tanaka et al. They showed that the use of siRNA to knock down Grb7 in pancreatic cell was associated with reduction of migratory potential of pancreatic cancer cell lines (Tanaka et al., 2006). In a recent experiment, Furuyuma and co-workers have examined the significance of FAK in pancreatic cancer formation to discover that FAK was expressed in up to 48 % of the studied cases and, importantly, its expression was found to relate to tumour size (Furuyama, et al., 2006). Since Grb7 is a binding partner of FAK and Grb7 over expression has been implicated in tumour size of other cancers, it might be the case that Grb7 has been co-implicated in the pancreatic tumour size. Genes on 17q12-q22 chromosomal region, which also includes the Grb7 locus, are noted to be amplified in some pancreatic tumours (Bashyam et al., 2005).

Finally, the druggability of Grb7 protein has been investigated by using a specific peptide inhibitor on different properties of cancer cells. With the use of cell migration experiments using a modified Boyden assay, a Grb7 peptide inhibitor was found to have a reduced the

migratory potential of a pancreatic cell line. This was specifically noted in pancreatic cancer cell lines that over expressed Grb7 (such as MiaPaca2 and PK8 cells), but was not found to reduce migration of other human pancreatic cancer cell lines that did not over express Grb7 (such as KLM1 cells) (Tanaka et al., 2006). Likewise, in an attempt to determine whether the Grb7 peptide inhibitor could arrest the metastasis of pancreatic cancer cells, Tanaka et al performed a peritoneal metastasis experiment to find out that treatment by the peptide resulted in a fewer peritoneal metastases of pancreas cancer cells as compared to the control. In addition, they report that the number as well as the total weight of tumour nodules per mouse was significantly reduced on treatment by the Grb7 selective peptide inhibitor.

3. Grb7 in other cancers

Apart from its role in pancreatic cancer, Grb7 has been extensively investigated as a target in a number of other human cancers including breast, gastric, hepatic, blood and testicular cancers. In breast cancer, for example, it is found to be over-expressed in a number of breast cancer cell lines. In particular, its co-over expression and co-amplification with ErbB2 is widely investigated (Shen et al., 2004). This might emanate from the fact that ErbB2 and Grb7 are found on the same chromosomal region at 17q12-q21, termed the erbB2 amplicon (Kauraniemi et al. 2006; Glynn et al., 2020). Moreover, Grb7 and ErbB2 form a functional association in growth factor dependent signalling (Holt et al., 2005) and are shown to synergistically enhance tumour formation. The mechanism of Grb7 dependent tumour formation as described in a recent paper (Chu et al., 2010) is proposed to involve Ras-GTPases which in turn promote phosphorylation of ERK1/2, thereby stimulating tumour growth. Moreover, co-overexpression of Grb7 and ErbB2 have also been associated with worse outcomes in some breast cancer subjects (Nadler et al., 2010). The fact that Grb7 is found within the core of the ErbB2 amplicon at 17q12 is what explains most of the co-implications in breast cancer. However, a study conducted to identify the contribution of co-amplified genes has demonstrated that Grb7 alone may be a factor in breast cancer carcinogenesis. With the use of RNA interference technology, it is has been shown that Grb7 knockout SKBR3 and BT474 breast cancer cell lines possessed decreased cell proliferation and cell-cycle progression (Kao et al., 2006). Furthermore, recent experiments involving siRNA have shown that removal of Grb7 by RNA-interference reduced the viability of BT474 xenograft cancer cells and increased the activity of the antitumour drug lapatinib (Nencioni et al., 2010).

Grb7 has also been identified as a culprit in other cancers. For example, in gastric cancer, over expression of Grb7 was found in up to 31% of esophageal carcinomas and that the over expression was shown to strongly correlate with extra mucosal invasive potential of gastric tumours (Tanaka et al., 1997). In a related study, more than 8-fold amplification and over expression of ErbB2 and Grb7 in primary gastric cancer cells was reported and the over expression was associated with the development of more aggressive gastric cancer phenotypes (Kishi et al., 1997). Similarly, up to 45% Grb7 overexpression was noted in some esophageal carcinomas as compared to normal mucosa (Tanaka et al., 2000) which was directly related with the development of lymph node metastases (Tanaka et al., 1997) suggesting that Grb7 overexpression is a major risk factor in such cancer populations. Itoh et al. showed that Grb7 overexpression was correlated with the level of FAK in Hep3B cells and that such over expression was a cause for invasive and metastatic potential exhibited by

the hepatocellular carcinoma (HCC) cell lines and that suppression of Grb7 expression delayed the onset of HCC tumour formation in mice (Itoh et al., 2007). Studies by Haran et al showed that Grb7 was not only over expressed but its expression correlated with the severity of the Chronic lymphocytic leukemia. They showed that up to 88 % of Grb7 expression was detected in Stage IV as compared to 18 % in the Stage I of leukemia (Haran et al., 2004). In other studies, mutations, copy number and expression levels of genes have shown Grb7 to be involved in the development of testicular germ cell tumours with up to a 63 % increase in Grb7 expression in primary tumour samples (McIntyre et al., 2005). These studies indicate the emergence of Grb7 as promising therapeutic target in a number of malignancies. The developments efforts made against Grb7 will be discussed below.

4. The molecular architecture of Grb7 protein

Human Grb7 protein is comprised of 532 amino acids. Its constituent residues are organized into a number of protein domains that serve different but complementary functions to the overall signalling role of Grb7 (Margolis et al., 1994). Grb7 domain components are well conserved across the species, and serve similar roles in different proteins (Han et al., 2001; Margolis et al., 1994). The modular structure of Grb7 is composed of a proline rich domain, a Ras-associating domain, a pleckstrin homology (PH) domain, Src homology 2 (SH2) domain and a BPS domain (between the PH and SH2 domains) (Filippakopoulos et al., 2009; Pawson, 1994). Fig. 3 illustrates the various domains of Grb7 together with the approximate amino acid residue bounds. The amino acid composition and the specific roles of each domain will be described in greater detail under each heading. .

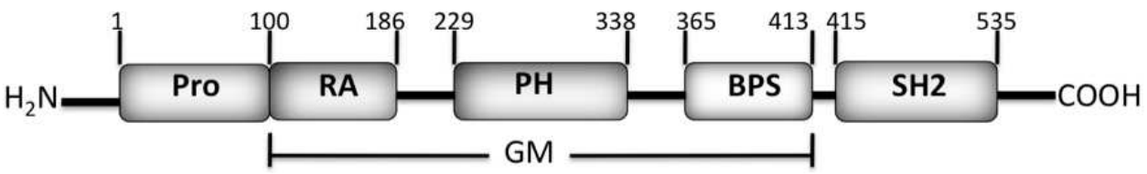

Fig. 3. The modular organization of Grb7 protein. Numbers indicate the residue number of the amino acids.

4.1 The N-terminal domain

The N-terminal domain of Grb7 comprises the first 100 residues of Grb7. It is a proline rich motif comprising a conserved sequence made of residues PS/AIPNPFPEL and is likely to exist as an unstructured domain in the absence of a binding partner. Up until now little has been known as to the binding partners of Grb7 via its N-terminal domain. Recently, however, experiments have indicated novel potential binding partners. For instance, mouse Grb7 has been shown to interact with HuR (Tsai et al., 2008), an RNA-binding protein important in regulation of nuclear-to–cytoplasmic shuttling of mRNA (Doller et al., 2008). The Grb7-HuR interaction is found to be mediated by the N-terminal domain of Grb7 (Tsai et al., 2008). Moreover, mouse Grb7 is also found to bind RNA via its proline rich N-terminal domain (Doller et al., 2008). These interactions have yet to be verified for human Grb7 and their physiological function remain to be elucidated.

4.2 The central GM region

The region of Grb7 bounded by the N- and C-terminal domains is what is referred to as the central GM, for Grb and Mig domain (Han et al., 2001). It consists of the longest stretch of Grb7 protein comprising about 300 amino acid residues. Its role in Grb7 signalling is much more studied and better known compared to the N-terminal domain. It characteristically displays more than 50% sequence similarity with the *Caenorhabditis elegans* protein Mig-10 (Manser et al., 1997; Ooi et al., 1995) from which it derives part of its name. Mig-10 (Migratory-10) is established to be critical for cell migration during embryogenesis (Manser et al., 1990). The presence of such a conserved sequence with known function lured researchers to investigate the role of Grb7 in cell migration, which predictably was proven to be the case. It is postulated that it is this domain that makes Grb7 an important cell migratory protein (Siamakpour-Reihani et al., 2009; Shen et al., 2002). The GM region is known to comprise three well conserved but non-contiguous domains: Pleckstrin homology (PH) domain, RA (Ras-associating) domain and a BPS (between PH and SH2) domain (Stein et al, 1994; Margolis et al., 1994).

The PH domain contains is a 110 amino acid long domain corresponding to residues 229-338 of Grb7. It is suggested to bind membrane bound phosphoinositides, thereby assisting Grb7's association to these molecules. Moreover, it is found to interact with FHL2, a signalling protein important in transcription regulation and cytoskeletal re-arrangement (Siamakpour-Reihani et al., 2009). Recently it is also reported to interact with Hax-1 (Hs-1 Associated protein X-1), another protein important in cell migration and apoptosis, and to explain a role for Grb7 dimerization in a head to tail manner (Siamakpour-Reihani et al., 2009, 2010). As a part of the GM region, it is proposed to play a role in cell migration

The RA domain of Grb7 is 87 amino acids long stretching from residues 100 to 186. Along with the PH domain, it is found to be important in the intramolecular dimerization of Grb7 by interacting with the SH2 domain, where the interaction is found to occur with micromolar affinity (Siamakpour-Reihani et al., 2010; Depetris et al., 2009). The phenomenon of dimerization is an important mechanism for the functioning of Grb7 (Porter et al., 2005). The RA domain is found in a number of proteins. It is also suggested to have a role in the involvement of Grb7 in Ras signalling pathway and for cell proliferation (Stein, et al, 2001). Together with the PH domain, the RA domain is reported to interact with the Hax-1 protein (Siamakpour-Reihani et al., 2010).

The BPS domain is a functional region of about 65 residues corresponding to residues 365-413 of Grb7. It is found between the PH and SH2 domain. The BPS region is thought to facilitate the interactions of SH2 domain to upstream partners of Grb7 (Stein, et al, 2001). Moreover, it is suggested the BPS domain could contribute to the specificity of Grb7 binding to its partners (Stein et al., 2003; Scharf et al., 2004). In the other Grb7 families such as Grb10 and Grb14, the BPS domain is found to interact with the activated IR and IGFR (He et al., 1998). It displays up to 60% sequence similarity among the Grb7 family members, The BPS domain is found to be intrinsically unstructured (Moncoq et al., 2003), though a very short structured stretch of about 9 residues was identified for Grb14 protein (Moncoq et al., 2004).

4.3 The C-terminal domain

By far the most widely investigated and thoroughly characterized region of Grb7 is the C-terminal Src homology 2 (SH2) domain (Pawson, 1994; Daly, 1997). It corresponds to residues 415-535 of the Grb7 protein. SH2 domains are phosphotyrosine peptide binding modules that are also found in a number of related proteins (Janes et al., 1997). Indeed, the discovery of Grb7 as an adaptor protein was dependent on this property of the SH2 domain. It is known to mediate the physical association Grb7 with a diverse array of membrane bound and cytoplasmic binding partners of Grb7 (Daly et al., 1998; Margolis et al., 1992). In particular, the SH2 domain is responsible for the recognition of specific phosphotyrosines (pTyr) residues via a well-described cationic pocket and surrounding peptide-binding cleft (Janes et al., 1997). The SH2 mediated association of Grb7 with its binding partners commences the first step in Grb7 dependent signal transduction. As such it forms an essential module for the variety of Grb7 mediated oncogenic transformations (Daly et al., 1998; Pero et al., 2003).

The binding specificity of the SH2 domain to upstream partners of Grb7 has been studied and characterized at length (Margolis et al., 1992;Daly et al., 1998). These studies have deciphered the sequence around the phosphorylated tyrosine recognized by Grb7 to be more or less conserved, from which a recognition motif of the sequence pYXN has been established. In other words, the presence of asparagine at a +2 position relative to phosphorylated tyrosine (pY) is what the SH2 domain of Grb7 specifically demands of its binding partners (Margolis et al., 1992; Daly et al., 1998). The position at +1 to the pY residue, indicated as X, is where any amino acid would be tolerated. This condition for recognition by SH2 domain is found in the great majority of established Grb7 binding partners including erbB2 (Stein et al., 1994) [60], Tek(Jones et al., 1999), c-Kit (Thömmes et al., 1999), SHPTP (Keegan et al., 1996), Shc(Frantz et al., 1997), PDGFR (Yokote et al., 1994). However, some exceptions has been noted where the +2 Asparagine is not required as in FAK (pYAE) (Han et al., 2001), EphB1(pYRD) (Han et al., 2002), cavoelin (pYRD)

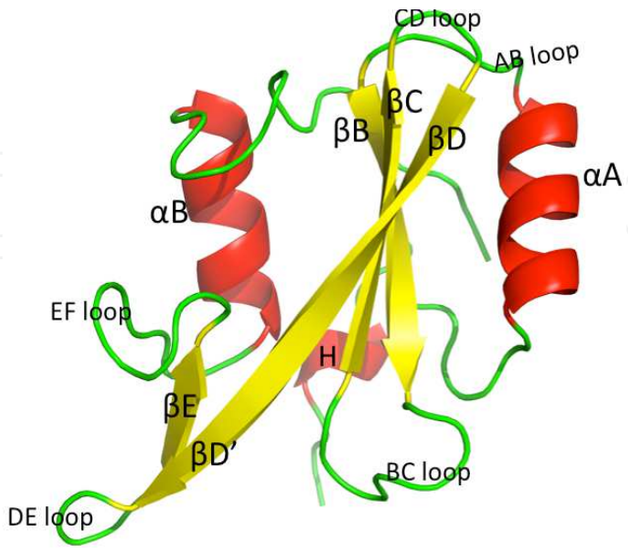

Fig. 4. Domain fold of Grb7 SH2 protein. Cartoon representation of the Grb7 SH2 domain shown as helix (red); B-sheet (yellow) and loops (green) and the structural motif labeling.

(Lee et al., 2000) or where the tyrosine does not have to be phosphorylated as in RndI(YDN)(Vayssière et al., 2000). Typically, the YXN recognition stretch is known to bind in a turn conformation as is established for a number of Grb2 antagonist peptides reported.

The experimental structure of Grb7 SH2 domain has been solved both by NMR and X-ray crystallography (Porter et al., 2007; Ivancic et al., 2003). The crystal structure is solved to 2.1 Å resolution with an overall tetrameric assembly by our group (PDB ID: 2QMS). As shown in Fig. 4, the Grb7 SH2 domain comprises two pairs of anti-parallel β-sheets flanked by a pair of α-helices (Porter et al., 2007). Such a structure is a general feature of SH2 domain proteins (Pawson, 1994; Margolis et al., 1994). According to the accepted nomenclature (Margolis et al., 1994), the central anti-parallel β-sheet is formed by the βB, βC and βD loops where as the two α-helices are labeled αA and αB (see Fig. 4 for details) which implies that the domain fold of Grb7 could be described as αAβBβCβDαB.

5. The development of Grb7 antagonists

Grb7 has become a promising target in pancreatic and other human cancers. Though Grb7 is a multidomain protein, most of the inhibitor development efforts are focused on the identification of agents that interact with its SH2 domain. This is because the SH2 domain commences the first and hence the fate determining step in the entire process of Grb7 dependent signalling (Holt et al., 2005). Moreover, the SH2 domain possesses a well defined and characterized binding pocket amenable to a variety of ligand design efforts (Porter et al., 2007). In addition, the requirement of the SH2 domain to bind to Grb7's myriad of upstream partners is generally conserved (Margolis et al., 1994; Han et al., 2001) where a minimal recognition motif is put forth. These factors endow the SH2 domain as an attractive module to target in the development of Grb7 based therapeutic agents. Hence all the Grb7 antagonists identified are specifically designed to act on the SH2 domain of the protein.

5.1 Polypeptide antagonists of Grb7

Inspired by the conservative motif requirement of Grb7 SH2 domain to bind its upstream binding partners, Pero et al conducted a PHAGE display experiment to identify the first polypeptide antagonist of Grb7 (Pero et al., 2002). The peptide was initially discovered as a 19 residue polypeptide flanked by Cys residues at positions 1 and 11. Interestingly, it was shown that the peptide was inactive in its open form and cyclization via disulphide formation was necessary for activity against Grb7. The original 19 residue structure was then simplified by removing residues outside the two Cys residues and the disulfide linkage was replaced with a thioether moiety to effect the ring closure. This 11 residue cyclic polypeptide, named G7-18NATE (sequence: WFEGYDNTFPC), was tested and proved to posses the same affinity as the larger disulfide containing form (Pero et al., 2002). An important attribute of this lead peptide is its selectivity for Grb7 and the fact that it is not phosphorylated. The chemical structure of G7-18NATE is displayed in Fig. 5.

The binding affinity of the G7-18NATE prototype peptide has been characterized extensively by isothermal titration calorimetry (Porter et al., 2007; Spuches et al., 2007; Ambaye et al., 2011a), surface plasmon resonance (Gunzburg et al., 2010) and ELISA assays (Luzy et al., 2008). Such investigations provide invaluable information that should guide the

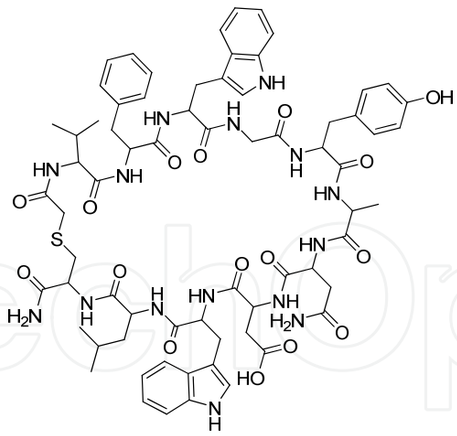

Fig. 5. Chemical structures of G7-18NATE lead polypeptide Grb7 antagonist.

further optimization of this lead polypeptide. The three ITC experiments concur on the affinity of the peptide for Grb7 SH2 domain (34.5 μM, 13.5 μM, 35.7 μM) indicating moderate affinity binding. Though this represents a breakthrough in Grb7 antagonist development, the affinity is not sufficient for animal experimentation. In other words, further optimization is necessary to transform the peptide into a clinical candidate. An important clue in this regard is afforded by isothermal titration calorimetry where deconvolution of the binding affinity into its components shows that the binding of G7-18NATE is enthalpically driven and entropically forbidden. This appears in line with the observation that the open form of G7-18NATE is devoid of any antagonistic activity (Pero et al., 2002). This knowledge could help in optimizing the lead peptide structure so as to improve the affinity.

Grb7 is an intracellular protein. Since G7-18NATE is a polypeptide, the plasma membrane represent a potential obstacle for it use in cellular systems. However, this has been overcome by the use of other peptides known to assist in crossing biological membranes. For this purpose, G7-18NATE was synthesized with a 19 residue long cell-penetrating sequence termed Penetratin for in vivo studies (see Fig. 6). The cell proliferation and migration inhibition assay conducted with this cell permeable derivative (G7-18NATE-Penetratin) demonstrate the combined effect of membrane crossing (Penetratin) and Grb7 inhibition (G7-18NATE) (Tanaka et al., 2006; Pero et al., 2007). Another cell-penetrating peptide with an 11 residue arginine rich sequence was also investigated for the cell permeablising effect (Pero et al., 2007). Both peptides were shown to have a synergistic effect with Doxorubicin in decreasing cancer cell proliferation. A related experiment conducted on pancreatic cancer cell migration effect clearly established the potential of G7-18NATE in diverse cancer cell lines (Tanaka et al., 2006). Our experience with G7-18NATE is that the length of the Penetratin tail can be cut short and still enter cells (Ambaye et al., 2011a). A Penetratin sequence consisting of only the last 8 residues was sufficient for membrane translocation of G7-18NATE. The cytoplasmic localization of G7-18NATE with this short penetratin was also confirmed (Ambaye et al., 2011a). Moreover, the possible interference on binding of G7-18NATE by the short Penetratin sequence was investigated by ITC and shown not to impede G7-18NATE binding. These experiments demonstrate that comparable in vitro-in vivo correlations can be achieved with the use of a shorter Penetratin.

Most recently the structure of the G7-18NATE peptide was determined in complex with the Grb7-SH2 domain using X-ray crystallography (Ambaye et al., 2011c). This revealed the

critical residues involved in binding the Grb7-SH2 domain, and their conformational arrangement. The same study also reported other phage-display derived Grb7-SH2 binding peptides with similar binding affinities for Grb7-SH2 domain as the lead G7-18NATE. These peptides all possessed the amino acid residues shown by the structure to be critical for binding by the structural study. This information will help to guide the design of future peptides with improved affinity and maintained specificity for Grb7.

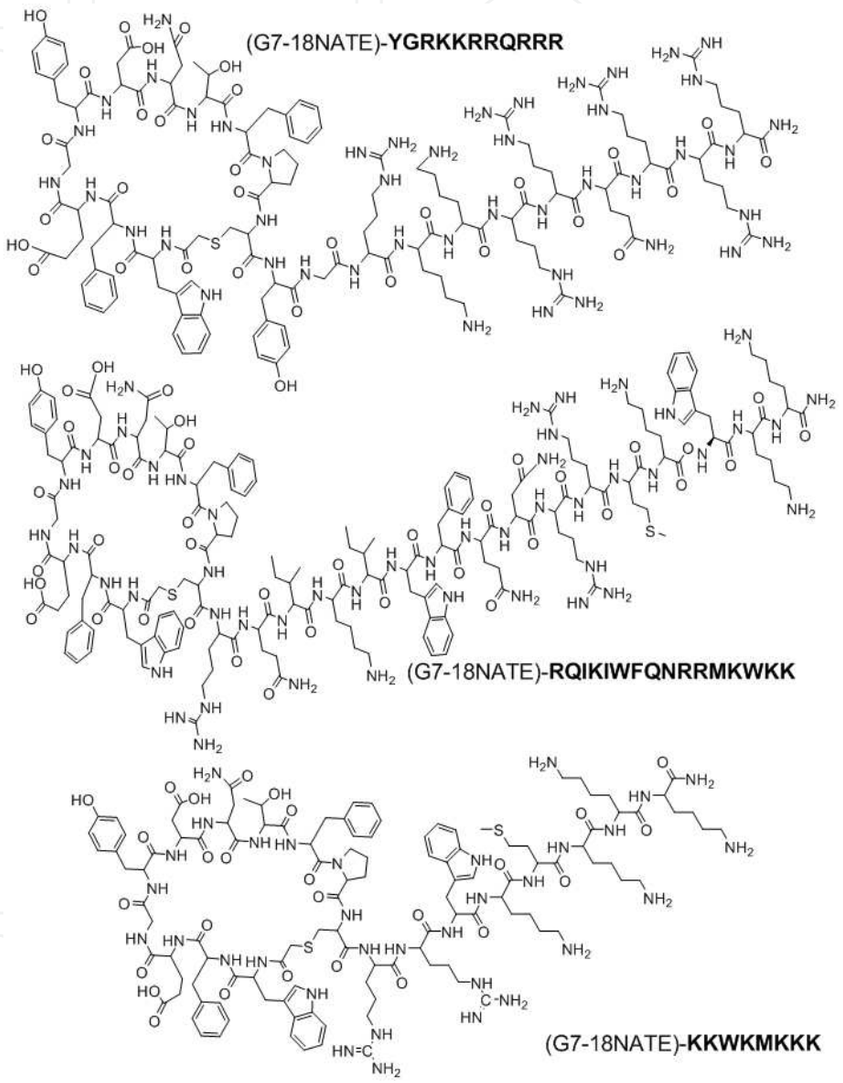

Fig. 6. Sequence and Chemical Structures of cell permeable G7-18NATE derivatives (Pero et al., 2007; Tanaka et al., 2007). One letter amino acid symbols in the sequence is indicated in bold fonts.

5.2 Short peptide antagonists of Grb7

Other short phosphorylated peptides based on the consensus recognition motif have also been developed and tested [Howl et al., 2007), see Fig.7. This includes peptides based on the sequence around the phosphotyrosine residue of erbB1, erbB2, erbB3 and ephB1 on Grb7

upstream binding partners. The phosphorylated peptides range from 6 to 11 residues with a dissociation equilibrium constant varying from 0.6 μM to 366 μM. Finally, peptides that were previously reported as Grb2 antagonists were tested for their inhibitory effect on Grb7. Interestingly, the results show that not only do the peptides retain the Grb7 inhibitory effect, but that the activity rank is maintained on both Grb2 and Grb7 antagonism, though a quantitative difference is observed (Spuches et al., 2007). These peptides, unlike G7-18NATE, do not show selectivity for Grb7.

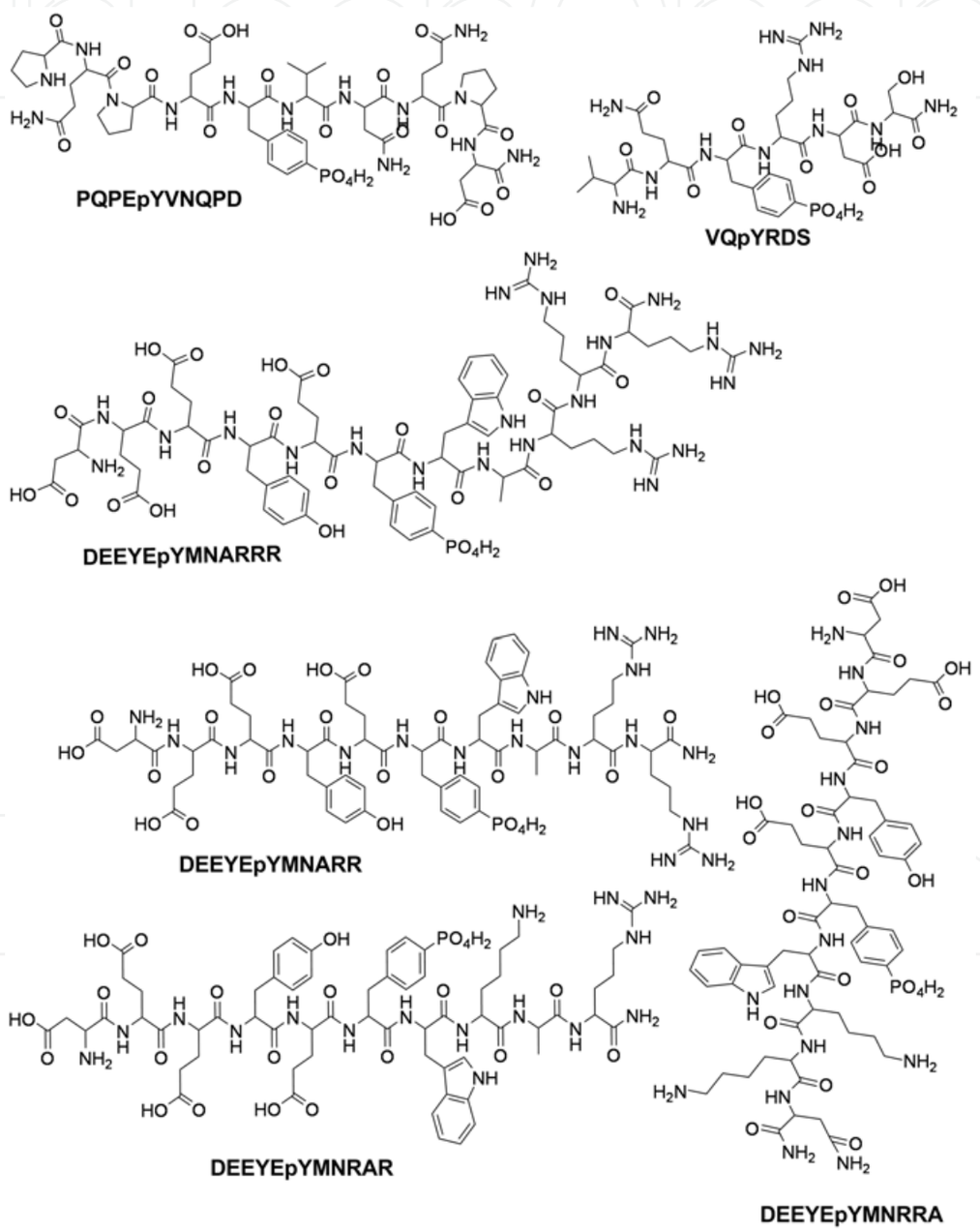

Fig. 7. Sequence and chemical structure of phosphorylated peptide antagonists of Grb7 (Howl et al., 2007). One letter amino acid symbols in the sequence is indicated in bold fonts.

5.3 Small molecule antagonists of Grb7

Recently, we embarked upon identifying small molecule antagonists of Grb7 (Ambaye et al., 2011b). In particular, the availability of the structure of peptides bound to SH2 domains has allowed us to apply series of computational chemistry approaches to idenitify potential antagonists of Grb7. Fig. 8 shows the structures of the most potent antagonists. The binding activity was examined first with ThermoFluor based denaturation followed by full thermodynamic characterization by isothermal titration calorimetry. This correlated with growth inhibition of Grb7 oversexpressing cancer cells. The result indicates near equivalent micromolar affinity values indicating the potential of non-peptide structures in cell based experiments. Fig. 8 show the benzopyrazine based antagonists of Grb7.

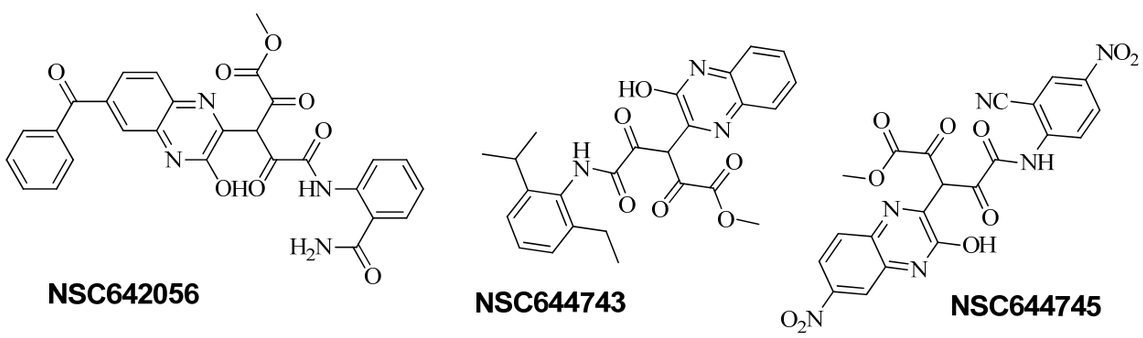

Fig. 8. Small molecule inhibitors of Grb7 (Ambaye et al., 2011b).

6. Conclusion and future outlook

Pancreatic cancer remains one of the leading causes of morbidity and mortality with the impact expected to rise in our aging societies. The limited efficacy and intolerable toxicity of available treatments means novel drugs with a novel mechanism of action are always sought. From the clinical standpoint, new targets provide novel drugs, novel mechanistic bases and potentially more efficacious means to treat diseases. Given its established role in malignancies such as pancreatic cancer and the fact that there is no drug that acts on it, Grb7 based drug development is likely to be a promising endeavour in the foreseeable future.

Grb7 is found a diverse array of signalling events critical for carcinogenetic transformation of human cells. Its druggablity is proven with the use of synthetic peptides, and an excellent start is made with the discovery of a peptide with specificity for Grb7. The major challenge for the use of peptide based drugs, however, comes from its intracellular localization. The recent development of cell permeable Grb7 antagonists is encouraging, and suggests that the permeability issue could be surmounted with little extra effort. Remaining issues to be

solved, however, include the need for a higher affinity peptide and the generally poor stability and metabolism of peptide-based drugs. Though several challenges are still ahead, the data obtained so far seem strongly encouraging to pursue Grb7 based anti-tumour drug development.

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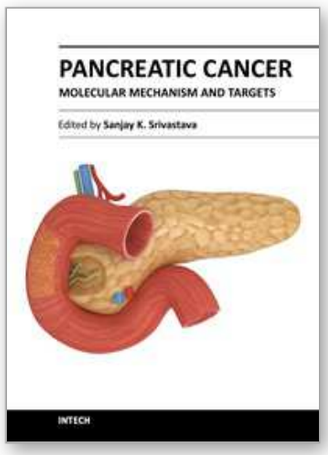

Pancreatic Cancer - Molecular Mechanism and Targets

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This book provides the reader with an overall understanding of the biology of pancreatic cancer, hereditary, complex signaling pathways and alternative therapies. The book explains nutrigenomics and epigenetics mechanisms such as DNA methylation, which may explain the etiology or progression of pancreatic cancer. Book also summarizes the molecular control of oncogenic pathways such as K-Ras and KLF4. Since pancreatic cancer metastasizes to vital organs resulting in poor prognosis, special emphasis is given to the mechanism of tumor cell invasion and metastasis. Role of nitric oxide and Syk kinase in tumor metastasis is discussed in detail. Prevention strategies for pancreatic cancer are also described. The molecular mechanisms of the anti-cancer effects of curcumin, benzyl isothiocyante and vitamin D are discussed in detail. Furthermore, this book covers the basic mechanisms of resistance of pancreatic cancer to chemotherapy drugs such as gemcitabine and 5-flourouracil.

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