International Doctorate Program in Molecular Oncology and Endocrinology
Doctorate School in Molecular Medicine

XIX cycle - 2003–2007
Coordinator: Prof. Giancarlo Vecchio

"INTERACTION BETWEEN THE INSULIN-LIKE GROWTH FACTOR 1 RECEPTOR AND PDK1 AS POTENTIAL THERAPEUTIC TARGET IN NEOPLASTIC CELLS"

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“Interaction between the Insulin-like Growth Factor 1 Receptor and PDK1 as potential therapeutic target in neoplastic cells ”

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LIST OF PUBLICATIONS

This dissertation is based upon the following publications:

1. Fiory F, Oriente F, Miele C, Romano C, Trencia A, **Alberobello AT**, Esposito I, Valentino R, Beguinot F, Formisano P. Protein kinase C-zeta and protein kinase B regulate distinct steps of insulin endocytosis and intracellular sorting. J Biol Chem 2004;279:11137-11145.

2. Fiory F, **Alberobello AT**, Miele C, Oriente F, Esposito I, Corbo V, Ruvo M, Tizzano B, Rasmussen TE, Gammeltoft S, Formisano P, Beguinot F. Tyrosine phosphorylation of phosphoinositide-dependent kinase 1 by the insulin receptor is necessary for insulin metabolic signaling. Mol Cell Biol 2005;25(24):10803-14.

3. Perfetti A, Oriente F, Iovino S, **Alberobello AT**, Barbagallo APM, Esposito I, Fiory F, Teperino R, Ungaro P, Miele C, Formisano P, Begiunot F. Phorbol esters induce intracellular accumalation of the antiapoptotic protein PED/PEA-15 by preventing ubiquitinylation and proteasomal degradation. J Biol Chem 2007;282(12):8648-57.

ABSTRACT

“INTERACTION BETWEEN THE INSULIN-LIKE GROWTH FACTOR 1 RECEPTOR AND PDK1 AS POTENTIAL THERAPEUTIC TARGET IN NEOPLASTIC CELLS”

The insulin-like growth factors (IGFs) play a crucial role in promoting cell survival, proliferation and facilitating invasion and metastasis. The insulin - like growth factor-1 receptor (IGF-1R) is the signalling receptor for both IGF-1 and IGF-2 ligands and is overexpressed in several human cancers. Recent epidemiological studies have shown that high concentration of serum IGFs are associated with increasing risk for several cancers. Therefore, IGF system represents an attractive antitumor target. Binding of IGFs to IGF-IR activates receptor tyrosine kinase activity, which activates a number of molecules involved in the Phosphatidylinositol 3-Kinases PI3-K signal transduction pathway. Phosphoinositides-Dependent Kinase-1 (PDK1) is a central molecule of PI3K pathway involved in cell survival and proliferation. In this study I demonstrated that IGF-1 induces tyrosine phosphorylation of PDK1 and co-precipitation with IGF-1R in MCF-7 cells. “Pull down” assays, performed with recombinant GST-PDK1 fusion protein and several PDK1 deletion mutants, reveled that PDK1 binds IGF1-R *in vitro*, and the region comprising aa 51-359 of PDK1 is necessary for this interaction. In cell-free assays, synthetic peptides corresponding to the IGF-1R C-terminus (aminoacids 1325-1367 [C43], 1346-1367 [C23] and similar peptides featuring tyrosine replacement with phenylalanine (Y→F peptide) [C43F and C23F]) are able to displace this interaction and to reduce PDK1 tyrosine phosphorylation by activated IGF-1R. The C43 peptide,which displays the stronger efficiency to compete IGF1-R/PDK1 interaction, has been used in further studies.Loading of (FITC) C43 peptide into MCF-7 cells reduces PDK1/IGF-1R interaction and activation of downstream substrates of PDK1 (Akt/PKB and PKCζ). Moreover, cell cycle analysis by flow cytometry reveled that C43 peptide, interfering with PDK1/IGF-1R interaction, is able to revert the protective effect of IGF-1 on cell death and to potentiate the effect of cell cycle arrest induced by campotothecin.

BACKGROUND

Cell cycle progression and cellular proliferation are regulated by a complex network of intrinsic factors and external stimuli. The very early events that rescue cells from cell cycle arrest are mediated through signals transmitted by a group of peptides, collectively known as growth factors (Cross M et al. 1991).

These molecules can be classified into two subgroups, namely the "competence" factors, such as the platelet- derived growth factor that enable cells to enter into the G1 phase, and the "progression" factors, such as the insulin-like growth factors (IGFs) that are required for progression from G1 into the S phase and, ultimately, cell division (Pardee et al. 1989).

Overexpression of growth factors and/or their receptors and expression of constitutively activated receptor are common events in malignancy and provide the underlying mechanisms for one of the hallmarks of cancer, namely uncontrolled proliferation (Hanahan et al. 2000).

Overexpression of growth factor receptors in the absence of up regulated ligand expression may, on the other hand, heighten tumor cell sensitivity and response to the appropriate ligand(s) and, thereby, regulate their growth in different microenvironments (Samani et al. 2007).

Tumor cells exhibit abnormal cellular activity, maintained by various peptide growth factors and among these, the IGFs play a crucial role in regulating cell proliferation and inhibiting apoptosis (Wu et al. 2003).

The IGF system

The IGF-1 system is important for normal human growth and development and is involved in the specialized functions of most physiologic systems (LeRoith et al. 1995).

IGF proteins regulate cell proliferation in an interconnected action via autocrine, paracrine and endocrine regulatory mechanisms. Consequently, any perturbation in each level of the IGF signaling proteins has been shown to be implicated in development and progression of numerous cancer types (Pavelic et al. 2007).

The IGF system consists of two ligands, IGF-I and IGF-II; three cell-membrane receptors, IGF-I receptor (IGF-IR), insulin receptor (IR), and IGF-II receptor (IGF-IIR); and six high-affinity IGF binding proteins, IGFBP-1 through -6 (Hasssan et al. 2002).

Most members of the IGF system are expressed by different cancer cells and may play an important role in the propagation of these malignancies (Leroith et al. 1995).

It was demonstrated that IGFs may enhance in vivo tumor cell formation, growth, and even metastasis and may reach tumors either from the circulation (endocrine) or as a result of local production by the tumor itself (autocrine) or by adjacent stromal tissue (paracrine) (Fig 1).

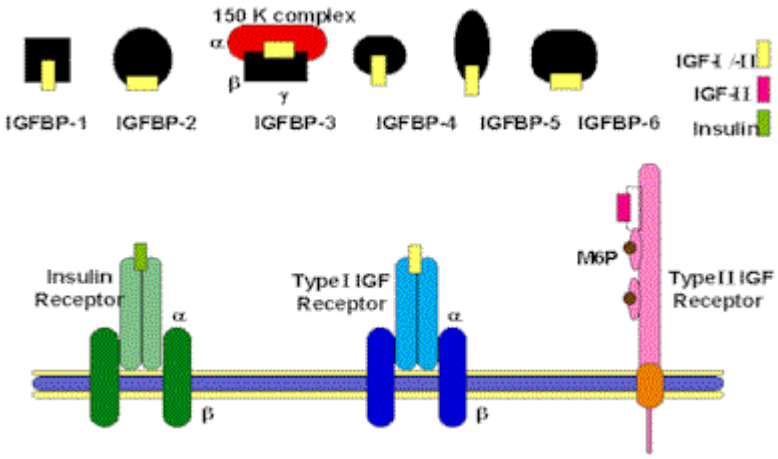

Fig 1: The IGFs, their receptos and their binding proteins.

IGFs

The IGFs are members of well-characterized family of insulin-related peptides that includes insulin, IGF-1 and IGF-2 (Wu et al. 2003).

IGF-1 and IGF-2 are small, single chain polypeptides synthesised as pre-pro molecules in a manner similar to insulin (Hasssan et al. 2002).

IGF-1 and IGF-2 share a 62% homology in amino acid sequence, and there is a 40% homology between the IGFs and proinsulin (Samani et al. 2007).

However, unlike insulin, IGF-1 and IGF-2 are expressed mainly from the liver during adult life and IGF-1 mediates most of the growth effect of growth hormone (Hasssan et al. 2002).

They are each encoded by single genes: the IGF-1 gene is located on chromosome 12 and the IGF-II gene is located on chromosome 11 (Yu et al. 2000).

IGF-1 is a trophic factor that circulates at high levels in the blood stream. Although the main source of IGF-1 in the serum is liver, many other tissues synthesize it and are sensitive to its action, especially during postnatal development (Pavelic et al. 2007).

Regulation of hepatic IGF-1 production is mostly mediated by growth hormone and insulin (Pavelic et al. 2007).

Growth hormone (GH) has a dominant role in upregulating IGF1 gene expression, but its stimulatory influence is markedly reduced by malnutrition. GH, in turn, is produced by the pituitary gland under the regulation of the hypothalamic factors somatostatin and growthhormone- releasing hormone (GHRH) (Pollak et al. 2004) (Fig. 2).

In turn, IGF-1 feeds back to suppress growth hormone and insulin release. In addition to growth hormone, developmental factors as well as nutrition status all modify IGF-1 production (Pavelic et al. 2007).

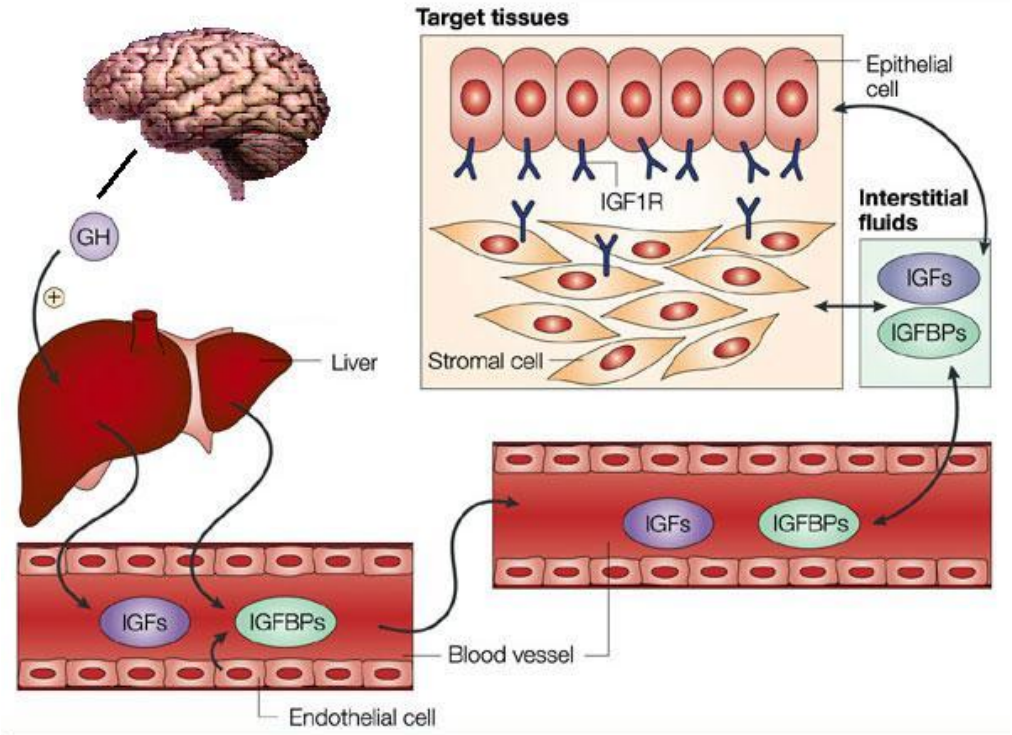

Fig 2: Regulation of circulating and tissue levels of insulin-like growth factors.
Most circulating insulin-like growth factors are produced in the liver. Hepatic IGF1 production is subject to complex regulation by hormonal and nutritional factors. Growth hormone (GH), which is produced in the pituitary gland under control of the hypothalamic factors growth-hormone-releasing hormone (GHRH) and somatostatin (SMS), is a key stimulator of IGF1 production. Various IGF-binding proteins (IGFBPs) are also produced in the liver. In IGF-responsive tissues, the ligands IGF1 and IGF2 as well as IGFBPs can be delivered through the circulation from the liver (an 'endocrine' source), but IGFs and IGFBPs can also be locally produced through autocrine or paracrine mechanisms. These mechanisms often involve interactions between stromal- and epithelial-cell subpopulations.

Total deletion of the IGF-1 results primarily in growth retardation and there are increasing evidence suggests that IGF-1 plays a role in glucose homeostasis, lipolysis, proteolysis and protein oxidation. To assess the role of circulating IGF-1 in metabolism, mice lacking of IGF-1 gene specifically in liver were generated (live IGF-1-deficient (LID) mice). These mice exhibit extremely low levels of circulating IGF-1 and high levels of growth hormone (GH). Interestingly, the LID mice showed hyperinsulinemia, which was associated with muscle insulin insensitivity. It was demonstrated that circulating low levels of IGF-1 that cause GH hypersecretion can affect insulin action in vivo. It was suggest that IGF-1 plays an important role in the hormonal balance between GH and insulin. This model provides solid evidence of the importance of the interrelations between IGF-1, GH, and insulin in maintaining normal glucose metabolism (Yakar et al. 2001).

IGF-I is a potent mitogen for a wide variety of cells and exerts its mitogenic action by increasing DNA synthesis and by stimulating the expression of cyclin D1, which accelerates progression of the cell cycle from G1 to S phase (Yu et al. 2000).

Complementary to their effects on cell proliferation, the IGFs can also inhibit cell death. The IGFs induce differentiation and stimulate differentiated functions in several cell types.

The synthesis of IGF-2 is relatively growth hormone independent. Its expression is much higher during foetal development than in postnatal life. It acts as a regulatory peptide; it is mitogenic for a number of cell types (Pavelic et al. 2007).

Genetic knock-out studies of the gene coding for IGF-2 in mice results in proportional growth retardation, suggesting that IGF-2 is principally an embryonic growth promoter (Hasssan et al. 2002).

The IGF-2 gene is transcribed from four different promoters (P1-P4). P2-P4 contains CpG islands, and transcription from these promoters is subject to imprinting (Pavelic et al. 2007).

Loss of imprinting is the most frequent mechanism for IGF-2 over-expression, as IGF-2 is normally expressed from the paternal allele only (Grimberg 2003).

Nevertheless, expression of IGFs is also influenced by various hormones, including estrogens, adrenocorticotropic hormone, thyrotropin, luteinizing hormone, follicle- stimulating hormone (FSH), and human chorionic gonadotropin, as well as by other growth factors, such as plateletderived growth factor (PDGF), epidermal growth factor (EGF), and fibroblast growth factor (FGF) (Yu et al. 2000).

Both ligands bind a group of at least six binding proteins, present in molar excess in the circulation and in tissues, which appear to control systemic and local bioavailability. IGFBP maintains IGF half-life in the circulation and extra-cellular space, and may have specific functions in the delivery of IGFs to signalling receptors at the cell surface.

Insulin-like growth factor receptors

The biological effects of IGF-1 and IGF-2 on a target cell are mediated by two types of cell surface receptors: IGF receptor of type 1 (IGF-1R) and IGF receptor of type 2 (IGF-2R) as well as through binding receptors for insulin (IR) (Fig. 3). IGF-1 binds to the type 1 receptor, and with a lower affinity to insulin receptor. IGF-2 binds with high affinity to the type 2 receptor and with low affinity to the type 1 receptor but it has no affinity for the insulin receptor. In general, most of the action of IGFs is mediated via IGF1-R (Pavelic et al. 2007).

Both IGF-1R and IGF-2R are glycoproteins and are located on the cell membrane. The two receptors, however, differ completely in structure and function (LeRoith et al. 1995, Stewart et al. 1996).

IGF1-R

IGF1R belongs to the receptor-tyrosine kinase (RTK) family (Li and Miller 2006).

Structurally, IGF-1R resembles the insulin receptor, and there is 60% homology between them (Herbert et al. 2000).

IGF-1R is expressed by all cell types with the exception of hepatocytes and T lymphocytes (Werner et al. 1991, Baserga et al. 1998).

The functional IGF-IR is a heterotetramer consisting of two identical α- and β-subunits, IGF-IR α and IGF-IR β, respectively, that are generated by proteolysis and glycosylation of the $\alpha\beta$ precursor encoded by a single gene (Lin et al. 2006).

The α- and β-subunits are linked by disulfide bonds to form αβ-half-receptor, which, in turn, is subsequently linked to an other αβ-half-receptor (by disulfide bonds between the α-subunit) to form the mature α2β2-holoreceptor (Jones and Clemmons 1995)

The *α*-subunits are entirely extracellular and are involved in ligand binding, ligand binding specificity is conferred by the cystein-rich regions of the α subunit (Keyhanfar et al. 2007).

Within the β-subunit, three major domains have been recognized: a juxtamembrane part, a tyrosine kinase domain and the C-terminus, each containing residues essential for different IGF-1R function (Surmacz 2000).

Ligand binding to the extracellular receptor triggers autophosphorylation of the β subunits and stimulates the tyrosine kinase activity. This sequence of events involves a conformational change in the catalytic loop domain of the tyrosine kinase region, binding of ATP to residue lys1003, and phosphorylation of residues tyr1131, tyr1135, and tyr1136. Each β subunit then transphosphorylates the other, leading to phosphorylation of a number of other tyrosines including, but not limited to, tyr950 in the juxtamembrane region, tyr1250, tyr1251 and tyr1316 in the carboxyl-terminal domain of the β subunit. Substitution of phenylalanine for tyrosine in each of these residues has resulted in a loss of function (LeRoith 2000).

Binding of IGF-I to the IGF-IR activates the tyrosine kinase of the receptor, which in turn triggers a cascade of interactions among a number of molecules involved in signal transduction (Lin et al. 2006).

IGF-IR activation causes rapid tyrosine phosphorylation of IRS-1 and -2 and intracytoplasmic assembly of a complex consisting of a variety of proteins that are responsible for stimulating diverse downstream signal transduction pathways (Jenkins and Bustin 2004).

The two main signalling pathways involve the PI-3K which promote suppression of apoptosis and the p21 ras/mitogen-activated protein kinase (MAPK) pathways which is associated with proliferation (Lin et al. 2006).

The importance of the IGF1R in normal mammalian development is clear from studies in mice lacking functional receptors. IGF1R null mice are 45% of the size of wild-type littermates at birth, and die shortly due to severe organ hypoplasia (Liu et al. 1993).

Mouse embryonic fibroblasts cultured from IGF1R null mice (R-cells) grow more slowly than wild-type fibroblasts, and are unable to proliferate under anchorage-independent conditions (Sell et al. 1994).

The different functions of the IGF-IR, including mitogenicity, protection from apoptosis, ability to transform cells, and ability to induce differentiation (Romano et al. 1999; Valentinis et al. 1999, Baserga et al. 1997), were examined with several mutant receptors and in different cell lines giving a map of the β subunit domains of the IGF-IR and their functions (Baserga 2000).

A mutation at lysine 1003 (the ATP-binding site) results in a receptor that has completely lost its functions suggesting that autophosphorylation of the receptor is a necessary component for its function (Baserga 2000).

A triple mutation in the tyrosine kinase domain (Y1131, Y1135 and Y1136), results in a receptor that is also seriously defective and fails to transmit a mitogenic signal (Gronborg et al. 1993; Li et al. 1994).

We can therefore say that the tyrosine kinase domain of the IGF-IR is necessary for its mitogenic signal. It is necessary, but it is also sufficient (with

lysine 1003), as other mutations at various residues have no effect on the mitogenicity of the receptor (Baserga 2000).

Tyrosine 950 and the C-terminus of the receptor are required for differentiation (Valentinis et al., 1999; Morrione et al., 2000), although they are dispensable for mitogenicity.

In the case of differentiation, however, the immediate substrates of the IGF-IR also play a very important role, as Shc proteins favour differentiation, while IRS-1 inhibits differentiation (Valentinis et al. 1999).

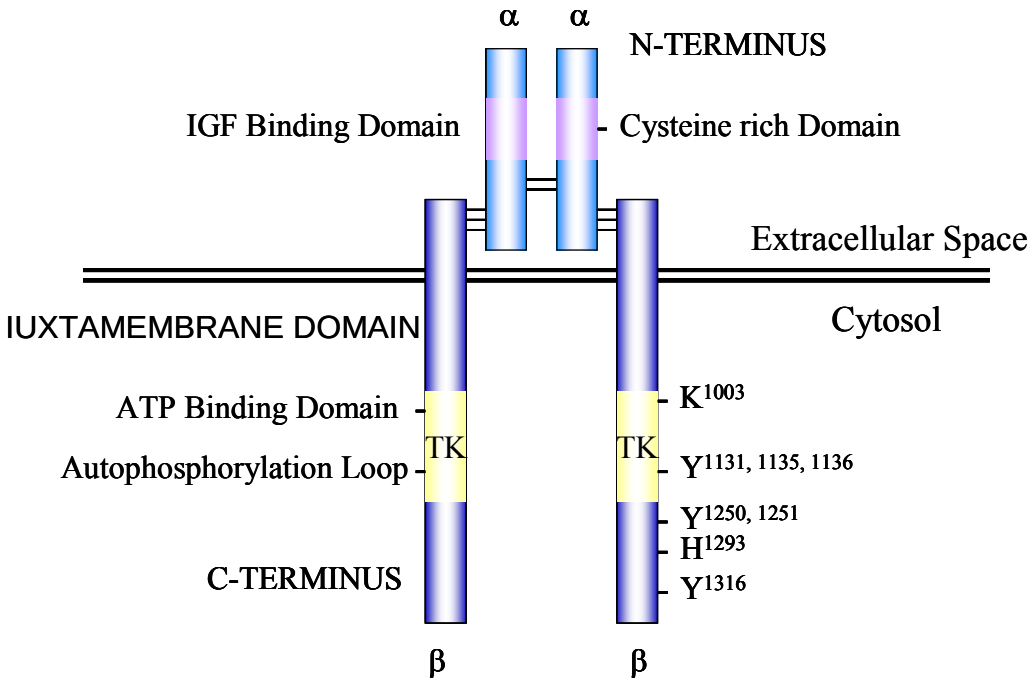

Fig 3: Structure of the Insulin-like growth factor I

For cell survival, the IGF-IR uses three different pathways originating from different domains (Peruzzi et al., 1999). The first domain is the tyrosine kinase domain, acting through the IRS-1/Akt/p70 pathway.

A second domain is based on activation of Shc, in which Y950 plays a preponderant role, which leads to the activation of MAPK (White 1998).

Finally, a third domain resides in a serine quartet at 1280-1283, which binds 14.3.3 (Craparo et al. 1997; Furlanetto et al. 1997) and activates Raf, promoting its mitochondrial translocation.

Interestingly, for survival, it is sufficient that any two of these three pathways be operative to exert protection from apoptosis. Regulation of cell size, like mitogenicity, requires the tyrosine kinase domain, understandably so, as cell size increase is a necessary pre-requisite for mitosis (Baserga 1999).

The transforming domain of the C-terminus is located between residues 1245 and 1310. In this region, at least three domains are involved, the tyrosine residue at 1251, the serine residues at 1280-1283, and, more weakly, the residues at 1293-1294 (Baserga 1999).

A mutational analysis has been performed to determinate whether various function of the IGF-1R. All studies demonstrated that a mutation in

ATP binding site produced “dead “ receptors incapable of signal transmission. Replacement of all three Tyr 1131, 1135, and 1136, or Tyr 1136 alone, with phenylalanine produced a receptor that was not mitogenic or transforming, but it still induced an efficient survival signal. Mutations in either Tyr 1131 or Tyr 1135 downregulated transformation without reducing cell growth (Hongo et al. 1996).

Deletion in the entire C-terminus at aa 1229 produced a receptor that retained a normal mitogenic function but was totally lacking transforming potential (Surmacz et al. 1995).

Interestingly, the IGF1-R terminus also appears to play a unique role in survival signalling. Mutants with a deleted C-terminus (at residues 1229 or 1245) retained or even amplified anti-apoptotic function, while single mutation in Tyr 1251, His 1293, and Lys 1294 reduced survival (O’Connor 1998).

Consequently, it has been suggested that the C-terminus is an intrinsic inhibitory domain of the IGF-1R, while the residues yr 1251, His 1293, Lys 1294 act as neutralizer of this pro-apoptotic function (O’Connor 1998).

To summarize, IGF-1R signals required for mitogenesis, transformation, and survival are distinct but partially overlap. For instance, no transforming activity is seen in the absence of mitogenic activity. Transformation also seems to have some common pathways with IGF-dependent survival. However, cell survival can be induced by a weak signal which is not sufficient for mitogenesis or transformation, while transformation requires strong IGF-1R activation and induction of specific signal originating at the C-terminus (Surmacz 2000).

IGF2-R

IGF-2R is monomeric receptor localizaed on cell surface and binds to mannose 6-phosphate residues on lysosomal enzyme (Nisseley et al. 1991).

Three ligand-binding regions are found in the extracellular domain of the receptor, one for IGF-2 binding and two for proteins containing mannose-6-phosphate (M6P) (Yu et al. 2000).

With regard to its role in IGF physiology, the IGF-2R may function primarily as a degradative pathway to remove IGF-2 from the extracellular environment (Oka et al. 1985).

Binding of IGF-2 to the IGF-2R causes internalization and degradation of the growth factor, this process is inhibited by lysosomal enzymes (Jones and Clemmon 1995).

IGFR2 does not have intracytoplasmatic signaling domain and is thought to be recycled between the plasma and different cellular compartments. The gene for IGF-R2 is located on chromosome 6q (Pavelic et al. 2006).

The function of the IGF-2R as scavenger receptor mediating the uptake and degradation of IGF-2 is well accepted. It is less clear whether any of the biological actions of IGF-2 are mediated by this receptor. Loss of this receptor in mice is associated with foetal death in utero. Signalling via the IGF-2R may involve GTP-binding protein activation. (Jones and Clemmon 1995).

Insulin receptor

Like the IGF1R also IR is synthesised as single-chain pre-proreceptors, with a 30-residue signal peptide that is cleaved co-translationally.

These assemble into disulphide linked tetramers comprised of two extracellular α subunits, and two β subunits that contain extracellular and transmembrane domains and a cytoplasmic region, which includes the tyrosine kinase domain (Ward and Garrett 2004).

IGF1R proreceptors can form heterodimeric complexes with insulin proreceptors, reflecting their close structural homology (Pandini et al. 1999).

The IR share significant structural homology with the IGF-1R. the kinase domains of these receptors are 80-90% identical. Also, Tyr 960 of the IR has the equivalent, Tyr 950, in the juxtamembrane domain in the IGF-1R. Importantly, the C-terminus regions of the receptors are quite different, sharing only approximately 40% homology. The equivalents of Tyr 1250 and 1251, Ser 1280-1283, and aa 1293-1310 are not present in the IR. Conseguently, it is believed that the differences between biological response of the IGF-1R and IR are associated with the induction of specialized signalling pathway arising from the C-terminus (Surmacz 2000).

IGF-binding proteins

The IGFs are found circulating complexed to binding proteins (IGFBPs) that are a family of six proteins with high affinity for the IGFs (Shimasaki and Ling 1991).

The IGFBPs share 40-60% amino acid sequence identity; all six have 16-18 conserved cysteine residues in the amino- and carboxy-terminal regions (Sachdev and Yee 2001).

Regulation of IGFBP gene transcription is complex and tissue specific (Herbert et al. 2000).

The genes for human IGF-BP are located on different chromosomes: genes for IGF-BPs 1 and 3 on chromosome 7, genes for IGF-BPs 2 and 5 on chromosome 2, the gene for IGF-BP 4 on chromosome 17 and the gene for IGF-BP 6 on chromosome 6 (Pavelic et al 2006).

A number of hormones, including estrogens, glucocorticoids, parathyroid hormone, FSH, GH, thyroid hormone, insulin, vitamin D, and cortisol, have been found to regulate the expression of IGFBPs. Growth factors, including FGF, EGF, TGFβ, PDGF, and IGFs themselves, as well as retinoic acid, are also involved in the regulation of expression of these binding proteins. The expression of IGFBP-1 is suppressed by insulin and IGFs (Herbert et al. 2000).

IGFBPs have multiple and complex functions, which can be either IGF dependent or IGF independent. With respect to IGF dependent function, IGFBPs are able to inhibit or to enhance the action of IGFs, resulting in either suppression or stimulation of cell proliferation (Clemmons 1997).

When binding to IGFs, IGFBPs play three major roles:

1) transporting IGFs
2) protecting IGFs from proteolytic degradation

3) regulating the interaction between IGFs and IGF-IR (Collett-Solberg and Cohen 1996).

IGF binding to IGFBP may be modulated by IGFBP modifications, such as glycosylation, phosphorylation and proteolysis, and by IGFBP association with cell surface or components of the extracellular matrix (Pavelic et al 2006).

The effects of IGFBP-2, IGFBP-3, and IGFBP-5 on IGFs are regulated by proteolysis of the binding proteins, whereas the effect of IGFBP-1 on IGFs is affected by phosphorylation (Kelley et al. 1996).

In addition to undergoing proteolysis and phosphorylation, IGFBP-1, IGFBP-2, IGFBP-3, and IGFBP-5 bind to specific cell membrane receptors or attach to the cell surface, which reduces their binding affinities for IGFs and results in the release of free IGFs (Yu et al. 2000).

Thus, the IGFBPs may play an important role in regulating IGF bioavailability and action, and add another level of complexity to the IGF system (Clemmons 1991).

They also have an IGF-independent function, for example IGFBPs interacts with the $\alpha 5\beta 1$ integrin to mediate cell mobility and type V transforming growth factor TGFb receptor (Jones et al. 1995).

IGF system and cancer

The insulin-like growth factor (IGF) signalling axis plays a pivotal role in normal growth and development (Liu et al. 1993), and is also implicated in mediating many aspects of the malignant phenotype in a variety of human malignancies (Reidemann et al. 2006).

Further evidence suggesting the importance of the IGF-1R pathway in cancer includes the finding that a variety of oncogenes require an intact IGF-1R for transforming activity (Sell et al. 1994).

A cell can enhance survival and proliferation while avoiding apoptosis by increasing IGF signalling. IGF signalling can be augmented through three possible mechanisms: increased ligand production, increased IGF1R or decreased amount of IGFBPs that competitively inhibit IGF/IGF1R binding.

However, increased expression of IGF-I, IGF-II, IGF-IR, or combinations thereof have been documented in various malignancies including glioblastomas, neuroblastomas, meningiomas, medulloblastomas, carcinomas of the breast, malignancies of the gastrointestinal tract, such as colorectal and pancreatic carcinomas, and ovarian cancer (Samani 2007).

The link between cancer and IGF signaling is also consistent with recent epidemiological studies showing an increased relative risk for the development of colon, prostate, breast, lung, and bladder cancers in individuals with circulating IGF-1 levels in the upper tertile of the normal range (Chan et al., 1998).

These findings were confirmed in animal models, where reduced circulating IGF-1 levels result in significant reductions in cancer development, growth, and metastases, whereas increased circulating IGF-1 levels are associated with enhanced tumor growth (Wu et al., 2003).

The effects of ligand over-expression were evident in two sets of transgenic mice. The first set over-expressed IGF-1 in the basal cells of the epidermis. Their phenotype included a slightly smaller birth size as well as skin

and ear morphologic changes. Epidermal hyperplasia, hyperkeratosis and increased labelling index attested to increased skin proliferation, and about half the older mice developed squamous papillomas, some of which converted into carcinomas. The second set of transgenic mice over-expressed IGF-1 in the basal epithelial cell of prostate. The mice developed prostatic hyperplasia by the age of 2-3 months and, atypical hyperplasia and prostatic intraepithelial neoplasia by 6-7 months. Well-differentiated adenocarcinomas were found in mice starting at age 6 months, and two of the older mice developed less differentiated carcinomas. Of all the mice 6 months of age or greater, 50% had prostate tumors (Grimberg 2003).

The IGF1R is frequently overexpressed in tumours, including melanomas, cancers of the colon, pancreas and, as we have shown, prostate and kidney (Hellawell et al. 2002, Bohula et al. 2003).

The role of the IGF-1R in malignant transformation rests on two fundamental observations. The first one is the singular resistance to transformation of mouse embryo fibroblasts with a targeted disruption of the IGF-IR genes. The second observation is that the downregulation of IGF-IR function causes massive apoptosis in tumour cells growing in anchorage independent conditions (Valentinis 2001).

The first inkling that the IGF-R plays a crucial role in malignant transformation was provided by experiments on R-cells, 3T3 cells originating from mouse embryos with a targeted disruption of the IGF-IR genes. It was found that R-cells could not be transformed by the SV40 large T antigen (Liu et al. 1993).

Mouse embryo fibroblasts (MEF), including 3T3 cells from several strains of mice, have a tendency to transform spontaneously in cultures (reduced growth factor requirements, foci formation in monolayer cultures and formation of colonies in soft agar), and the SV40 T antigen is, by itself, a strong transforming agent in MEF. The failure of R-cells to become transformed by the SV40 T antigen indicated a role of the IGF-IR in the transformation of cells in culture (Baserga 2000).

IGF1R activation or overexpression is associated with n increased propensity for invasion and metastasis. This effect is mediated by multiple signalling intermediates that influence invasive potential. IGF-induced phosphorylation of IRS-1 can influence the interaction between E-cadherin and β-catenin, enhancing β-catenin trascriptional activity and disconnecting E-cadherin from the actin cytoskeleton (Playford et al. 2000).

Loss of E-cadherin expression or function is well recognised as causing disruption of cell–cell contacts and release of invasive tumour cells from primary epithelial tumours (Hazan et al. 2004).

Similarly, tumour cell motility and invasive potential are influenced by crosstalk between the IGF axis and integrins (Shen et al. 2006), and by IGF induced secretion of matrix metalloproteinases (Zhang et al. 2003).

The importance of these functions is highlighted by the finding that IGF1R overexpression confers an invasive, metastatic phenotype in a murine model of pancreatic cancer (Lopez and Hanahan 2002).

Furthermore, IGF1R inhibition is capable of inhibiting metastasis in vivo (Dunn et al. 1998, Samani et al. 2001).

The increased expression of IGF1R results in an enhanced response to IGF-I that is manifested in greater downstream signaling through PI3-K and MAPK.

Traditionally, the Ras/Raf/MAP kinase pathway was thought to primarily mediate the cell proliferative response to growth factors such as the IGFs, whereas the PI3-kinase pathway, which activates AKT/PKB, was primarily implicated in mediating the antiapoptotic effects of the IGFs. However, recent studies have demonstrated a role for both pathways in mediating both responses (Kulik et al. 1997).

The role of these pathways in oncogenesis has been extensively investigated and altered expression or mutation of many components of these pathways have been implicated in human cancer (Vivanco and Sawyers, 2002).

Signal transduction pathways

Upon ligand binding, the intrinsic tyrosine kinase of the IGF-IR is activated, and this results in autophosphorylation of tyrosines on the intracellular portion of the β-subunit, including tyrosine residues in the juxtamembrane and C-terminal domains. Once phosphorylated, tyrosine 950 in the juxtamembrane domain can serve as a docking site for several adapter molecules, termed insulin receptor substrate (IRS) (Samani 2006).

These events lead to recruitment of receptor the substrate IRS-1, IRS-2, Shc proteins and Grb10, which, in turn connect to other proteins that are responsible for stimulating diverse downstream signal molecules (Sachdev and Yee 2001).

Binding of IRS proteins to the IGF-I receptor occurs via the NPXpY motif, which includes tyr950 (LeRoith 2000), and all three Shc proteins (66, 52, and 48 kDa) bind to IGF-IR through the Src-homology 2 (SH2) or PTB domain (Pelicci et al. 1992, Kavanaugh and Williams 1994, Tartare-Deckert et al. 1995).

After binding to the receptor, these proteins become phosphorylated on tyrosine residues, presumably by the IGF-I receptor (LeRoith 2000).

IRS-1 has 18 tyrosine phosphorylation sites and thus serves to connect to several downstream pathways to transduce IGF signal (Sun et al. 1993).

Diverse downstream molecules interact with IRS-1 and Shc proteins through their SH2–binding domains. IRS-1 binds the p85 regulatory subunit of PI3K (Tartare-Deckert et al. 1996) and activates the PI3K pathway, while phosphorylated Shc proteins interact with other signaling molecules like Grb2/Sos and activate downstream Ras-MAPK signaling cascades (Pelicci et al. 1992, Skolnik et al. 1993, Giorgetti et al. 1994).

Grb2 (*g*rowth factor *r*eceptor-*b*ound protein 2), an adapter protein, binds both Shc and IRS via its SH2 domain and via its SH3 domain to mSOS(*m*ammalian *S*on *o*f *S*evenless) (LeRoit 2000).

mSos is a guanine nucleotide-exchange protein that loads GTP onto the small G protein Ras, and thereby activates the Ras/ Raf/MAP kinase pathway. (LeRoith 2000).

The phosphotyrosine residues on IRS-1 also form docking sites for additional signaling molecules Syp, Nck (Lee et al. 1993) and Crk (Beitner-Johnson et al. 1996) (Fig 4).

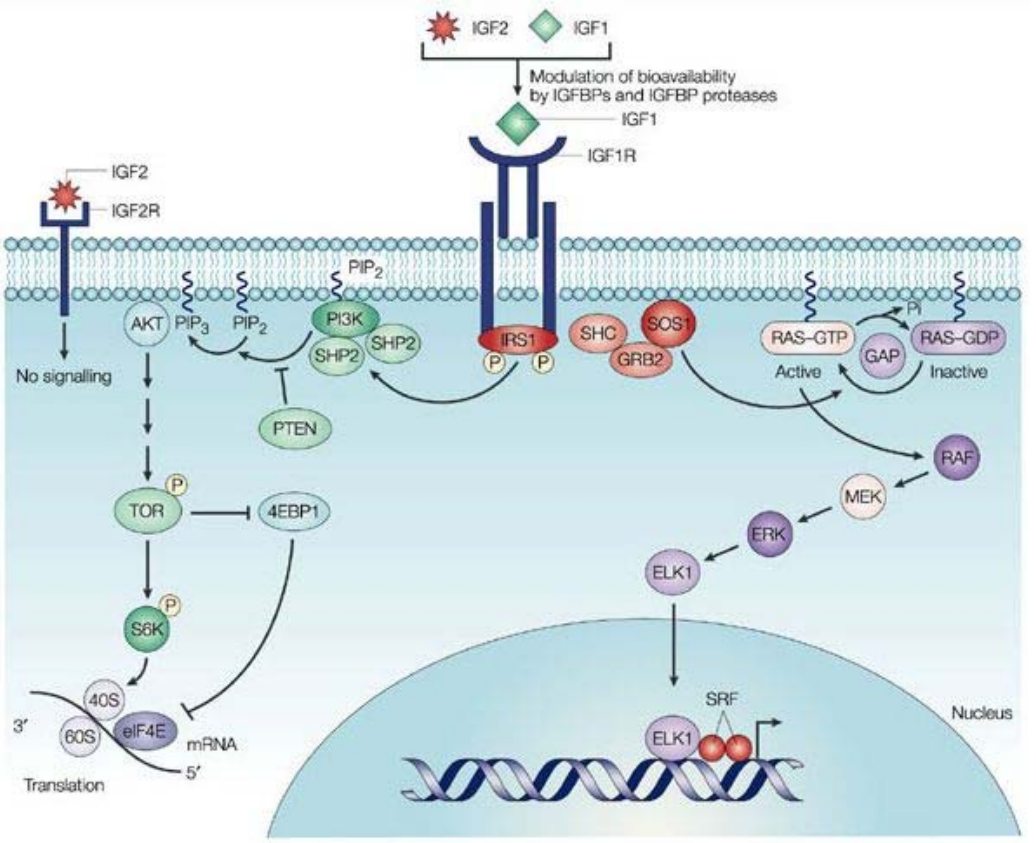

Fig 4: Overview of insulin-like growth factor 1 receptor activation and downstream signalling.
The insulin-like growth factor 1 receptor (IGF1R) is a tyrosine kinase cell-surface receptor that binds either IGF1 or IGF2. The local bioavailability of ligands is subject to complex physiological regulation and is probably abnormally high in many cancers. Ligands can be delivered from remote sites of production through the circulation or be locally produced. IGF-binding proteins (IGFBPs) and IGFBP proteases have key roles in regulating ligand bioavailability.The IGF2R binds IGF2, but has no tyrosine kinase domain and appears to act as a negative influence on proliferation by reducing the amount of IGF2 available for binding to IGF1R. Following ligand binding to IGF1R, its tyrosine kinase activity is activated, and this stimulates signalling through intracellular networks that regulate cell proliferation and cell survival. Key downstream networks include the PI3K–AKT–TOR system and the RAF–MAPK systems. Activation of these pathways stimulates proliferation and inhibits apoptosis. For many cell types, the key effects of signalling downstream to AKT relate to regulation of cell survival and mRNA translation, while the principal effect of signalling downstream to RAS involves regulation of cellular proliferation.

PI3K pathway

The phosphatidylinositol 3-kinases (PI3Ks) are a family of enzymes (Classes I, II and III) (Cooray 2004).

The class I PI3K signaling pathways represent a widespread signal transduction network that is used by a huge variety of cell surface receptors to regulate complex cellular phenomena such as cell growth, survival, proliferation, and movement (Cooray 2004)

Class IA PI3Ks can be activated through binding of the Src homology (SH2) domain in the adaptor subunit to autophosphorylated tyrosine kinase receptors (Fig. 1) or alternatively to non-receptor tyrosine kinases in the cytoplasm, such as the Src family kinases or JAK kinases (Andrews et al. 2007).

Class I PI3Ks are heterodimeric proteins consisting of a catalytic subunit (110 kDa, p110) and an adaptor/regulatory subunit (85kDa, p85) (Cooray 2004).

The regulatory p85 subunit consists of several domains including the SH3 domain, two proline rich fragments, and two SH2 domains separated by the iSH2 (inter SH2) sequence. The iSH2 domain provides the interaction between the p85 and p110 subunits, and the two SH2 domains are responsible for binding of the p85/p110 heterodimer with receptor tyrosine kinases (Krasilnikov 2000).

The catalytic p110 subunit of PI3K is homologous to protein kinases and possesses both serine.threonine protein kinase and phosphoinositide kinase activities (Krasilnikov 2000).

At the membrane, PI3K phosphorylates phosphatidylinositol-4,5-bisphosphate (PIP2) at the 3′ position on its inositol ring and converts PIP2 to PIP3. Subsequently, PIP3 recruits other downstream molecules, particularly the serine-threonine kinases Akt and PDK1, via binding to their pleckstrin omology (PH) domains.

In turn, the production of PtdIns(3,4,5)P3 is regulated by the phosphatase PTEN, which catalyses the dephosphorylation of PtdIns(3,4,5)P3 to PtdIns(4,5)P2 (Maehama & Dixon, 1999).

The rise in concentration of these two lipids coordinates the localization and function of multiple effector proteins, which bind these lipids through their specific pleckstrin homology (PH) domains. This binding facilitates the recruitment of these proteins to the plasma membrane and their subsequent activation (Andrews et al. 2007).

It is now well established that many of the diverse metabolic, proliferative and survival effects triggered through activation of PI3-K are mediated by the activation of a subgroup of AGC (cAMP-dependent, cGMPdependent, protein kinase C (PKC)) family of protein kinases (Mora et al. 2004) .

These comprise isoforms of protein kinase B (PKB, also known as Akt), p70 ribosomal S6-kinase (S6K), serum and glucocorticoid responsive kinase (SGK), p90 ribosomal S6 kinase (RSK) and PKC (Komander et al. 2004).

The phosphoinositide-dependent kinase (PDK-1)1 plays a pivotal role in cellular signaling by regulating the activation state of these protein kinases (Gao et al. 2001).

PDK1

The 3-phosphoinositide-dependent protein kinase-1 (PDK-1) is a 64-kDa protein, of 556 amino-acid, comprised of a Ser/Thr kinase domain near the N-terminus, a C-terminal pleckstrin homology (PH) domain, and an ATP-binding site located between the two domains (Lim et al. 2003; Biondi et al. 2002).

This pivotal kinase plays a crucial role in mediating signal transduction downstream of phosphatidylinositol 3-kinase (PI3- kinase) in response to mitogen stimulation (Lim et al. 2003).

PDK1 phosphorylates and activates several protein kinases from the AGC group (which includes PKA, PKG and PKC), to which PDK1 also belongs (Biondi 2004).

AGC family protein kinases possess over 40% identity within their kinase catalytic domain (Alessi 2001) (Fig 5).

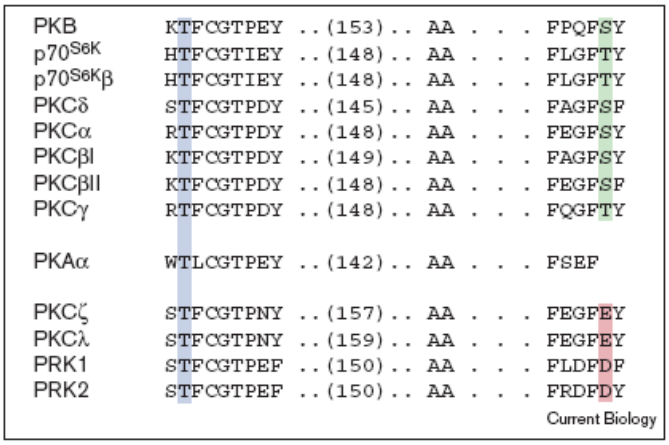

Fig 5: Alignment of the conserved sequences surrounding the kinase domain activation loop and the carboxy-terminal phosphorylation sites of several AGC kinases. The number of amnio acid residues between the activation loop and the carboxy-terminal phosphorylation site are indicated for each kinase. The activation loop phosphorylation site are phosphorylated by PDK1 and are highlighted in blue. The carboxy-terminal phosphorylation sites, are highlighted in green. The pseudo substrate sites present in the atypical PKCs and the PKC-related Kinases are highlighted in in red.

A common regulatory mechanism of kinases is through phosphorylation of segment near the entrance to the active site, the activation loop, and a second phosphorylation site at the carboxyl terminus, in the hydrophobic motif (Toker and Newton 2000).

Phosphorylation of both residues is required for the maximal activation of these enzymes (Mora et al. 2004).

The activation loop phosphorylation sites of these kinases are phosphorylated by PDK1 (Peterson and Schreiber 1999)

PDK1, like all other AGC kinases, requires phosphorylation at its own T-loop residue (Ser 241) in order to be activated (Mora et al. 2004).

PDK1 has been shown to possess the intrinsic ability to phosphorylate itself. Phosphorylation of Ser241 may be mediated by an intermolecular mechanism suggesting that dimerization and *trans*-phosphorylation in the activation loop may regulate PDK1 activity in cells (Wick et al. 2003).

Other mechanisms of regulating PDK1 activity have been proposed: Insulin/IGF-1 treatments stimulate plasma membrane and nuclear localization of PDK1 and tyrosine phosphorylation has also been shown to activate PDK1 (Riojas et al. 2006).

In fact there is evidence that PDK-1 undergoes tyrosine phosphorylation in response to several growth factors. Studies with the tyrosine phosphatase inhibitor pervanadate indicate that full activation of PDK-1 requires phosphorylation at Tyr373/376 in 293 cells overexpressing insulin receptors (IRs) (Fiory et al. 2005).

The first identified and best characterized PDK1 substrate is the proto-oncogene Akt (Feldman et al. 2005).

Numerous studies have found a high level of activated PKB/Akt in a large percentage (30–60%) of common tumor types, including melanoma and breast, lung, gastric, prostate, hematological, and ovarian cancers. When activated in tumor cells, Akt has multiple effects that promote disease progression, including suppression of apoptosis and stimulation of tumor cell proliferation, metabolism, and angiogenesis (Feldman et al. 2005).

The PDK1/Akt signaling pathway thus represents an attractive target for the development of small molecule inhibitors that may be useful in the treatment of cancer (Feldman et al. 2005).

PKB/Akt is a 57 kDa Ser/Thr kinase with a PH domain that preferentially binds PtdIns(3,4,5)P3 and PtdIns(3,4)P2. Two specific sites, one in the kinase domain (Thr308) in PKB and the other in the C-terminal regulatory region (Ser473) in PKB, need to be phosphorylated for full activation of this kinase (Fig 6).

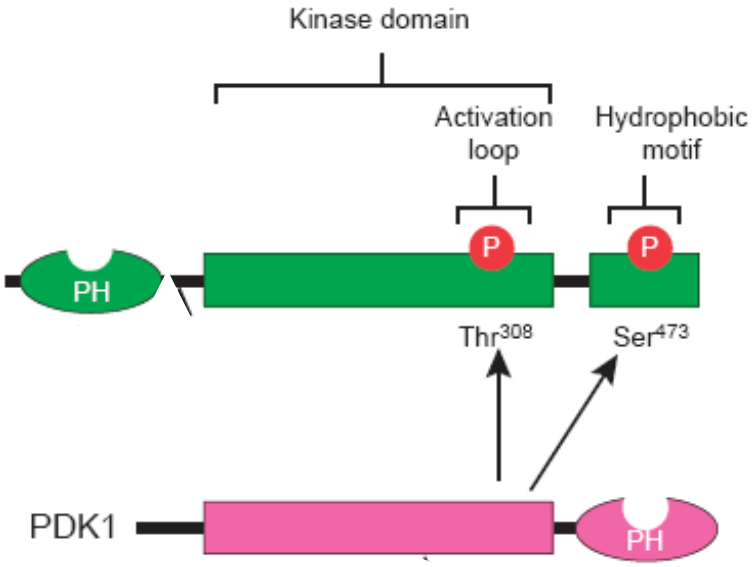

Fig 6: Protein architecture of Akt PKB and PDK1. Akt/ PKB requires for full activation phosphorylation on Threonine 308 in the activation and phosphorylation on Serine 473 at C-terminus region.

PKB is cytosolic in unstimulated cells, and some of it translocates to the plasma membrane upon activation of PI3K, where it becomes activated (Vanhaesebroeck and Alessi 2000).

Recent structural and cellular studies have also indicated that binding of PKB to PtdIns(3,4,5)P3 induces a major conformational change (Mora et al, 2004) that is likely to be required for PKB activation by PDK1. Importantly, binding of PtdIns(3,4,5)P3 to the PDK1 PH domain does not directly affect activity of the PDK1 kinase domain, but stimulates PKB activation by inducing the co-localization of PKB and PDK1 (Komander et al. 2004) (Fig 7).

PDK-1 is also the upstream kinase for p70 S6 kinase and both atypical and conventional isoforms of protein kinase C.

PKCs are involved in cell proliferation and possibly, therefore, carcinogenesis.

Furthermore, PDK-1 directly phosphorylates PKCζ at the activation-loop Thr410 residue leads to activation of the enzyme, both *in vitro* and *in vivo.*

Recent studies indicate that PDK1 serves as an important effector of mammary epithelial cell growth and invasion in the transformed phenotype.

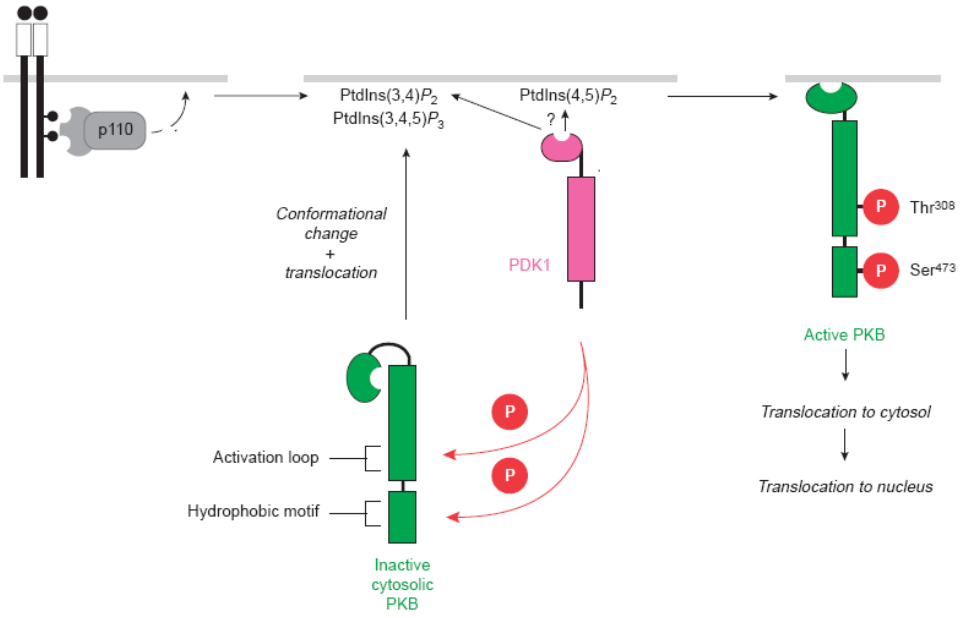

Fig 7:Model of the activation mechanism of Akt/PKB by PI3K and PDK1. PI3K increase intracellular concentration of PtdIns(3,4,5)P3 that bind to PH domain of PKB inducing a conformational change. PKB translocates to the plasmamembrane where it is phosphorylated by PDK1 on Threonine 308. PKB, once activated trnslocates to the cytosol and to the nucleus.

PDK1 is highly expressed in a majority of human breast cancer cell lines.

The presence of increased PDK1 expression in the majority of invasive breast cancers suggests its importance in the metastatic process (Xie et al. 2003).

The activation of PDK1 can lead to mammary tumorigenesis, and PDK1 expression may be an important target in human breast cancer. Recently, it was found that antisense oligonucleotides targeting PDK1 could block the proliferation of U-87 glioblastoma cells by promoting apoptosis (Zeng et al. 2002).

Forced expression of PDK-1 induced anchorage-independent growth *in vitro*, a hallmark of cellular transformation, and isografts of PDK-1 transforming cell lines into syngeneic mice induced the formation of poorly differentiated mammary carcinomas, indicating that dysregulation of PDK-1 may be involved in tumorigenesis (Lim et al. 2003).

Tissue microarray analysis of human invasive breast cancer indicated that PDK1 pSer241 was strongly expressed in 90% of samples.

PDK1 has been found to serve as an effective therapeutic target for inhibition of cancer growth.

AIM OF THE STUDY

In many cancer cells, the increased activity and/or expression of growth factors and their receptors contribute to the abnormal proliferation and/or to the decreased cell death.
The insulin-like growth factor (IGF) signalling axis is implicated in normal growth and development but is also involved in mediating many aspects of the malignant phenotype in a variety of human malignancies. The IGF axis has been recognised as a drug target and it could be potentially blocked at several distinct levels.
IGF survival and proliferation signals are largely mediated by the engagement of the PI3K pathway and a pivotal role for the induction of this signal cascade is played by PDK1.
PDK1 regulates a multiplicity of cellular functions by regulating the activation state of diverse protein kinases, such as the AGC superfamily of serine/threonine protein kinases, which, in turn, play crucial roles in metabolism, growth, proliferation and survival.
Thus, similar as growth factor receptors abnormalities, in PDK1 expression or function might contribute to the deregulation of cell growth in cancer cells.
The aim of this study is to identify the molecular mechanism by which the IGF-1R controls cell survival in cancer cells focusing on formation of molecular complexes by the activated IGF-1R. In particular the work aims to demonstrate that PDK1 is directly activated by IGF-1R and to test the possibility that selective disruption of this interaction will inhibit survival signals induced by IGF-1 and facilitate killing upon targeting of cancer cells

MATERIALS AND METHODS

Cell culture and transfection

The MCF7 human breast cancer cells were available in host laboratory and the NIH 3T3 cells stably expressing *IGF1R* cDNA have been previously described (Hermanto et al. 2002).The MCF7 and NIH 3T3 cells were grown in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum, 100 IU/ml penicillin, 100 IU/ml streptomycin, and 2% L-glutamine in a 5% humidified CO2 incubator.

Western Blot analysis

MCF7 and NIH 3T3-IGF1R cells were lysed in lysis buffer (50mM HEPES pH 7.6, 150mM NaCl, 10mM EDTA, 10mM $Na_4P_2O_7$, 2mM Na_3VO_4, 100mM NaF, 10% glycerol, 1mM PMSF, 100 IU/ml aprotinin, 20μM leupetin, 1% Triton X-100), for 2 h at 4 °C and lysates were centrifuged at 14,000 x g for 15 minutes to remove cellular debris. Cell lysates were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and analyzed by western blot as previously described (Laemmli 1970). Nitrocellulose membranes were probed with antibodies to IGF1R α-subunit (UPSTATE Biotechnology), PDK1 (UPSTATE Biotechnology), phospho PDK1 (Ser241) (CELL-SIGNALING Techology), phospho PDK1 P-$Tyr^{373/376}$ (CELL-SIGNALING Techology), PKCζ (SANTA CRUZ), phospho PKCζ P-Thr410 (SANTA CRUZ), Akt/PKB α (UPSTATE Biotechnology), phospho Akt/PKB P-Thr^{308}, GST (UPSTATE Biotechnology). Immunoreactive bands were detected by enhanced chemiluminescence according to the manufacturer's instructions (GE Healthcare, NJ).

Co-immunoprecipitation

Lysates from MCF7 and NIH 3T3-IGF1R cells were incubated with IGF1R α-subunit antibody (UPSTATE Biotechnology) at 4 °C for 16 hours. After incubation the mixture was incubated with protein A sepharose resin (Sigma-Aldrich, MO) pre-equilibrated with HNT buffer (50mM HEPES pH 7.5, 150mM NaCl, 0,1 % Triton X-100) for 2 hours at 4 °C with vibrant shaking. The bound antibody-antigen complexes were washed three times with HNT and were then eluted in SDS sample buffer (Laemmli 1970). Total elute was separated by SDS-PAGE followed by immunoblotting with appropriate antibodies.

Pull-down experiment

PDK1-glutatione *S*-transferase (GST) fusion proteins were generated as described by Alessi et al. 1997. NIH 3T3-IGF1R cell lysates (500 μg of protein) were incubated in the presence of Sepharose-bound GST-PDK1 (5 μg) for 2 h at 4 °C. The beads were washed four times with HNT buffer and then resuspended in SDS sample buffer followed by boiling for 5 min and centrifugation at 25,000 x g for 3 min. Supernatants were separated by SDS-PAGE followed by immunoblotting with appropriate antibodies

Densitometric and statistical analysis

Densitometric analysis was performed using Scion Image Analyzer. Data were analyzed with Statview software (Abacus-concepts) by one-factor analysis of variance. *p* values of less than 0.05 were considered statistically significant.

Receptor Purification

Confluent monolayers (corresponding to 6-8 X 10^7 cells) of NHI3T3 overexpressing IGF-1R were solubilized in 1% Triton X-100, 50 mM HEPES, pH 7.6, 150 mM NaC1, 10 pg/ml leupeptin, 10 pg/ml bacitracin, and 1 mM phenylmethylsulfonyl fluoride. The insoluble material was separated by ultracentrifugation at 100,000 x g for 1 h at 4 "C. The supernatant was applied to a wheat germ agglutinin (WGA) Sepharose column pre-equilibrated with buffer containing 0.1% Triton X-100,50 mM HEPES, pH 7.6,150 mM NaCl and the protease inhibitors described above. The column was extensively washed using the same buffer, and bound glycoproteins were eluted in the same buffer containing 0.3 M N-acetylglucosamine.

***"In Vitro"* phosphorylation**

Aliquots of WGA-purified receptors were incubated in the absence or the presence of 100 nM IGF1 for 1 h at room temperature. Then the receptor was incubatede with GST-PDK1 fusion protein and phosphorylation was initiated by the addition of 1.6 mM CTP, 9.7 μM ATP, 0.01M Hepes pH 7.4, 0.02% TRITON, 5mM $MnCl_2$, 6.6 mM $MnCl_2$. After 20 min at room temperature, the reaction was stopped by the addition of 800 μl of ice-cold stopping solution (50 mM HEPES, pH 7.4, 10 mM $Na_4P_2O_7$, 100 mM NaF, 4 mM EDTA, 2 mM Na_3VO_4, 2 mM phenylmethylsulfonyl fluoride, 0.2 mg/ml aprotinin (14 trypsin inhibitor units/mg) and 1% Triton X-100). Proteins were separated on 7.5% polyacrylamide gels and detected by autoradiography.

Immunofluorescence microscopy

Cells were grown on to gelatin-treated glass coverslips in 60 mm dishes, and were allowed to adhere for 48 h. Cells were fixed with ice-cold methanol and permeabilized with 0.2% Tween 20 in TBS. Cells were blocked with 10% FBS in TBS-T buffer [Tris-buffered saline (TBS) containing 0.1% (v/v) Tween 20] for 15 min. Cells were washed with TBS-T and then incubated with a 1:200 dilution of fluorescein-tagged goat anti-mouse secondary antibody (Santa Cruz Biotechnology). After washes with TBS-T, the coverslips were mounted on to a microscope slide using a 90% solution of glycerol in TBS and analysed with a Zeiss Axioplan2microscope.

Flow cytometry

Cells were fixed with ethanol and routinely kept at -20°C overnight. Cells were washed twice with TBS and permeabilized with TBS, 4% fetal bovine serum, 0.1% Triton X-100 for 10 minutes on ice. Cells were washed twice with TBS-0.1% Tween 20, resuspended in TBS and incubated with ioduro propidum and RNasi for 20 minutes. After cells were analyzed using a flow cytometer (FACScan, Becton Dickinson, Mountain View, CA). Data were analyzed with ModFit/LT (Verity Software, Topsham, ME).

RESULTS AND DISCUSSION

IGF-1 induces PDK1 activation and coprecipation with IGF-1R

3- Phosphoinositide-dependent protein kinase-1 (PDK1) plays a central role in signal transduction pathways induced by phosphoinositide 3-kinase PI-3K) (Park et al 2001).

PDK1 works as a master upstream kinase controlling the activation of numerous AGC (cAMP-dependent, cGMP-dependent, protein kinase C) kinase members (Mora et al 2004).

It is well described that PDK1 is tyrosine-phosphorylated in response to treatment of cells with H_2O_2 and vanadate and that tyrosine phosphorylation is correlated with increased enzymatic activity (Prasad et al. 2000).

Three tyrosine phosphorylation sites of PDK1 were identified using *in vivo* labeling and mass spectrometry: Tyr 9 and Tyr 373/376. Using site-directed mutants, it was demonstrated that phosphorylation on Tyr 373/376 is important for PDK1 activity; phosphorylation on Tyr 9 has no effect on the activity of the kinase (Park et al. 2001)

I studied whether endogeneous PDK1 was tyrosine phosphorylated following insulin-like growth factor-1 (IGF-1) treatment. These studies were performed in human breast cancer cells (MCF-7) using antibody that specifically recognizes pTyr 373/376 on PDK1. I first verified that PDK1 was phosphorylated on tyrosine residues in response to IGF-1 treatment. These experiments were performed in the absence of serum with or without supplementation of IGF-1. Therefore, MCF-7 cells have been treated with IGF-1 (100ng/ml) for 5 and 30 minutes. As shown in Fig 8, IGF-1 rapidly induces the tyrosine phosphorylation of endogeneous PDK1 on Tyr 373/376 after 5 minutes of treatment, while after 30 minutes the tyrosine phosphorylation is comparable to the control cells.

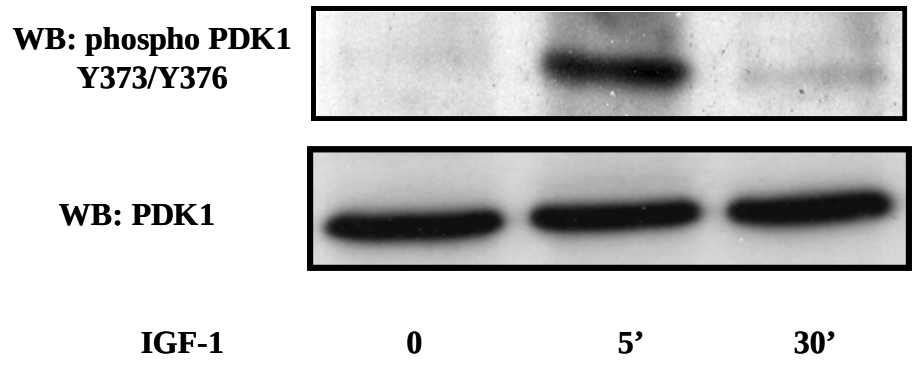

Fig 8: PDK1 tyrosine phosphorylation upon IGF-1 treatment. MCF-7 cells were starved for 16 hrs and stimulated with 100ng/ml IGF-1 for the indicated times. Cell lysates were separated on SDS-PAGE and immuno-blotted with phospho PDK1 (Y373/Y376) antibody and reblotted with anti PDK1 antibidy.

It was demonstrated that in response to insulin, PDK1 directly binds to the insulin receptor (IR) C terminus and it is tyrosine phosphorylated by insulin receptor kinase (Fiory et al. 2005)

Accordingly I investigated whether IGF-1 induces the coprecipitation of PDK1 with IGF-1R. To verify the interaction between PDK1 and IGF-1R, MCF-7 cells were stimulated with IGF1 (100ng/ml) for 5 and 30 minutes in absence of serum. Lysates from MCF-7 cells, were precipitated with anti α-subunit of IGF1-R antibody and the immunoprecipitated proteins were analyzed by western blot with specific antibody against PDK1. The chemiluminescence (ECL) revealed increased levels of PDK1 associated to IGF-1R after 5 minutes of stimulation with IGF-1, compared to the control cells. As in the case of tyrosine phosphorylation, the interaction is poorly detectable after 30 minutes of IGF1 stimulation (Fig 9).

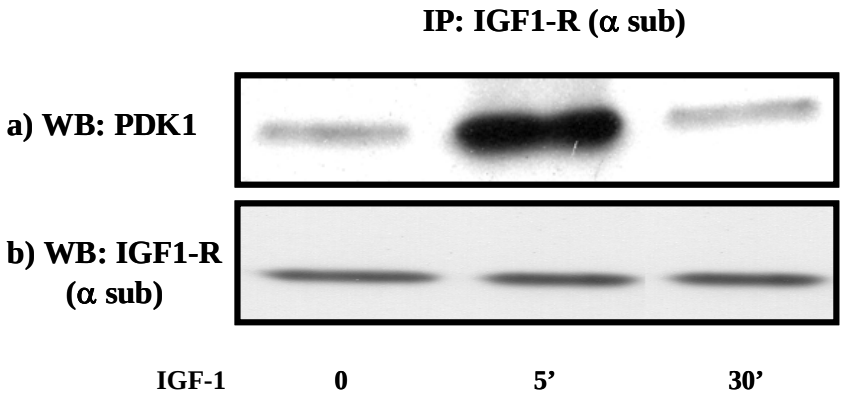

Fig 9: PDK1/IGF-1R co-precipitation in response to IGF-1 stimulation. MCF-7 cells were starved for 16 hrs and stimulated for the indicated times with 100ng/ml IGF-1. The cells were solubilized, and equal amounts of proteins (200 μg) were immunoprecipitated (I.P.) with IGF1-R α-subunit antibodies, followed by Western blotting (WB) with PDK-1 **(a)** or IGF1-R α-subunit antibodies **(b)**.

PDK1 requires phosphorylation on Serine 241 of its activation loop for catalytic activity, most probably due to PDK1 autophosphorylation (Gao et al. 2006).

I verified whether PDK1 was autophosphorylated upon IGF-1 stimulation. To this aim, MCF-7 cells were stimulated with IGF-1 for 5 and 30 minutes in the absence of serum. Lysates were immunoprecipitated with anti IGF-1R α subunity antibodies and PDK1 activation was analyzed by Western blot with antibody that specifically recognizes phospho-serine 241. As shown

in Fig 10, IGF1 induces PDK1 precipitation with IGF1-R and its autophosphorylation.

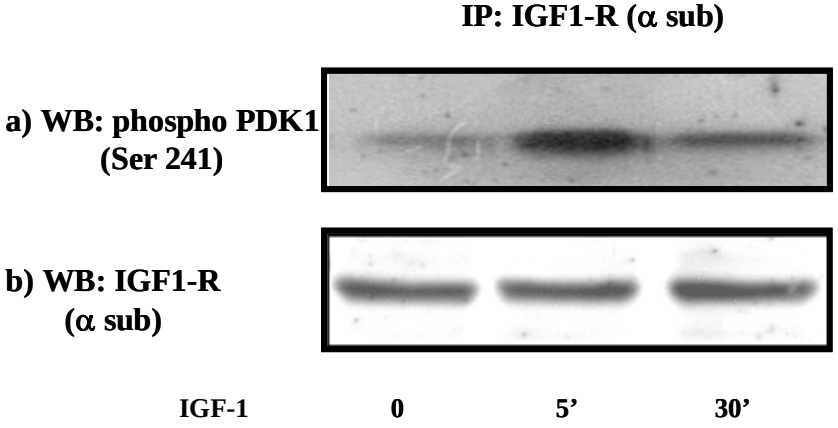

Fig 10: PDK1 interacts with IGF-1R and is activated in response to IGF-1 treatment. MCF-7 cells were starved for 16 hrs and stimulated with 100ng/ml IGF-1 for the indicated times. Lysates were immoprecipitated with IGF-1R (α subunit), immoprecipitated proteins were separated on SDS-PAGE and analyzed by Western blot with phospho PDK1 (Ser 241) **(a)** and with anti IGF1-R (α subunit) **(b)** antibodies.

PDK1 phosphorylates and activates several other protein kinases from the AGC group (which includes PKA, PKG and PKC), to which PDK1 also belongs (Biondi et al. 2004). A common regulatory mechanism of these kinases is through phosphorylation of the activation loop, and of a second phosphorylation site at the carboxyl terminus, in the hydrophobic motif (Toker et al. 2000).

The best characterized cellular substrate for PDK1 is Akt/PKB which is activated by phosphorylation at Threonine 308 (Alessi et al. 1997). Moreover PDK1 phosphorylates the activation loop of PKCζ on Thr 410 leading to the activation of the enzyme, both *in vitro* and *in vivo* (Chou et al. 1998).

Thus, phosphorylation of Akt/PKB and PCKζ in response to IGF-1 was verified in MCF-7 cells lysates using phospho-specific antibodies that only recognize phosphorylated Thr 308 on Akt and phosphorylated Thr 410 on PCKζ, the sites of PDK1 phosphorylation.

Stimulation of serum-starved cells with IGF-1 (100ng/ml) for 5 and 30 minutes induces an increase of phosphorylation of the T-loop residues of Akt/PKB and PCKζ (Fig 11 a ,b)

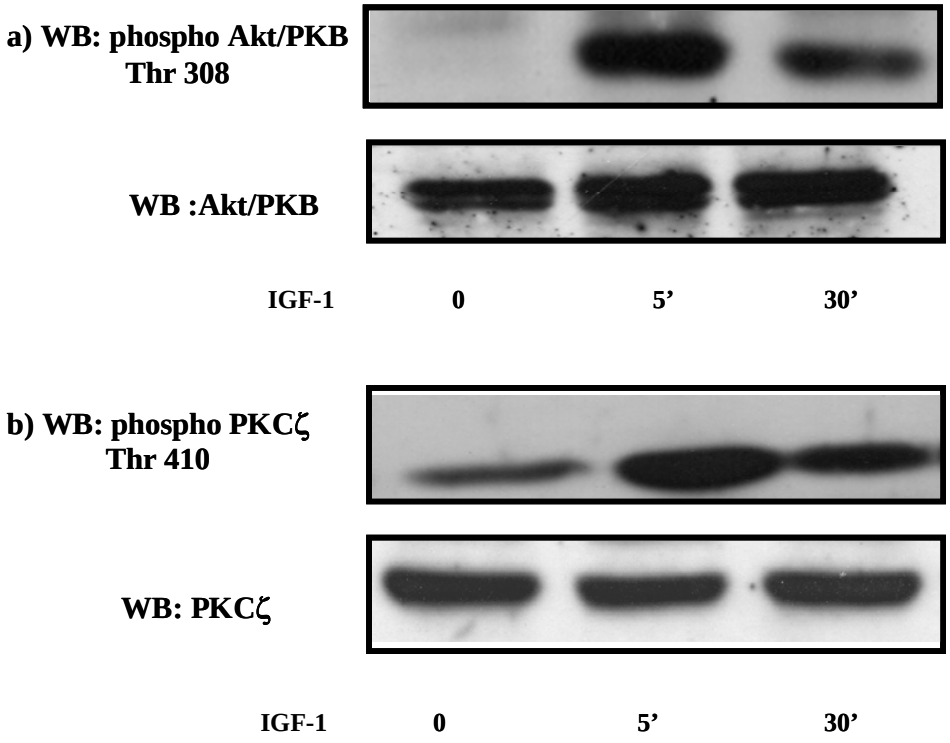

Fig 11: Akt/PKB and PKCζ phosphorylation in MCF-7 cells upon IGF-1 stimulation. a) MCF-7 cells were exposed to 100 ng/ml IGF-1 for the indicated times and then solubilized as described in Materials and Methods. Cell lysates were blotted with specific phospho-threonine308–Akt/PKB antibody (p-Thr308 Akt/PKB) and then reblotted with Akt/PKB antibodies **b)** MCF-7 cells were exposed to 100 ng/ml IGF-1 for the indicated times and then solubilized as described in Materials and Methods. Cell lysates were blotted with specific phospho-threonine410-PKCζ antibody (p-Thr410 PKCζ) and then reblotted with PKCζ antibodies.

Pull down experiments

In order to determine the minimal interaction fragments on PDK1 with IGF1-R, PDK1 deletion mutants were fused to GST and used in pull-down experiments with lysates obtained by NIH3T3 cells overexpressing IGF1-R.

In particular, I generated fusion proteins, with PDK1 full-length (residues 1-556), two PDK1 mutants lacking the PH domain corresponding to 1-359 aa and 51-359 aa, respectively, and with the PH domain alone (residues 408-556) of PDK1.

The recombinant proteins were generated as described in Materials and Methods and the expression of fusion proteins was detected by Western blot analysis with anti-GST antibody (Fig 12).

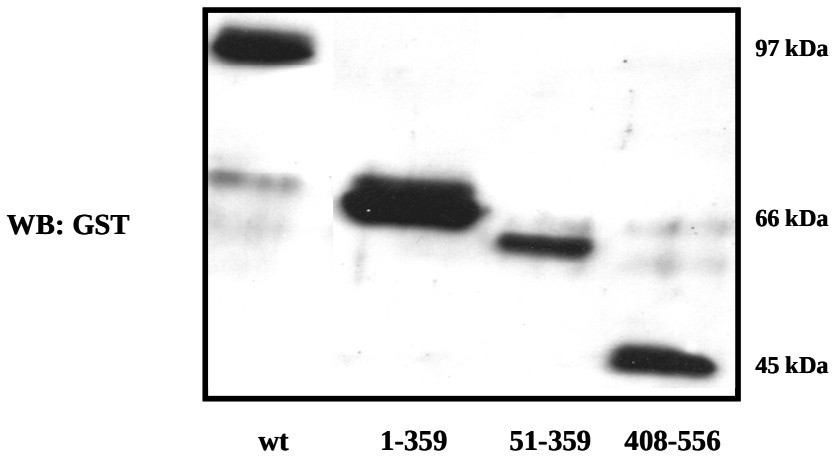

Fig 12: Recombinant GST-PDK1 fusion protein expression. The recombinant proteins were generated as described in Materials and Method, separated on SDS-PAGE and then expression of fusion proteins was detected by Western blot analysis with anti-GST antibody.

The ability of IGF-1R to bind PDK1 was assessed by Western blot with antibody against anti β-subunit of IGF-1R.

As expected, IGF-1R binds PDK1 in pull down experiments. Surprisingly this interaction is stronger for the fusion protein 51-359 and 1-359 than the full length fusion protein while it is almost completely abolished with the fusion protein 408-556 (fig 13).

Thus, I demonstrated that IGF1-R binds PDK1 "in vitro" and the region encompassing the residues 51-359 of PDK1 seems to be important for this interaction while the region corresponding to the PH domain of the protein is not necessary for the interaction and could have negative effect on the association.

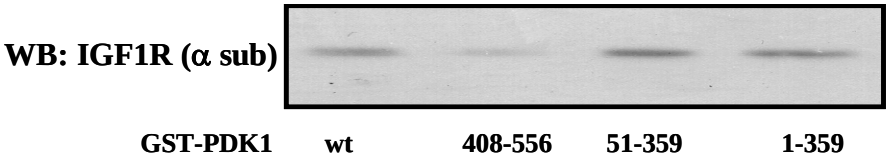

Fig 13: GST-PDK1 pull down of IGF-1R. NIH3T3 overexpressing IGF1R were starved for 16 hrs and stimulated for 5 minutes with 100ng/ml IGF1. Cell lysates were incubated with recombinant proteins and the interaction analyzed by Western blot with anti IGF-1R (α sub) antibody.

Peptides displace interaction and inhibit *in vitro* tyrosine phosphorylation

In a previous study (Fiory et al. 2005) it was observed that the region corresponding to the insulin receptor (IR) C-terminus is critical for the interaction with PDK1 and its activation. In particular the truncation of the IR C-terminus 43 amino acids dramatically abolishes this interaction.

With this background, I wanted to prove if synthetic peptides corresponding to the region of IGF-1R C-terminus would inhibit PDK1 tyrosine phosphorylation in vitro and its interaction with IGF-1R.

Thus, four peptides corresponding to the aminoacids 1325-1367 of IGF-1R (C43), 1346-1367 (C23) and similar peptides featuring tyrosine replacement with phenylalanine (Y→F peptide) (C43F and C23F) were generated (in collaboration with Dr. M. Ruvo, IBB-CNR, Naples, Italy)..

To verify the ability of synthetic peptides to displace the binding IGF-1R/PDK1, I performed pull-down assays with WGA-purified IGF-1R and GST-PDK1 protein in presence of synthetic peptides at increasing concentration. All the four peptides reduce the association of PDK1 with IGF-1R in vitro. In particular, C43 peptide at 60nM concentration is capable to reduce PDK1 interaction with IGF-1R by 50% (fig 14)

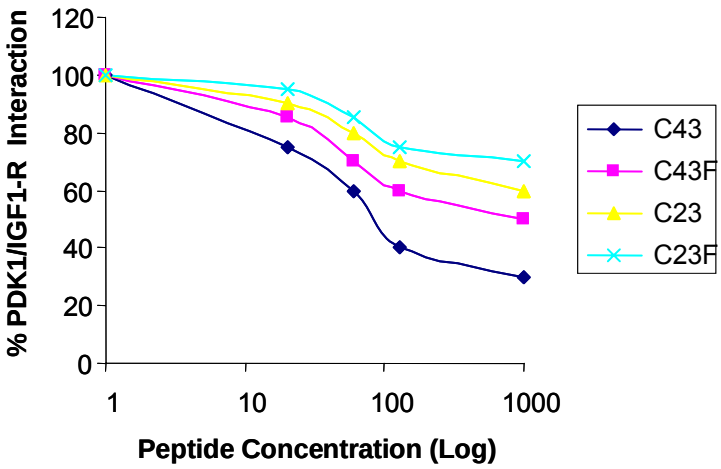

Fig 14: Effect of synthetic peptides on PDK1/IGF1-R interaction. IGF1-R was partially purified from NHI3T3 cells overexpressing IGF1-R as described in Material and Methods and then it was incubated for 2 hrs at 4° C with Sepharose-GST bound recombinant PDK1 (GST-PDK1) or GST alone, in the presence or in the absence of synthetic peptides (C43, F43, C23 and F23) at increasing concentration as indicated. Pulled down proteins were blotted with anti IGF1-R (β subunit) antibody and the results quantitated by laser densitometry.

Next, the ability of IGF-1R to directly phosphorylate PDK1 was verified in vitro assays.

Activated-IGF-1R caused a five fold increase in PDK1 phosphorylation at tyrosine residues 373/376. Addition to the phosphorylation reactions of the peptides at 60nM concentration caused a 65% reduction of PDK1 phosphorylation (Fig 15) but had no effect on IGF-1R phosphorylation (data not shown). As in the case of PDK1 /IGF-1R interaction, the substitution of tyrosine with phenialanine on C43 peptide (Y→F) had a lower effect on PDK1 phosphorylation.

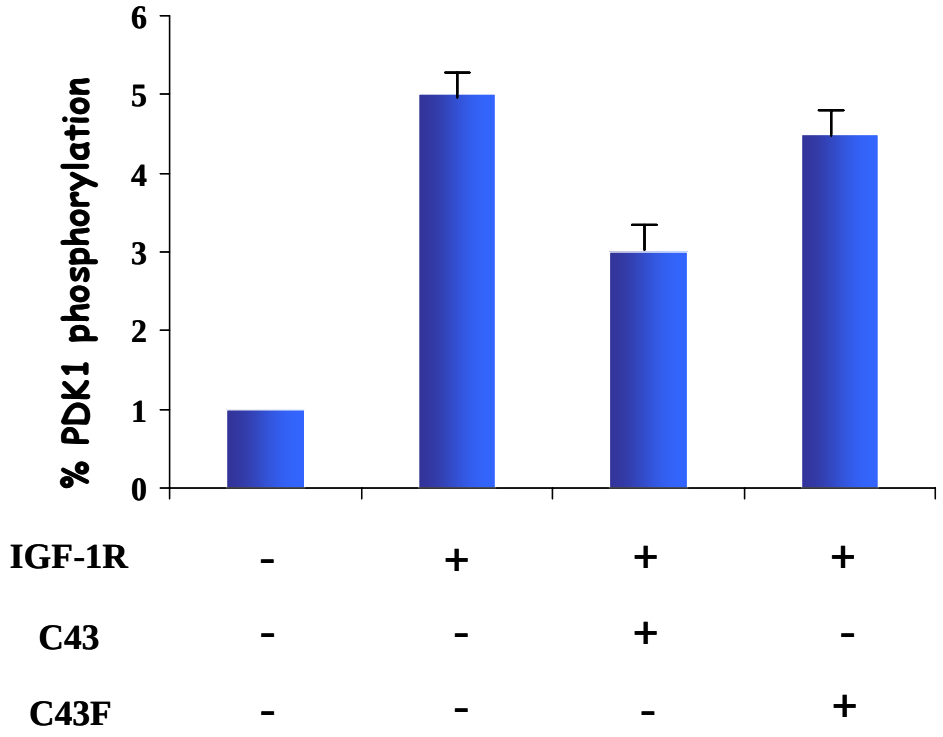

Fig 15: Effect of C43 peptide on PDK1 phosphorylation. IGF1-R was partially purified from NIH3T3 cells overexpressing and IGF-1R was activated with IGF-1 in *vitro*. Then IGF-1R was incubated with PDK1-GST fusion protein in the absence or in the presence of C43 peptide and F43 peptide at 60nM concentrations. After migration on SDS-PAGE, Western blot analysis was performed with anti phospho PDK1 (Y373/Y376) antibody and the results quantitated by laser densitometry.

Peptide loading in MCF7 and NIH3T3 cells

To evaluate the effect of C43 peptide on PDK1/IGF-1R interaction and on downstream signalling in intact cells, MCF-7 and NIH-3T3 IGF-1R cells, were incubated with the fluorescein isothiocyanate-conjugated (FITC) C43 peptide at increasing concentration. Intracellular loading of synthetic peptide

was achieved by means of cationic lipid mixture. As revealed by fluorescence microscopy, FITC-C43 peptide was relatively efficiently transduced in both MCF-7 and NIH-3T3 IGF-1R cells at 10μM concentration (40%) (Data not shown).

Next, I performed co-immunoprecipitation experiments in MCF-7 and NIH-3T3 IGF-1R cells to evaluate the effect of C43 peptide on PDK1/IGF-1R interaction.

MCF-7 and NIH3T3-IGF-1R cells, after (FITC) C43 peptide loading, were stimulated for 5 minutes with 100ng/ml IGF-1 in the presence or in the absence of serum. Cell lysates were precipitated with anti α- subunit IGF-1R antibody and the association with the protein was revealed with specific antibody against PDK1. As shown in fig 16, C43 peptide was able to decrease PDK1/IGF-1R association in response to IGF-1 as compared to control cells, both in MCF-7 and NIH3T3-IGF1-R cells.

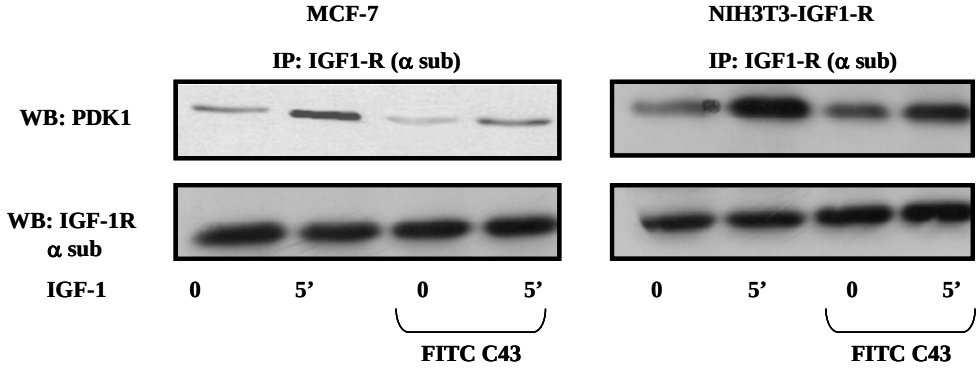

Fig 16: Effect of C43 peptide on PDK1/IGF1-R interaction in intact cells. MCF-7 and NIH3T3-IGF1-R treated or not with (FITC) C43 peptide, were starved for 16 hrs and stimulated for indicated time with 100ng/ml IGF-1. Cell lysates were immunoprecipitated with anti IGF-1R (α sub) antibody, then the immoprecipitated proteins were separated on SDS-PAGE and Western Blot analysis was performed with anti PDK1 and anti IGF1-R α subunit antibodies.

To test the ability of C43 peptide to interfere with IGF-1R signalling I analyzed the activation state of Akt/PKB and PKCζ. MCF-7 and NIH3T3 cells were stimulated with IGF-1 in the absence or in the presence of the (FITC) C43 peptide and the lysates were analyzed by Western blot with anti-phospho-Akt/PKB (Thr 308) antibody and anti phospho-PKCζ (Thr 410). In both cell types (FITC) C43 peptide reduced Akt/PKB and PKCζ activation in response to IGF-1 (Fig 17).

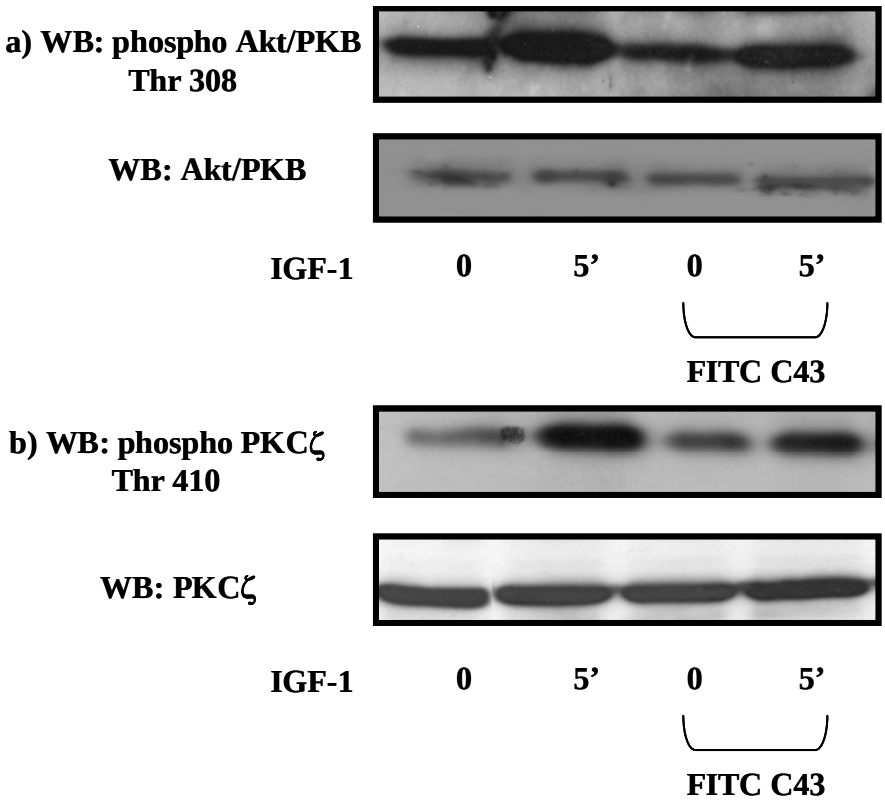

Fig 17: Effect of C43 peptide on PDK1 substrates phosphorylation. MCF-7 cells, treated or not with (FITC) C43, were starved for 16 hrs and then stimulated for indicated times with 100ng/ml IGF-1. Cell lysates were separated on SDS-PAGE and Western blot analysis was performed with anti phospho Akt/PKB (Thr 308) and reblotted with anti Akt/PKB antibodies **(a)** and phospho PKCζ (Thr 410) and reblotted with PKCζ antibodies **(b)**.

Peptides reverts IGF1 protection from apoptosis and cell growth arrest.

Recent studies have shown that the IGF-1R plays a crucial role in the induction and maintenance of malignant phenotype (Samani 2007). Overexpression and/or constitutive activation of IGF-1R lead to acquisition of transformed phenotype and tumorigenic potential in various cell types.

The available data suggest that targeting of the IGF system in vivo may inhibit cancer progression and / or cause cancer regression directly by inducing apoptosis and cell growth arrest.

Various strategies have been used to target components of this system in established animal and tumor cell lines and animal models of cancer, and some of these strategies may be advancing to clinical use. Among them are down-regulation of IGF-1R by antisense oligonucleotides, antisense RNA, small interfering RNA, single chain antibody, full humanized anti-IGF-1R monoclonal antibodies and specific kinase inhibitors (Samani 2006). Some of these molecular strategies to target the IGF1 system have failed for the magnitude of toxicity, particularly glucose-intolerance, due to the cross reactivity with the insulin receptor. Moreover even if IGF1R inhibitors prove to

have limited activity as single agents, it will be important to evaluate effects on disease progression and chemosensitivity, given the important role of IGF1R in invasion and metastasis, and in protecting from killing induced by cytotoxicity drugs (Reidemann and Macaulay 2006).

Therefore, I wanted to prove the principle that disrupting the interaction of the IGF-1R with PDK-1, IGF-1 protective effect on cell death could be reverted.

At first, I investigated the effect of IGF-1 on cell cycle and proliferation in NIH-3T3-IGF-1R cells. To this aim NIH-3T3 cells, overexpressing human IGF-1R, were exposed to serum-free media in the absence or in the presence of IGF-1 and the induction of apoptosis/necrosis was analyzed by a spectrum of flow cytometric assays. The phases of the cell cycle and the occurrence of a sub-G_1 peak of apoptotic cells were determined with propidium iodide staining.

The untreated cells present a normal distribution among the phases of cell cycle while, 16h of serum-free media treatment induces significant accumulation of cells in the sub-G1 phase that is indicative of induction of cell death.

Interestingly, the addition of IGF-1 to serum-free media for 16 h protects cells from serum starvation induced apoptosis.

Then, I evaluated the efficiency of the four transduced peptides to inhibit the pro-survival function of IGF-1 on cells.

To this aim, each FITC-peptide (C43, C43F, C23 and C23F) was loaded into NIH-3T3-IGF1-R cells and after 16h of treatment with IGF-1 in the absence of serum, I analyzed cell cycle variations and in particular the ratio of cells in sub-G1 phase.

The transduction efficiency of FITC-peptides into the cells was measured by flow cytometry analysis. An example of fluorescence distribution derived from incubation with FITC-C43 peptide is shown in (Fig 18). For FITC-C43 peptide, greater than 20% of live cells exhibit fluorescence greater than 10^1, which is adjusted for background fluorescence in untreated cells. Distribution of fluorescence is generally narrow and reproducible for the other FITC-peptides.

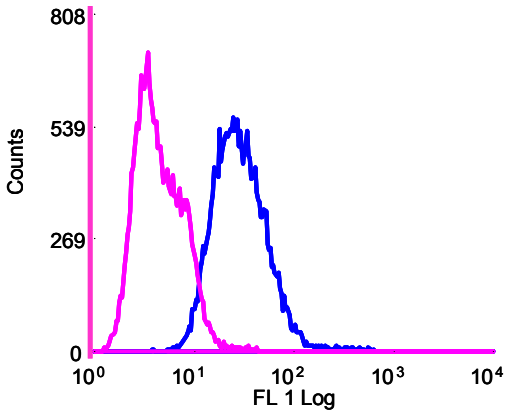

Fig 18: Peptide loading into NIH3T3-IGF1R cells

Cell cycle analysis by flow cytometry was preformed only on cells that are positive for fluorescence. Results from cytometry analysis indicated that serum starvation of NIH-3T3-IGF-1R cells induces an increase of apoptosis/necrosis of cells compared to the starved cells treated with IGF-1 for 16h. In particular, the ratio of sub-G1 phase in NHI-3T3-IGF-1R changed from 6,4 in starved cells to 1,3 in presence of IGF-1 100ng/ml indicating that IGF-1 protects cells from cell death.

However, peptides pre-treatment, reduced cell proliferation, causing cell-cycle arrest in the sub-G1 phase. In fact the ratio of sub-G1 phase is 7 for C43 peptide, 6,7 for C23 peptide, 6,4 for C43F peptide and 5,7 for C23F peptide. (Fig 19). These data indicated that C43 peptide, compared to the others, more efficiently reverted the protective effect of IGF-1 (Fig 19).

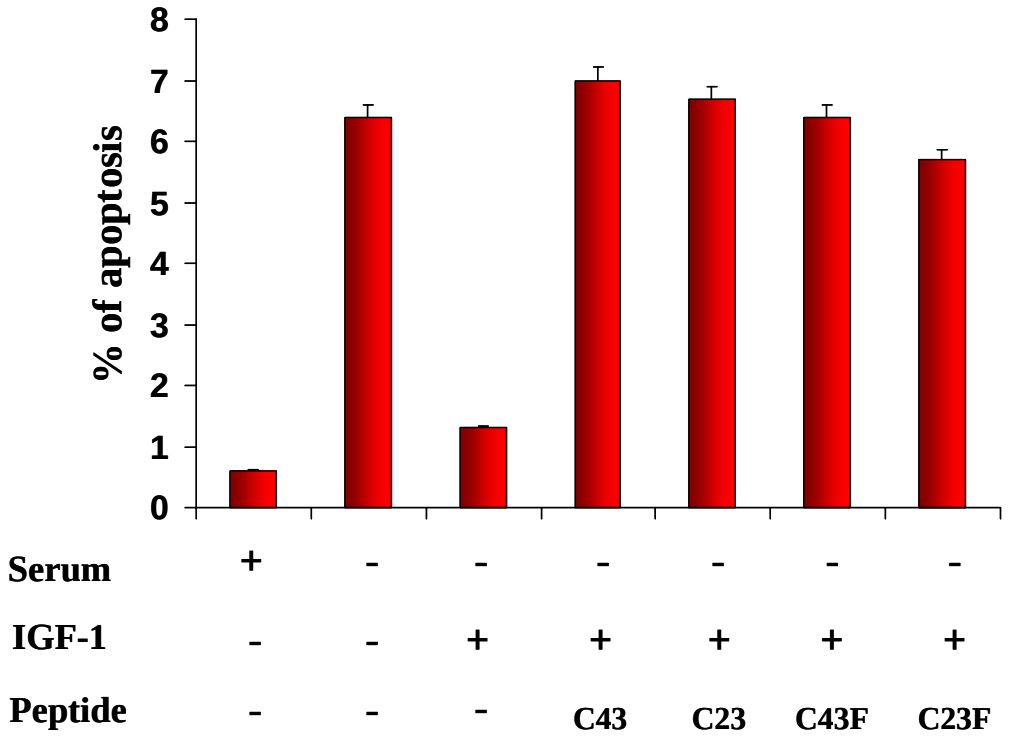

Fig 19: C43 peptide reverts the protective effect of IGF1 on starved cells. Four peptides (C43, C23, C43F and C23F) were loaded into NIH3T3-IGF1-R cells and then staved for 16 hrs and incubated in the presence of IGF-1. Cells were treated for with propidium iodide staining and ratio of sub-G1 phase was analyzed by flow cytometric assays.

For this reason the next experiments were performed only with C43 peptide.

Then I evaluated the perturbation of cell cycle and the arrest of proliferation of cancer cells, MCF-7 cells, in presence of camptothecin a cytotoxic drug that is normally used as inhibitor of nucleic acid synthesis and a potent inducer of strand breaks in chromosomal DNA. Camptothecin treatment induces cell cycle arrest with an accumulation of cells in G2 phase. Cell cycle analysis by flow cytometry in MCF-7 reveals that addition of camptothecin to induces significant accumulation of cells in G2/M phase. The ratio of G2/M phase change from 3.1 in starved cells to 45.87 in presence of camptothecin. This effect is partially reverted by simultaneous treatment with IGF-1. In dead, the ratio of G2/M in this condition is 29.60. When FITC-C43 peptide (10μM) was loaded into MCF-7 in presence of IGF-1 and camptothecin was detected an increased accumulation of cells in G2/M, which raising to 58.30. Thus, C43 peptide is able not only to revert the protective effect of IGF-1 but also to potentiate the effect of camptothecin (Fig 20)

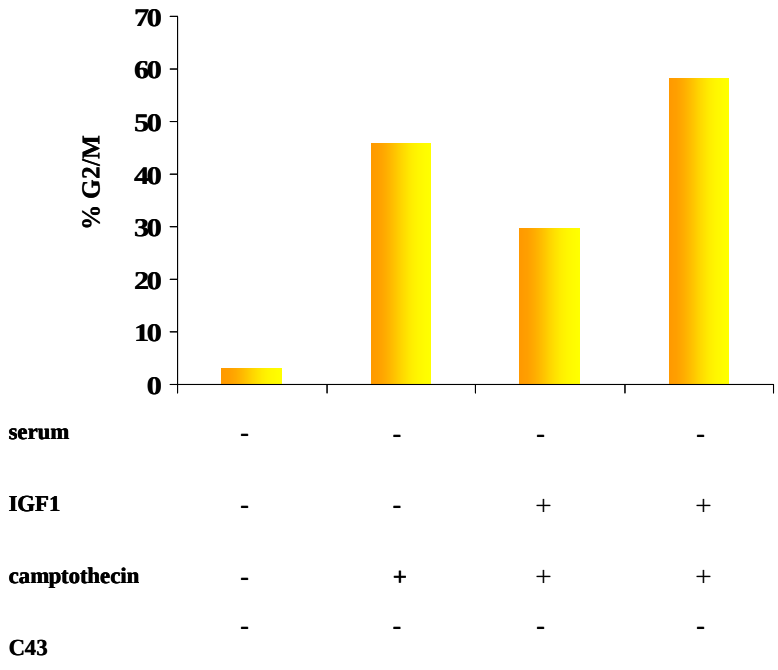

Fig 20: C43 peptide increases the effect of campotothecin in G2/M cell cycle arrest. C43 peptide was loaded into MCF-7,then cells were starvaed for 16 hrs and treated with campotothecin in the presence or in absence of IGF1. MCF-7 cells were treated with propidium iodide and the cell cycle arrest in G2/M phase was analyzed by flow cytometric assays.

CONCLUSIONS

PDK1 is tyrosine phosphorylated and co-precipitates with IGF1-R in response to IGF-1 in MCF-7 cells. “Pull down” experiments I revealed that PDK1 binds IGF-1R through its N-terminus region (aa 51-356) while the the PH domain is not necessary for this interaction. The C43 synthetic peptide corresponding to the C-terminus region of IGF-1R is capable to reduce PDK1/IGF-1R interaction both and *in vitro* and in intact cells. The displacement of this association by C43 peptide loading into MCF-7 cells interferes with IGF-1R signalling, In fact, threonine phosphorylation of PDK1 substrates (Akt/PKB and PKCζ) in response to IGF-1 is drastically reduced. Moreover, C43 peptide reverts the IGF-1 protection from apoptosis and potentiates the effect of cell cycle arrest induced by camptothecin. This represents, the best of my knowledge, the first proof-of-priciple that disrupting the interaction of IGF1-R with PDK1 may provide the molecular basis for targeting strategies in cancer cells.

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