From the Department of Oncology and Pathology
Cellular and Molecular Tumor Pathology
Cancer Center Karolinska,
Karolinska Institutet, Stockholm, Sweden

REGULATION OF INSULIN-LIKE GROWTH FACTOR-1 RECEPTOR EXPRESSION AND SIGNALING

Radu Vasilcanu

M. D.

Karolinska
Institutet

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Doctors are men who prescribe medicine of which they know little, to cure diseases of which they know less, in human beings of whom they know nothing.

Voltaire 1760

To Mara

ABSTRACT

Insulin-like growth factor-1 receptor (IGF-1R), a member of the insulin receptor tyrosine kinase family is a broadly expressed transmembrane receptor that plays a key role in malignant cell growth. IGF-1R transmits information provided by extracellular stimuli into intracellular signaling pathways resulting in the subsequent regulation of various effector systems. Under normal cellular conditions IGF-1R signaling network is tightly regulated. The most prominent regulator of IGF-1R signal termination is desensitization of receptors by the removal of activated receptors from the cell surface mediated by accelerated endocytosis. For some membrane receptors the signal mediating receptor internalization/downregulation is constituted by ubiquitination. Recently, we showed that IGF-1R undergoes ubiquitination following ligand stimulation. The proto-oncogene MDM2 was identified as an E3 ligase involved in IGF-1R ubiquitination.

Studies on new events involved in IGF-1R downregulation and intracellular signaling constitute the subject of the present thesis.

β-arrestins are ubiquitously expressed cytosolic proteins generally known to be involved in the regulation of endocytosis and signaling elicited by G protein-coupled receptors (GCPRs). We provide evidence that the two widely co-expressed isoforms of β-arrestin, bind to the IGF-1R and, by serving as adaptor proteins bring the oncoprotein E3 ligase MDM2 to the receptor. Thus, β-arrestins promote ubiquitination but also degradation of the receptor. In this respect, β-arrestin 1 is more potent then isoform 2. Actually, β-arrestins are an absolute requirement for interaction between MDM2 and IGF-1R, indicating their relevance for cell growth and cancer.

We also investigated the role of β-arrestin 1 and MDM2 in intracellular signaling. We found that both MDM2 and β-arrestin 1 also are necessary for IGF-1 stimulated phosphorylation of ERK1/2 but not of Akt. In addition, the modulating effect of MDM2 and β-arrestin 1 on ERK activation has consequences on cell cycle progression. Thus, MDM2 and β-arrestin 1 do not only induce ubiquitination and degradation of IGF-1R but also influence cell growth by modulating the activity of ERKs.

The cyclolignan PPP is an inhibitor of phosphorylation of IGF-1R and activation of downstream molecules, without interfering with the highly homologous insulin receptor (IR). Further, PPP has well established anti-tumor effects on several in vivo tumor models. We could demonstrate that PPP also causes downregulation of IGF-1R. Furthermore, the PPP-induced downregulation of IGF-1R required the expression of wild type MDM2 E3 ligase, indicating that MDM2-dependent ubiquitination and degradation of IGF-1R represents an important mechanism in this respect. Our data also suggest that this effect of PPP plays a role in induction of apoptosis.

Finally, we demonstrated that PPP in fact induces IGF-1R ubiquitination, but also temporarily activates ERK1/2. This effect is IGF-1R-specific since PPP does not affect ERK phosphorylation in IGF-1R negative cells. Moreover, in the absence of MDM2, PPP-induced activation of ERK did not occur. The temporary MDM2-dependent ERK phosphorylation induced by PPP may contribute to the apoptotic effect of this compound.

Key words: IGF-1R, β-arrestins, MDM2, ubiquitination, internalization.

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LIST OF ABBREVIATIONS

14.3.3	Adaptor scaffolding protein
aa	Aminoacids
Akt	Protein kinase B
A-loop	Activation loop of the receptor
ARF	Alternative reading frame (protein)
ASK-1	Apoptosis signal-regulating kinase 1
ATP	Adenosine triphosphate
Bad	Bcl associated death promoter
Bak	Bcl-2 Homologous Antagonist-Killer Protein
Bax	Bcl-2-Associated X Protein
Bcl	B-cell leukemia protein
β2AR	β2 adrenergic receptor
Cbl	Cellular product of cbl oncogene
c-Crk	Adaptor protein in Ras pathway
CDK	Cyclin-dependent protein kinase
cDNA	Complementary DNA (DNA copy of mRNA)
DNA	Deoxyribonucleic acid
E2F	Transcription factor activating adenovirus E2 gene
EGF	Epithelial growth factor
ERK1 / 2	Extracellular signal-regulated kinase 1 / 2
FGF	Fibroblast growth factor
GH	Growth hormone
GPCR	G protein-coupled receptor
Grb2	Growth factor receptor-bound protein 2
Grb10	Growth factor receptor-bound protein 10
GSK	Glycogen synthase kinase
GTP	Guanosine triphosphate
IGF-1	Insulin-like growth factor 1
IGF-1R	Insulin-like growth factor 1 receptor
IGF-2	Insulin-like growth factor 2
IGF-2R	Insulin-like growth factor 2 receptor
IGFBP	Insulin-like growth factor binding proteins
IL-3	Interleukin-3
IR	Insulin receptor
IR-A/B	Insulin receptor isoform A/B
IRR	Insulin receptor related receptor
IRS1-4	Insulin receptor substrate 1-4
JAK	Janus protein tyrosine kinase
JNK	Jun N-terminal kinase
kDa	Kilo Dalton
Lys	Lysine
MAPK	Mitogen activated protein kinase
mTor	Mammalian target of rapamycin (FK506 binding protein)
MDM2	Murine double minute 2
MEK	MAP kinase kinase
mRNA	Messenger ribonucleic acid

Nedd4	Neuronal precursor cell-expressed developmentally downregulated
N	Nitrogen
PARP	Poli(ADP-ribose) polymerase
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PDGF	Platelet derived growth factor
PI3K	Phosphatidylinositol-3'-kinase
PKB	Protein kinase B
PPP	Picropodophyllin
PTB	Phospho-tyrosine binding domain
Raf	Protein-serine/threonine kinase (encoded by the raf oncogene)
Ras	Human homologue of Rat sarcoma
Rb	Retinoblastoma protein
rDNA	Ribosomal DNA
RNA	Ribonucleic acid
rRNA	Ribosomal RNA
RTK	Receptor tyrosine kinase
RT-PCR	Reverse transcription polymerase chain reaction
Ser	Serine
SH	Src homology
Shc	Src homology and collagen
SHP	SH2-containing phosphatase
siRNA	Small interference RNA
Sos	Son of the sevenless
Src	Protein encoded by src proto-oncogene
STAT	Signal transducer and activator of transcription proteins
TK	Tyrosine kinase
UBF	Upstream binding factor
V2R	Vasopresin-2 receptor
VEGF	Vascular endothelial growth factor
wt	Wild type
Y	Tyrosine

1 INTRODUCTION

1.1 CANCER AND THE IGF SYSTEM

Roughly 650 millions years ago, when the first metazoans appeared, they carefully programmed their cells to participate in constructing the diverse tissues that make organismic survival possible. Most types of cells in the metazoan body carry a complete genome, retaining ability to grow and divide long after tissue development has been completed.

The risk is that individual cells may gain access to information in their genomes that is usually denied to them. Their DNA may be subject to corruption by various mechanisms that alter the structure and the information content of the genome. The resulting changes may be alterations in cellular growth programs, and these in turn can lead to the appearance of large populations of cells that not longer follow the rules governing normal tissue. These cells appear to only to make more copies of themselves, turning into a disruptive cell multiplication that we call cancer.

Tumor transformation, the process by which normal cells evolve into cells with neoplastic phenotypes, is driven by a sequence of randomly occurring mutations and epigenetic alterations of DNA which disrupt the normal cellular control of defense, repair and apoptosis. Tumor development is a multi-step process formally analogous to Darwinian evolution, in which a succession of genetic changes, each conferring one or another type of growth advantage, leads to the progressive conversion of normal cells into cancer cells (Foulds 1954; Nowell 1976).

Highly advanced human cancer cells share a number of essential attributes that they have acquired: a reduced dependence on exogenous growth factors; an acquired resistance to growth inhibitory signals; an ability to multiply indefinitely (immortalization); a reduced susceptibility to apoptosis; an ability to generate new blood vessels (angiogenesis); an acquisition of invasiveness and metastatic capability; an aptitude to evade elimination by the immune system; and an acquisition of genomic instability (Hanahan and Weinberg 2000).

In almost all cells within the organism exist a similar molecular machinery regulating their proliferation, differentiation and death. In the process towards a malignant phenotype, transformed cells exploit the multiple molecules involved in normal extracellular signaling pathways to create growth advantage over normal cells. In particular, the complex signaling networks mediating cell growth are regulated in part by polypeptide growth factors that can act (by autocrine and/or paracrine mechanisms of action) as positive or negative modulators.

The growth factors are unable to cross the cell membrane and they exert the effects via binding to cell surface receptors, most of which possess intrinsic tyrosine kinase activity. Following interaction of polypeptide growth factors with their specific transmembrane receptors, a cascade of intracellular signals resulting in the activation or repression of various subsets of genes occurs. One of the most important players in this setting is insulin-like growth factor 1 receptor (IGF-1R). IGF-1R is involved in transformation and proliferation of malignant cells (Baserga 1995; Baserga 2000; Girnita, Wang et al. 2000; Girnita, Girnita et al. 2003), in prevention of apoptosis and in maintanence of the malignant phenotype of tumor cells as well as it has an important function in tumor cell protection against antitumor therapy (Baserga 1995; Baserga 2000; Yu and Rohan 2000; Baserga 2005).

1.2 THE EVIDENCE FOR THE INVOLVEMENT OF IGF SYSTEM IN MALIGNANCY

The insulin-like growth factor (IGF) family, consisting of ligands, binding proteins and receptors, is an important system implicated in the development of the organism and the maintenance of normal function of many cells of the body. During postnatal development and longitudinal growth, the main functions of growth hormone (GH) are mediated via IGF-1. During puberty, elevated sex steroid levels (particularly estrogens) stimulate GH production, leading to activation of the GH/IGF-1 axis (Christoforidis, Maniadaki et al. 2005).

Although serum IGF-1 levels decline progressively after puberty, significant levels of circulating IGF-2 are detectable throughout adult life. IGF-1R mRNA levels also decline after puberty, but remain high in some tissues such as the brain and kidney. However, increased expression of IGF-1, IGF-2, and IGF-1R has been documented in various malignancies.

Whereas the IGFs and the IGF-1R are not by themselves oncogenes, experimental and epidemiological evidence suggest that they may enhance proliferation of preneoplastic and neoplastic cells (Baserga 1999) and that IGF-1R expression is a requirement for transformation by oncogenes (Baserga 2005).

The evidence that higher IGF-1 levels might be associated with higher risk of cancer diagnosis was recently reviewed by Pollak *et al*. (Pollak, Schernhammer et al. 2004). So far the best correlations can be found in prostate cancers, colon cancer and some special groups of breast cancers.

Very strong experimental data support that high IGF-1 levels increase proliferation of tumor cells and constitute a candidate risk factor in cancer development (Baserga 1994; Valentinis, Porcu et al. 1994; Werner and LeRoith 1996). Since 1998 several prospective studies have suggested that high IGF-1 level in circulation is associated with an increased risk of developing prostate cancer (Cohen, Peehl et al. 1998), and this association was especially clear in younger men (Harman, Metter et al. 2000; Stattin, Bylund et al. 2000). This is consistent with the hypothesis that high serum IGF-1 levels in younger men predict the incidence of advanced prostate cancer several years later, while IGF-1 levels at the time of diagnosis are not especially informative. This hypothesis suggests that long-term exposure of prostate epithelial cells to high levels of IGF-1 increases the probability of initiating hyperplasia at this level (LeRoith and Roberts 2003).

In 1998 Holly (Holly 1998) reported that premenopausal women with the highest tertile of serum IGF-1 levels had a significantly increased risk of developing breast cancer. Ma *et al.* (Ma, Pollak et al. 1999) and Palmquist *et al*. (Palmqvist, Hallmans et al. 2002) have reported positive associations between serum IGF-1 and colorectal cancer risk in US, Greek and Swedish cohorts, while Probst-Hensch *et al*. (Probst-Hensch, Yuan et al. 2001) found an association between IGF-1 or IGFBP-3 levels and colorectal cancer risk. In a Chinese cohort Yu *et al.* (Yu, Spitz et al. 1999) reported a positive association between high IGF-1 and low IGFBP-3 levels (but not IGF-2) and lung cancer risk.

Increased expression of IGF-1, IGF-1R or both has been documented in glioblastoma, neuroblastoma, melanomas, rhabdomyosarcoma, and leukemias (Belfiore, Pandini et al. 1999; Hakam, Yeatman et al. 1999; Xie, Skytting et al. 1999; Girnita, Girnita et al. 2000; All-Ericsson, Girnita et al. 2002).

During tumorigenesis, overexpression of the IGF-1R increases the cellular responsiveness to the IGFs in terms of proliferation and inhibition of apoptosis. Several oncogenes have been shown to affect IGF-1 and IGF-1R expression (Baserga 1994; Werner, Shalita-Chesner et al. 2000).

IGF-1R is involved not only in the induction of cell transformation but also in the maintenance of the transformed phenotype (LeRoith, Baserga et al. 1995). IGF-1R was identified as a potent regulator of the invasive/metastatic phenotype and IGF-1 was confirmed as a paracrine growth-promoting factor for liver metastasis (All-Ericsson, Girnita et al. 2002).

The IGF-1 receptor is commonly, though not always, overexpressed in many cancers and signaling pathways emanating from the IGF-1R affect cancer cell proliferation, adhesion, migration and cell death (critical functions for cancer cell survival and metastases). In contrast, IGF-1R is not an absolute requirement for normal cell growth (LeRoith, Werner et al. 1995; Yu and Rohan 2000). A better understanding of the IGF system will enable development of novel approaches to diagnose and treat various human cancers.

1.3 THE IGF SYSTEM

Normal cells receive growth stimulatory signals from their surroundings. Without an effective intercellular communication, the behavior of individual cells could not be coordinated. Such communication depends on the capability of some cells of emitting signals and of others of receiving them. Much of this constant chatter is conveyed by growth factors (small proteins that are released by some cells make their way through intercellular space, carrying with them precise biological messages). Growth factors mediate biological responses by binding and activating cell surface receptors with intrinsic protein kinase activity (Aaronson 1991). To date, 58 receptor tyrosine kinases (RTKs), belonging to 20 different receptor families, have been identified (Hubbard and Till 2000; Robinson, Wu et al. 2000). The interaction of a growth factor with its receptor activates a cascade of intracellular biochemical events which are ultimately responsible for the biological response observed.

The IGF system consists of two ligands (IGF-1 and IGF-2), three cell membrane receptors (IGF-1R, IR and IGF-2R) and six high-affinity IGF binding proteins (IGFBP-1 - -6). Additionally, new members of the IGF family have been described, like the insulin receptor related receptor (IRR) (Dandekar, Wallach et al. 1998) (Zhang and Roth 1991) and the IGF-1R/IR hybrid receptor (Treadway, Morrison et al. 1989; Frattali, Treadway et al. 1992) but their activation mechanisms and functions are still largely unknown.

The IGF ligands

IGF-1 and IGF-2 share a 62% homology in amino acid sequence, and there is a 40% homology between the IGFs and proinsulin (Furstenberger and Senn 2002). Several lines of evidence suggest that the binding sites for IGF-1 and IGF-2 on the receptor may be distinct (Steele-Perkins and Roth 1990). Recent receptor binding affinity assays, using a recombinant high-affinity form of the IGF-1R, revealed a 4-fold difference in affinities of IGF-1 and IGF-2, and this agreed closely with cell based assays (Forbes, Hartfield et al. 2002). However, ligand binding affinities may vary with cell type and specific experimental conditions. It is of interest that the IGF-2 concentrations are 5- and 3.5- fold higher than than the IGF-1 levels in human fetal and adult sera, respectively (Bennett, Wilson et al. 1983)

IGF-1 and IGF-2 are major growth factors, while insulin mainly regulates glucose uptake and cellular metabolism.

IGF-1 is a 70–amino acid peptide with a molecular mass 7.5 kDa. The liver is the most important site of IGF-1 production, but this growth factor may be synthesized by almost any tissue in the body (Rosen and Pollak 1999). The autocrine or paracrine production of IGF-1 plays a major role in tissue growth (Cohen and Rosenfeld 1994). Serum IGF-1 levels are affected by many factors and growth GH is the principal regulator of IGF-1 production in the liver and secretion into the bloodstream. IGF-1 in the bloodstream then exerts feedback regulation on the hypothalamus and pituitary gland, reducing GH secretion from the anterior pituitary gland. Serum IGF-1 levels also change substantially with age, increasing slowly from birth to puberty, surging during puberty and declining with increasing age thereafter. As IGF-1 is produced at only low levels during the embryonic period, it is considered to be more important for postnatal growth and development. Most effects of IGF-1 result from the activation of IGF-1R.

IGF-2 is a polypeptide with a molecular mass of about 74 kDa. IGF-2 is produced in various tissues. Serum concentration of IGF-2 remains stable after puberty and is not regulated by GH. On the other hand, IGF-2 plays a fundamental role in embryonic and fetal development, whereas its role in the postnatal period appears to be less important as it can largely be replaced by IGF-1 (Yu and Rohan 2000). Deletion of the paternally imprinted *IGF-2* gene, which is normally expressed in the trophoblast, results in placental insufficiency and low fetal weight (Constancia, Hemberger et al. 2002). IGF-2 interacts with IGF-1R, IGF-2R and IR (mainly with the IR molecular isoform IR-A).

IGF receptors

IGF-1R and IR posses tyrosine kinase activity and mediate the biological effect of the three ligands. Therefore, IGF-1 functions primarily by activating the IGF-1R, insulin by activating IR, whereas IGF-2 can act through either the IGF-1R or through the IR-A isoform. Most of the biological actions of IGF-1 and IGF-2 are mediated by IGF-1R. There is a high homology (70%) between the IGF-1R and IR amino acid sequences especially within the tyrosine kinase domain (84%) (Ullrich, Gray et al. 1986; Sepp-Lorenzino 1998), whereas IGF-1R and IGF-2R differ completely in structure.

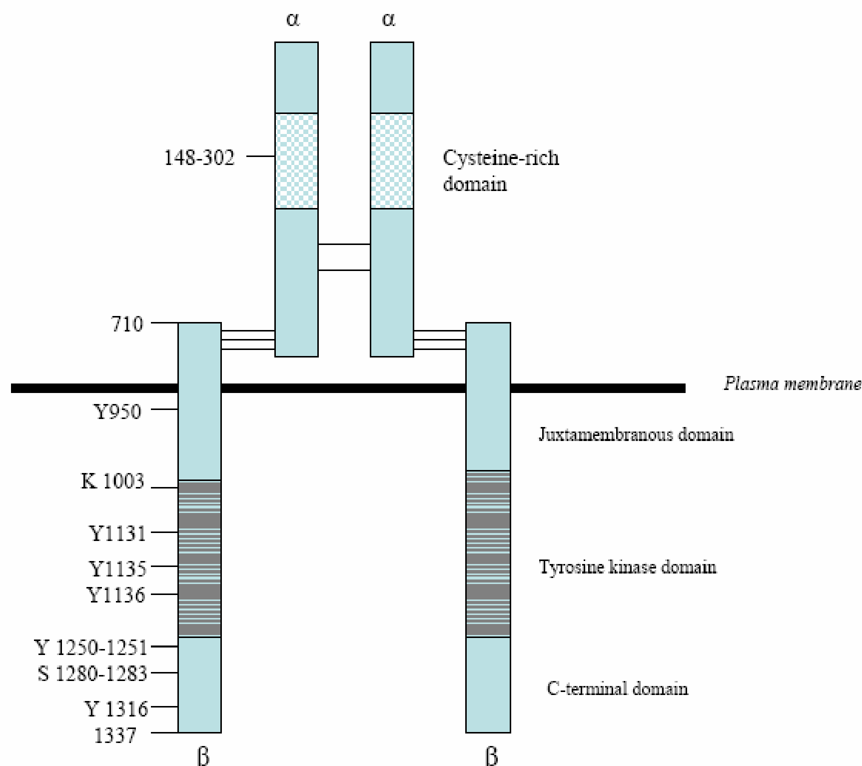

Figure 1. **Schematic representation of the IGF-1 receptor**. Domains and amino acid residues important for tyrosine kinase activity and signal transduction are indicated.

The human IGF-1R cDNA contains an open reading frame of 4101 nucleotides that encode a protein of 1367 amino acids. The IGF-1R is synthesized as a single chain pre-propeptide with a 30-amino acid signal peptide that is cleaved after translation. The propeptide is then glycosylated, dimerized and transported to the Golgi where it is processed at a furin cleavage site to yield α- and β-subunits. These subunits, through disulfide bonds, form a tetramer (β-α-α-β) that is transported to the plasma membrane It has been shown that N-linked glycosylation of IGF-1R is necessary for its translocation to the cell surface (Carlberg, Dricu et al. 1996; Jansson, Hallen et al. 1997; Wang, Xie et al. 1999). This translocation may be rate-limited by the availability of dolichyl phosphate in the endoplasmic reticulum (Carlberg, Dricu et al. 1996). The mature cell membrane-bound IGF-1R consists of two 130- to 135-kDa α-chains and two 90- to 95-kDa β-chains, with several α–α and α–β disulfide bridges (Massague and Czech 1982). The α-subunits, which are entirely extracellular, form the ligand-binding domain that binds one ligand molecule.

The α-subunit contains 706 amino acids and has in its structure two homologous domains, L1 and L2, separated by a cyteine-rich domain, containing 25 or 27 cysteines in three repeating units. The α-subunit is entirely extracellular and forms a dimer with the other/homologue α-subunit. The cysteine-rich domain (aa 148-302) is also conserved in the IR (Andersen, Kjeldsen et al. 1990; Gustafson and Rutter 1990; Kjeldsen, Andersen et al. 1991; Schumacher, Mosthaf et al. 1991; Zhang and Roth 1991) and the ligand binding pockets of IGF-1R and IR are formed by the extracellular α-subunits and possibly some extracellular portions of the β-subunits. Differences in receptor ligand specificities are likely to be dictated by sequence differences within this region, and indeed lower homology was found in the amino-acids sequences of the extracellular cysteine-rich domains (48%), C-terminal of the α subunits (47%) and N-terminal portion of the β-subunits (41 %). These regions are the most hydrophilic sequences of the extracellular domain and are likely to be exposed on the surface of this domain and function in defining ligand specificity (Ullrich, Gray et al. 1986). The extracellular subunit ends with three fibronectin tipe III (FnIII) domains.

The β-subunit contains 627 amino acid residues, spans the plasma membrane and has three domains, being the extracellular, transmembranous and intracellular domains. The extracellular domain of the β-subunit is 196 amino acids in length, while the transmembranous domain is 24 amino acids in length (located at position 906-929). The intracellular part of β-subunit can be divided in a juxtamembranous, a tyrosine kinase (TK) and a C-terminal domain. The homology between IGF-1R and IR at these levels are different. The TK domain exhibits the highest homology between the two receptors (84%) and the juxtamembranous domain shares 61% of homology, whereas the C-terminal domain shares only 44% (Ullrich, Gray et al. 1986). Despite this high degree of homology, experimental evidences suggest that the two receptors have distinct biological roles. The Insulin receptor is known to be a regulator of glucose transport and biosynthesis of glycogen and fat (Patti and Kahn 1998), whereas the IGF-1R is a potent regulator of cell proliferation and differentiation (Lammers, Gray et al. 1989; Blakesley, Scrimgeour et al. 1996). An important clue here could be the three discrete IGF-1Rs regions of low-homology sequence, following residues 986, 1072 and 1208. The presence of a highly heterogeneous sequence within otherwise highly conserved tyrosine kinase domains of gene family members appears highly significant and indicates a possible function of this subdomain in definition of specific receptor function. The carboxy-terminal receptor domain may, in conjunction with the nonapeptide sequence at position 1073-1081 and the divergent membrane-proximal region between residues 933 and 955, be responsible for receptor-specific, ligand-induced, intracellular signal generation (Ullrich, Gray et al. 1986).

Within the TK domain a cluster of three tyrosine residues, located at position 1131, 1135 and 1136, is critical for receptor autophosphorylation (LeRoith, Werner et al. 1995). Also, the presence of the catalytic region, containing the ATP binding motif (Gly-XXX-Gly-XXX-XXX-Gly) at position 976-981 and a catalytic Lys in position 1003 are essential for the ATP binding (Hanks, Quinn et al. 1988).

IGF-1R signaling is transmitted by an intracellular domain consisting of a binding site for phosphorylated substrates at tyrosine residue 950, a tyrosine kinase domain that containing the ATP-binding site at lysine 1003 and three critical tyrosines at positions 1131, 1135 and 1136 and a C-terminal domain containing several tyrosines and serines, such as tyrosines 1250, 1251, and 1316 and serines 1280–1283 that are phosphorylated and play a role in IGF-1R signaling. The contributions that these C-

terminal domain amino acids make to IGF-1R function in normal and malignant cells are not fully understood.

Mutation at Y 950 decreases the effectiveness of the receptor, which is, however, still mitogenic in response to IGF-1. The lysine at 1003 is the ATP binding site. Mutation at the lysine 1003 results in a non-functional receptor. Mutations at the three tyrosine residues (Ys) of the tyrosine kinase domain result in an almost but not completely inactive receptor (Baserga 2000). Mutations of the ATP binding site that ablate kinase activity or at Y950 (the major binding site for IRS1) abolish both proliferation and transformation (Gronborg, Wulff et al. 1993; Coppola, Ferber et al. 1994; Li, Ferber et al. 1994), clearly demonstrating that these residues are required for both mitogenic and transformation signaling.

Autophosphorylation of the IGF-1R β-subunit is unaffected by replacement of the C-terminal tyrosine residues. The total level of IGF-1R phosphorylation as well as the phosphorylation of adaptor proteins IRS1 and Shc are unaffected by the C-terminal mutated IGF-1R. Furthermore Grb-2 association with phosphorylated IRS1 and SHC was similar in cells expressing the wild type or the mutated IGF-1Rs. In conclusion, the tyrosine residues in the C-terminus of the receptor do not significantly mediate signals that use the MAP kinase or PI3-kinase pathways (Esposito, Blakesley et al. 1997). However, the C-terminal region of the IGF-1R is required for transformation (Surmacz, Sell et al. 1995).

Regarding the antiapoptotic function of IGF-1R, the studies performed by O'Connor *et all* (O'Connor, Kauffmann-Zeh et al. 1997) suggested that the residues important for protection against apoptosis are distinct from those involved in mitogenesis and that partially overlap with those mediating cell transformation. Thus, point mutation of some residues within C-terminal domain such as Y1250F/Y1251F and H1293F/K1294R ablate antiapoptotic function, whereas IGF-1R C-terminal truncation mutants d1229 and d1245 IGF-1Rs retain anti-apoptotic activity (O'Connor, Kauffmann-Zeh et al. 1997). Therefore, this describes an alternative antiapoptotic pathway that originates from the serines at positions 1280 to 1283, probably through the intervention of 14.3.3 protein resulting in translocation of Raf-1 to mitochondria (Peruzzi, Prisco et al. 1999).

IGF-2 can also bind to a second receptor, **IGF-2R**, which is identical to the cation-independent mannose-6-phosphate receptor and serves as a scavenger receptor (Moschos and Mantzoros 2002) Interestingly, IGF-2 can also bind to the insulin receptor subtype A (IR-A), with an affinity similar to that of insulin. IR-A is more mitogenic than subtype B (Sciacca, Costantino et al. 1999), the latter having a metabolic function. IR-A is expressed in certain tumors, such as mammary cancers, and the IGF-2/IR-A interaction may play a role in cancer growth. In addition, **hybrid** heterodimeric receptors consisting of insulin and IGF-1 receptor subunits may form and could play a role in receptor signaling in normal and abnormal tissues. For example, one study, examining eight human breast cancer cell lines and 39 human breast cancer specimens, found that the hybrid receptor content exceeded the IGF-1R content in over 75% of the specimens. In the human breast cancer cell line MDA-MB157 these hybrid receptors were autophosphorylated in response to IGF-1. This response exceeded IGF-1R autophosphorylation and led to increased proliferation, suggesting that the hybrid

receptors were the major mediators of IGF signaling in these cells (Pandini, Vigneri et al. 1999).

IGF binding proteins (IGFBPs)

The physiological activities of the IGFs are modulated by their association with the IGFBPs. These include a structurally related superfamily of secreted proteins consisting of BP1–6 that bind the IGFs with different affinities (*e.g*., IGFBP-6 binds IGF-2 with a 20- to 100-fold higher affinity than IGF-1) and several related proteins that bind ligand with lower affinities (Clemmons 1998; Rosenfeld, Hwa et al. 1999)

The IGFBPs regulate the biological accessibility and activity of the IGFs in many ways. They transport IGFs from the circulation to peripheral tissues (*e.g*., IGFBP-1, -2, and -4), maintain a reservoir of IGFs in the circulation (IGFBP-3), potentiate or inhibit IGF action and mediate IGF-independent biological effects.

1.4 IGF-1R ACTIVATION

The mechanism which underlies the operation of RTKs, transphosphorylation, has been elucidated first for EGF receptor but subsequently for all tyrosine-kinase receptors.

One of the major features, distinguishing the IGF-1R from most other tyrosine-kinase receptors, is that it requires domain rearrangements rather than receptor oligomerization for cellular signaling. When ligand binds to the extracellular subunit of the IGF-1R it induces conformational changes in the transmembrane subunits, resulting in trans-autophosphorylation of the TK. The crystal structure of the inactive and phosphorylated kinase domain of the IGF-1R has provided a molecular model of the IGF-1R catalytic activity (Favelyukis, Till et al. 2001). In the unstimulated state, the activation loop (A-loop), containing the critical tyrosine (Y) residues 1131, 1135 and 1136, behaves as a pseudosubstrate that blocks the active site. Y1135 (being the first tyrosine to be phosphorylated) in the A-loop is bound in cis position in the active site, thus preventing the substrate access and occluding the ATP binding site as well. After ligand binding, the three tyrosines of the A-loop are transphosphorylated by the dimeric subunit partner. The first site of autophosphorylation is Y1135, followed by 1131 and then by Y1136. Phosphorylation of Y1135 and Y1131 destabilizes the autoinhibitory conformation of the A-loop, whereas phosphorylation of Y1136 stabilizes the catalytically optimized conformation of it (Favelyukis, Till et al. 2001). Moreover, kinetic experiments on autophosphorylation of the purified 0P, 1P, 2P and 3P forms of IGF-1R kinase indicate that each phosphorylation event causes an increase in catalytic efficiency. The overall increase in catalytic efficiency from 0P to 3P is over 120-fold (Favelyukis, Till et al. 2001).

The kinase activity is at a low basal level, but sufficient to induce trans-autophosphorylation once stimulated. The substitution of the Y1135 has relatively small inhibitory effect on receptor autophosphorylation (Stannard, Blakesley et al. 1995). The same effect is obtained by modifying the Y1131 (Li, Ferber et al. 1994). In contrast, substitution of the Y1136 impaired the function of the receptor (Li, Ferber et al. 1994). More interestingly, double substitution of tyrosines 1131/1136 or 1135/1136

reduces autophosphorylation level by 50%, whereas substitution of tyrosines 1131/1135 blocks any detectable autophosphorylation (Hernandez-Sanchez, Blakesley et al. 1995).

The changes of the A-loop conformation allow the substrate and ATP access to the kinase active site. Autophosphorylation also occurs at tyrosine residues in the juxtamembranous and carboxyl-terminal domains (flanking the TKR domain) and creates docking sites for downstream signal transduction molecules.

1.5 SIGNALING PATHWAYS

As a consequence of ligand-induced transphosphorylation, a receptor molecule will acquire and display a characteristic array of phosphotyrosine residues on its cytoplasmic tail. These phosphotyrosines become attractive homing sites especially for various SH2-containing cytoplasmic proteins (specifically, proteins that are free to move from one location to another in the cytoplasm). Consequently, shortly after becoming activated by IGF binding, IGF-1R becomes decorated with a specific set of partner proteins that are attracted to its various phosphotyrosines, leading to activation of signaling cascades. The multiple cellular responses that IGF-1 elicits could be explained by the fact that IGF-1R is able to activate a specific combination of downstream signaling pathways. The ultimate targets of the MAPK and PI3K pathways (the major signal transduction cascades) include members of the Ets and Forkhead transcription factor families. Regulation of transcription factors provides a mechanism by which IGF action at the cell surface can elicit changes in gene expression that eventually mediate the proliferative, differentiative and apoptotic effects of IGFs.

MAPK Pathway

Following activation of the IGF-1R kinase, SH2-containing protein Shc becomes activated by phosphorylation and makes a complex with the second adaptor protein, the growth factor receptor-binding protein 2 (Grb2). Grb2 can be activated also by binding directly to phosphorylated IRS1 via its SH2 domain. Grb2 interacts with the Sos (son of sevenless, a guanine nucleotide exchange protein). Sos stimulates the release of GDP and subsequent binding of GTP to the low-molecular-weight protein Ras.

Ras protein is able to interact physically with several alternative downstream signaling partners. The first of Ras effectors is the Raf kinase. Like the great majority of protein kinases in the cell, Raf phosphorylates substrate proteins on their serine and threonine residues. The activation of Raf by Ras depends upon the relocalization of Raf within the cytoplasm (Ras proteins are always anchored to the inner surface of the plasma membrane through their C-terminal hydrophobic tails).

During the time it is anchored to Ras, Raf becomes phosphorylated, acquires active signaling powers and proceeds to phosphorylate and activate a second kinase known as MEK (MAPKK). MEK, a kinase which can phosphorylate serine/threonine residues as well as tyrosine residues, will phosphorylate two other kinases, the *e*xtracellular signal-*regulated k*inases 1 and 2, commonly referred to as ERK1 and ERK2. (Boulton, Nye et al. 1991; Robbins, Cheng et al. 1992). Phosphorylated and activated each of ERKs then phosphorylates substrates that, in turn, regulate various cellular processes.

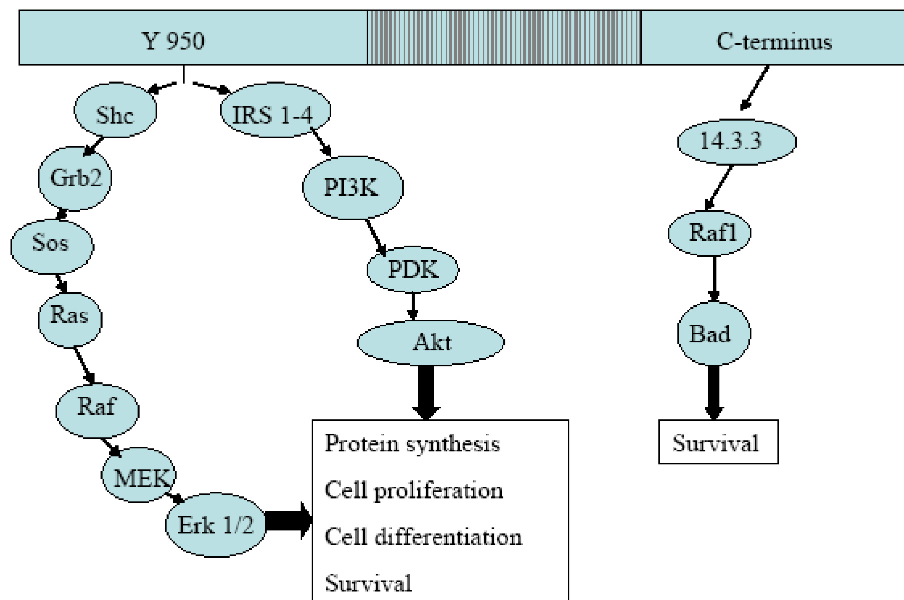

Figure 2. **Signalling pathways of the IGF-1R.** Schematic diagram of the three main signalling pathways originating fron the IGF-1R.

This signaling cascade is called MAPK (mitogen-activated protein kinase) pathway. ERK1 and ERK2 are considered as MAPKs. The kinase responsible for phosphorylation of a MAPK is termed generically a MAPKK. The kinase responsible for phosphorylation of a MAPKK is therefore called MAPKKK (Raf is classified as a MAPKKK).

Once activated, ERK kinases proceed to phosphorylate cytoplasmic substrates and can also translocate to the nucleus, where it causes the phosphorylation of several transcription factors (Ets, Elk-1 and SAP-1) directly and, in addition, phosphorylate and activate other kinases, resulting in activation of other transcription factors (Seger and Krebs 1995; Lenormand, Brondello et al. 1998). At the same time, the Mnk1 kinase, a cytoplasmic substrate of ERK1 and ERK2, activates the translation initiation factor eIF4E, thereby helping to activate the cellular machinery responsible for protein synthesis.

ERKs are involved in spindle formation and mediate also transcriptional induction of the *cyclin D1* gene; stimulate phosphorylation of the pRb protein and release of the E2F-1 transcription factor (Peeper, Upton et al. 1997). The free E2F-1 can activate in its turn the transcription of p14 ARF (Bates, Phillips et al. 1998). ARF

was shown to inhibit the p53-MDM2 association that maintains p53 in its inactive form (Sherr and Weber 2000). In its turn p53 can directly interfere with the MAPK cascade by inactivating ERK2/MAPK via caspase/mediated cleavage (Marchetti, Cecchinelli et al. 2004).

In the cytoplasm MAPK regulates microtubule dynamics by phosphorylating microtubules associated proteins. Cyclin B is also a substrate for MAPK and its phosphorylation on MAPK sites is important for the translocation of CyclinB/Cdc2 complex to the nucleus where is activated by CDC 25C.

IRS1-PI3K Pathway

IRS1 is one of the first molecules that reach full activation after phosphorylation of tyrosine residues of intracellular subunit of IGF-1R. IRS1 has three domains: an N-terminal pleckstrin homology (PH) domain, a phosphotyrosine-binding domain (PTB) and a C-terminal domain (with more than 20 potential phosphorylation sites interacting with SH2 domain-containing proteins) (Wang, Myers et al. 1993). Grb2, SH-PTP2 (a tyrosine phosphatase), p85 and other adaptor proteins like Crk and Nck contain SH2 domains and interact with activated IRS1. Among other molecules involved in interaction with IRS1, secondary to IGF-1R activation are β1-integrins (important in cell adhesion to laminin) (Goel, Fornaro et al. 2004).

IRS1 interact with p85 (a regulatory subunit of PI3K), leading to activation of catalytic subunit p110 of PI3K and inducing phospholipid products as a downstream signal. P85 can also bind direct to the phosphorylated intracytoplasmic region of IGF-1R. These phospholipids function as ligands for pleckstrin-homology (PH) domain-containing proteins. Akt/PKB is a serine threonine kinase which interacts with these phospholipids causing its translocation to the inner membrane and activation by the 3-phosphoinositide-dependent protein-kinases (PDKs). IGF-1 stimulation of the PI3K pathway induces phosphorylation of the Thr308 and Ser473 residues on Akt and activates this kinase (Alessi, Andjelkovic et al. 1996; Balendran, Currie et al. 1999). Active Akt is in its turn phosphorylates and inhibits several proapoptotic proteins such as Bad (del Peso, Gonzalez-Garcia et al. 1997) and caspase 9 (Cardone, Roy et al. 1998). At least three other Akt effectors have been described. These are the survival transcription factor cyclic AMP response element binding protein (CREB), the proapoptotic effector proteins glycogen synthase kinase-3β (GSK-3β) and winged-helix family of forkhead transcription factors (Zheng, Kar et al. 2002; Leinninger, Backus et al. 2004).

The activated Akt can phosphorylate and dissociate Bad from Bad-Bcl2 complex. Thus, while Bad is sequestered into a complex by 14.3.3, Bcl2 is released to produce antiapoptotic effect. In the absence of Bad phosphorylation, cytochrome c is released from mitochondria by proapoptotic proteins like Bax, and activates caspases 3 and 9 (Bouchard, Rouleau et al. 2003) .

Akt activation can also induce stimulation of mTOR that will lead to phosphorylation of the 40S ribosomal S6 protein by the p70S6 kinase (Dupont, Pierre et al. 2003), affecting protein-synthesis machinery and transition from G0 to G1 of the cell cycle. At the same time mTOR activation can induce (via 4E-BP phosphorylation) translation of cyclin D1 (Blume-Jensen and Hunter 2001). In some cell lines mTOR will also induce activation of MMP2 with effects on cell migration and metastasis potential (Zhang, Bar-Eli et al. 2004).

Another effect of Akt activation is phosphorylation of MDM2 on serine 166 and serine 186 (necessary for translocation of MDM2 from the cytoplasm into the nucleus) (Mayo and Donner 2001).

An important cross-talk between Akt and MAPK pathway at the level of Raf and ERK has been described in MCF7 breast cancer cells. Akt antagonizes Raf activity by direct phosphorylation of Ser259 and creates a binding site for the 14.3.3 protein with negative effect on Raf activity (Zimmermann and Moelling 1999). Other experiments suggest that the IGF-1 signal leading to stimulation of DNA synthesis of MCF7 cells is transduced to ERK through PI3K, but only when the cells are anchorage-deficient (Suzuki and Takahashi 2000).

Other emerging pathways

14.3.3 proteins (a family of proteins that bind to serine/threonine-phosphorylated residues) interact with IRS1 or directly to the phosphoserine residues within the COOH-terminus of the IGF-1R. 14.3.3 proteins regulate key molecules involved in different physiological processes such as intracellular signaling (e.g., MEKK, PI3K,IRS1), cell cycling (e.g. Cdc25, CDK2, centrosomes), apoptosis (e.g. Bad, ASK-1) and transcription regulation (e.g. FKHRL1, p53, TAZ, TLX-2). Remarkably, 14.3.3 proteins in many cases alter the function of the target proteins, allowing them to serve as direct regulators of their targets (Tzivion, Shen et al. 2001). Activated 14.3.3 proteins antagonize the activity of associated pro-apoptotic proteins, including Bad and ASK-1. Thus, expression of 14.3.3 inhibitor peptides in cells is sufficient to induce apoptosis. These 14.3.3 antagonist peptides can sensitize cells for effective killing by anticancer drugs such as cisplatin (Masters, Subramanian et al. 2002). Activated Akt can also induce the association of Bad and 14.3.3. The prevention of Bad-related death by growth factor regulation of Bad phosphorylation is a major mechanism by which IGF-1R induces cell survival.

The IGF-1R activates the JAK and STAT pathways (the IGF-1R phosphorylates JAK1, JAK2 and then STAT3 (Emanuelli, Peraldi et al. 2000; Zong, Chan et al. 2000))

C-Crk, an adaptor protein in the Ras pathway, that associates with mSOS and C3G, appears to be an IGF-1R specific substrate (Beitner-Johnson and LeRoith 1995). There are suggestions that IR and IGF-1R are activating alternative pathways by acting as G protein-coupled receptors which engage different G-proteins (Dalle, Ricketts et al. 2001).

In conclusion, the specific pathways predominantly activated by IGF-1R stimulation depend on the cell type. However the mechanisms that underly this specificity are still unknown, but may be determined by expression levels of downstream substrates such as IRS or Shc.

1.6 FUNCTIONS OF IGF-1R

IGF-1R stimulation induces signaling via different pathways and the selected pathway determines the specific effects. Signaling through IRS1 promotes mostly mitogenesis and protection from apoptosis, while signaling through Shc proteins favors

differentiation if IRS1 is absent or very low. In the absence of IRS1, the IGF-1R has alternative pathways for survival that center on Raf1 and its translocation to mitochondria (Peruzzi, Prisco et al. 1999).

Mitogenic function of IGF-1R

The involvement of the IGF system in the cell cycle progression was demonstrated by the group of Renato Baserga (Baserga and Rubin 1993; Rubin and Baserga 1995). These studies showed that the interaction between IGF-1 and IGF-1R is sufficient for most cells to progress through the cell cycle. IGF-1R expression is the critical determinant that causes cells to switch from a 'non-mitogenic' to a 'mitogenic' model. In accordance with this hypothesis, Balb/c-3T3 cells stably transfected with an expression vector encoding the IGF-1R can grow in the sole presence of IGF-1. When both the receptor and ligand are expressed, cells are able to grow in the absence of any exogenous growth factor (Pietrzkowski, Lammers et al. 1992). For comparison, growth of parental Balb/c-3T3 cells requires supplementation of the growth media with PDGF and EGF. According to this hypothesis, IGF-1 acts in concert with initiation factors such as EGF and PDGF to induce cell cycle progression (Coppola, Ferber et al. 1994; DeAngelis, Ferber et al. 1995; Baserga, Hongo et al. 1997). Experimental evidence showing that competence factors such as PDGF and FGF increase the expression of the IGF-1R gene by stimulating its promoter activity supports this concept (Rubini, Werner et al. 1994; Hernandez-Sanchez, Werner et al. 1997).

IGF-1R stimulates mitogenesis in many different cell types, a function localized mainly to the tyrosine kinase and in some systems to the C-terminal domain of the β-subunit (Hongo, D'Ambrosio et al. 1996; Esposito, Blakesley et al. 1997).

IGF-1R and the cell cycle

Cell cycle progression can be regulated by IGF-1R through control of several cycle checkpoints. The IGF-1R can facilitate G0-G1 transition through activation of p70 S6K, leading to phosphorylation of the S6 ribosomal protein and an increased ribosomal pool necessary for entry into the cycle (Dupont, Pierre et al. 2003). It can promote G1-S transition by increasing cyclin D1 and CDK4 gene expression, leading to retinoblastoma protein phosphorylation, release of the transcription factor E2F, and synthesis of cyclin E (Dupont, Karas et al. 2003). The IGF-1R-induced increase in cyclin D1 synthesis can be mediated through other mechanisms. It may be transcriptionally regulated through the ERK pathway or it mediated through increased mRNA stability in a PI3K/Akt-dependent manner. PI3K/Akt signaling can also increase cyclin D1 levels through enhanced mTOR-mediated protein translation and inhibition of GSK-mediated cyclin D1 phosphorylation (Hamelers, van Schaik et al. 2002). In addition, IGF-1R can also downregulate the transcription of the cyclin-dependent kinase (CDK) inhibitor (CDKI) p27KIP1 or alter its processing and nuclear localization through PI3K/Akt.

The IGF-1R may also exert a regulatory role in G2-M transition, possibly by increasing cyclins A and B and cdc2 synthesis (Furlanetto, Harwell et al. 1994),

Taken together, the data suggest that the IGF-1R/IGF axis can positively regulate cell cycle progression at several phases, but its major direct effect is probably exerted at the G1-S transition.

Effects on cellular differentiation

Under certain conditions, myoblasts, osteoblasts, adipocytes, oligodendrocytes, neurons and hematopoietic cells can be induced to **differentiate** by IGF-1R (Petley, Graff et al. 1999). Myoblasts in cultures are undifferentiated cells, which can grow indefinitely in serum, but differentiate into myocytes if the serum is removed or decreased. If the cells are incubated with IGF-1 after serum removal, they proliferate for shorter time and is followed by differentiation (Navarro, Barenton et al. 1997). Experiments in 32D cells (murine hematopoietic cells of myeloid lineage undergoing apoptosis within 24 hours after withdrawal of Interleukin-3, IL3) have pointed out that they survive in the absence of IL3 (when they overexpress the IGF-1R) after addition of IGF-1. However, after 48 hour growth, the cells begin to differentiate along the granulocytic pathway (Valentinis, Romano et al. 1999).

The IGF system of ligands, receptors and binding proteins is undoubtedly a major player in normal cellular growth and differentiation, as well as in aberrant growth seen in neoplastic disorders. Whereas the IGFs and the IGF-1R are not by themselves oncogenes, experimental and epidemiological evidence suggest that they may enhance proliferation of preneoplastic and neoplastic cells (Baserga 1999). Furthermore, down-regulation or functional inactivation of IGF-1R sensitizes tumor cells to apoptosis or reverses tumor cell phenotype.

Antiapoptotic function of IGF-1R

The antiapoptotic function of the IGF-1R allows IGF-1R to function as a cell survival agent. Accordingly, the domains of the IGF-1R required for its antiapoptotic function are different from those required for its proliferative role (O'Connor, Kauffmann-Zeh et al. 1997). Important for apoptosis prevention are tyrosine residues 1250 and 1251, histidine 1293 and lysine 1294, all of which are localized in the C-terminus of the β-subunit (O'Connor, Kauffmann-Zeh et al. 1997).

The capacity of the IGF-1R to protect cells from programmed death has been demonstrated in many different systems (Rodriguez-Tarduchy, Collins et al. 1992) and *in vivo* models (Werner and Le Roith 2000). These studies proved IGF-1R to be the major single factor determining cell survival. The obvious implication of these findings is that activation of the IGF-1R may rescue cells, tagged for elimination, from apoptosis in the absence of IGFs (Sell, Baserga et al. 1995).

The main signaling pathway for IGF-1R-mediated protection from apoptosis has been previously elucidated and consists in the activation of PI3K and Akt/protein kinase B as well as phosphorylation and inactivation of Bad, a member of the Bcl-2 family of proteins (Datta, Dudek et al. 1997). In its unphosphorylated state, Bad is localized at the mitochondrial membrane where it interacts with Bcl-2 and prevents Bcl-2 from performing its anti-apoptotic functions. Once phosphorylated by Akt/PKB on Ser 126, Bad associates with the cytosolic protein 14.3.3 and becomes unable to interfere with Bcl-2 (Zha, Harada et al. 1996). If Bad is not phosphorylated, proapoptotic proteins (e.g. Bak and Bax) are released from the inhibitory control of

Bcl-2, become activated and cause cytochrome c release from mitochondria. This will results in caspase-9 and subsequently caspase-3 activation (Hanahan and Weinberg 2000). Active caspase-3 cleaves and inactivates/activates its specific substrates. For example, the poly(ADP-ribose) polymerase (PARP), which plays an important role in maintenance of the DNA integrity (Bouchard, Rouleau et al. 2003) is inactivated by cleavage. The final result of caspase-3 activity is extensive proteolysis and degradation of DNA, which represent the final steps in the apoptotic process. Despite the PI3K/Akt antiapoptotic pathway being shown to be shared by both IR and IGF-1R for mitogenesis and/or survival, the treatment with inhibitors of PI3K in mouse embryo fibroblasts suggests that IGF-1R has alternative pathways in this respect (Prisco, Romano et al. 1999). One alternative pathway is the MAPK pathway (Parrizas, Gazit et al. 1997; Peruzzi, Prisco et al. 1999), propageted at least in part through Shc proteins (Pronk, McGlade et al. 1993; Scheid and Duronio 1998). Shc binds to the 950 tyrosine residue of IGF-1R and induces Ras activation (Ceresa and Pessin 1998). Activation of this pathway results also in Bad phosphorylation at a serine residue. This was demonstrated using an MEK inhibitor that caused loss of Bad phosphorylation and apoptosis (Peruzzi, Prisco et al. 1999).

Finally, a third pathway was proposed by Peruzzi *et al*. (Peruzzi, Prisco et al. 1999), based on the integrity of a serine quartet at residues 1280–1283 of IGF-1R (Li, Resnicoff et al. 1996). These serines are known to bind isoforms of the 14.3.3 protein (Craparo, Freund et al. 1997; Furlanetto, Dey et al. 1997) and promote mitochondrial translocation of Raf-1 (Peruzzi, Prisco et al. 1999). Interestingly, 14.3.3 binds to IRS1 even better than it binds to the IGF-1R (Kosaki, Yamada et al. 1998). 14.3.3 proteins have been implicated in many cellular functions, among which are stabilization of phosphorylated Bad (Zha, Harada et al. 1996), the enhancement of Raf kinase activity (Freed, Symons et al. 1994; Li, Janosch et al. 1995) and stabilization of activated Raf-1 (Dent, Jelinek et al. 1995). Targeting of Raf-1 to mitochondria also results in inhibition of apoptosis (Wang, Rapp et al. 1996; Salomoni, Wasik et al. 1998).

According to the studies of Baserga *et al*, IGF-1R has at least three pathways for protection of 32D cells from apoptosis induced by IL-3 withdrawal and the combination of any two of these pathways are sufficient for cells survival (Peruzzi, Prisco et al. 1999; Navarro and Baserga 2001).

Higher receptor levels correlate with increased clonogenic survival under cell culture conditions characteristics for solid tumors (e.g. hypoxia, low pH, low glucose level). This suggest that overexpression of IGF-1R may be an important survival factor for cancer cells in vivo. Experiments using reporter gene assays demonstrated induction of the IGF-1R promoter by hypoxia and low pH (Peretz, Kim et al. 2002). Western blot analyses confirmed accumulation of IGF-1R in cells under hypoxic conditions. The tumor microenvironment can stimulate cells to overexpress IGF-1R, and cells overexpressing the receptor have a slight survival advantage, leading in time to the evolution of the tumor cell population. This pathway may provide one explanation for the frequent observations of IGF-1R overexpression in a variety of human tumors.

Role in cell transformation

The IGF-1R plays an important role in the transformation of cells and the first evidence came with the observation of Sell *et al*. (Baserga and Rubin 1993; Sell, Rubini

et al. 1993). R- mouse embryo fibroblast cells with targeted disruption of IGF-1R gene were refractory to transformation by the SV40 large T antigen (Baker, Liu et al. 1993; Liu, Baker et al. 1993). Since mouse embryo fibroblasts, including 3T3 cells, have a strong tendency to transform spontaneously in cultures, the fact that R- cells were resistant to transformation by c-src and other viral and cellular oncogenes (Coppola, Ferber et al. 1994; Sell, Dumenil et al. 1994; D'Ambrosio, Keller et al. 1995; DeAngelis, Ferber et al. 1995; Miura, Surmacz et al. 1995; Morrione, DeAngelis et al. 1995; Steller, Zou et al. 1996; Toretsky, Kalebic et al. 1997; Valentinis, Morrione et al. 1997) was remarkable. However, there is two exceptions, one being v-src, which is able to induce transformation of R- cells by directly activating the PI3K and MAPK pathways (Penuel and Martin 1999). The second exception is mutated G *alpha* 13 (Valentinis and Baserga 2001).

The wild-type IGF-1R, when overexpressed, can transform cells in culture (colony formation in soft agar) (Kaleko, Rutter et al. 1990; Pietrzkowski, Lammers et al. 1992).

Mutation experiments showed that C-terminal truncated IGF-1R is still a functional receptor with mitogenic (Surmacz, Sell et al. 1995; Hongo, D'Ambrosio et al. 1996) and anti-apoptotic properties (O'Connor, Kauffmann-Zeh et al. 1997; Prisco, Romano et al. 1999). However, R- cells with this mutant lacked transforming capability (D'Ambrosio, Keller et al. 1995; Surmacz, Sell et al. 1995; Tanaka, Ito et al. 1996).

The transforming domain can be tentatively located between residues 1245 and 1310, where at least three domains seem to be involved, the tyrosine residue at 1251, the serine residues at 1280-1283 and, more weakly, the residues at 1293-1294 (Baserga and Morrione 1999).

In conclusion, IGF-1R, with few exceptions, is not an oncogene but its expression is a requirement for transformation by oncogenes (Baserga 1999).

Regulation of cell size

Increase in cell size is an important process required for cell proliferation, from G1 to G2 phase. The cell must double its DNA amount as well as its size (Fraser and Nurse 1978). The growth of cell and body size is largely controlled by the activity of RNA polymerase I, which is in turn regulated by a number of proteins at the rDNA promoter, including the upstream binding factor 1 (UBF1) (Grummt 1999). RNA polymerase I activity regulates ribosome biogenesis, the major determinant factor in cell size (Jorgensen, Nishikawa et al. 2002).

There is a lot of evidence for a role of the IGF axis in regulation of cell and body size (Razzini, Ingrosso et al. 2000). Recent studies have shown that IRS1 can be translocated to the nucleus in cells stimulated with IGF-1 (Prisco, Santini et al. 2002; Tu, Batta et al. 2002; Sun, Tu et al. 2003; Tu, Baffa et al. 2003). All experiments support that IRS1 interacts with and co-precipitates the UBF protein and increases rRNA synthesis (Tu, Batta et al. 2002; Sun, Tu et al. 2003; Wu, Tu et al. 2005). There is evidence that PI3K can be located in the nucleus (Neri, Borgatti et al. 2002) and nuclear PI3K has been shown to directly phosphorylate and activate UBF1 (Drakas, Tu et al. 2004). Accordingly, IRS1 activates UBF1 and the rDNA promoter through

indirect phosphorylation (by PI3K) of specific UBF1 residues and/or by preventing the degradation of UBF1 following IGF-1 stimulation (Baserga 2005).

The IGF-1 induced activation of the ribosomal DNA promoter (Surmacz, Kaczmarek et al. 1987) is further supported by the finding that p70S6K knockout mice are somewhat smaller than their wild type littermates (Shima, Pende et al. 1998). However, the importance of IRS1 and p70S6K in cell size regulation was demonstrated more rigorously by the recent reports that homologues of both IRS1 and the S6 kinase regulate cell size in *Drosophila* (Bohni, Riesgo-Escovar et al. 1999; Montagne, Stewart et al. 1999). The position of Akt in the pathway is less clear, as Akt can also be activated in the absence of IRS1 (Songyang, Baltimore et al. 1997), but a dependence on the PI3K is established through p70S6K inactivation (Cantley 2002).

The role of the IGF-1 axis in growth control is, however, well established. The pioneer work of Estratiadis and collaborators established the role of IGF axis in normal growth *in vivo* (Ludwig, Eggenschwiler et al. 1996). Even though other pathways are involved, a fraction of IGF-1R signaling is non-redundant and cannot be replaced by other growth factors. Subsequent experiments, *in vivo* and *in vitro*, have shown that the residual growth occurring in the absence of the IGF-1R but in the presence of IGF-2, is due to the activation of the IR by IGF-2 (Louvi, Accili et al. 1997; Morrione, Valentinis et al. 1997), specifically the A isoform of the IR (Sciacca, Costantino et al. 1999).

1.7 REGULATION OF IGF-1R

Transcriptional regulation of the IGF-1R gene

The levels of expression of the IGF-1R gene are determined, to a large extent, at the transcriptional level (Werner and Roberts 2003). Molecular characterization of the IGF-1R gene regulatory region revealed a number of features whose presence provides clues as to the molecular basis for the transcriptional control of this gene. The IGF-1R promoter lacks canonical TATA and CAAT sequences, two promoter elements that are generally required for accurate transcription initiation, and that are absent in many cases of 'housekeeping' genes (Werner, Bach et al. 1992). Transcription of this gene, however, starts from a unique site contained within an 'initiator' motif, a discrete promoter element able to direct initiation in the absence of a TATA box. Similar to other widely expressed genes, the IGF-1R promoter is extremely GC-rich (80%) and contains several binding sites for members of the Sp1 family of zinc-finger nuclear proteins. Analysis of physical and functional interactions of Sp1 at the IGF-1R promoter revealed that Sp1 is a potent transactivator of the IGF-1R gene (Beitner-Johnson, Werner et al. 1995). Thus, basal IGF-1R promoter activity was extremely low in Sp1-null *Drosophila*-derived Schneider cells, while cotransfection of an Sp1 expression vector significantly enhanced promoter activity. Recent studies have identified the Kruppel-like factor-6 (KLF6), a zinc-finger transcription factor mutated in prostate cancer and other malignancies, as a potent transactivator of the IGF-1R gene (Rubinstein, Idelman et al. 2004). Combined, these studies accentuate the important role of zinc-finger proteins in stimulation of IGF-1R expression.

In addition, transcription of the IGF-1R gene is negatively regulated by a number of tumor suppressors, including the breast cancer gene-1 (BRCA1), p53, and the Wilms' tumor protein-1 (WT1). Transcriptional suppression of the IGF-1R gene may be responsible for keeping IGF-1R levels below a certain threshold. Moreover, interactions between stimulatory and inhibitory transcription factors seem to determine the level of expression of the IGF-1R gene and, consequently, the proliferative status of the cell.

The product of the tumor suppressor gene *p53* is capable of suppressing the activity of the IGF-1R promoter as well as lowering the endogenous levels of IGF-1R mRNA (Werner, Shalita-Chesner et al. 2000). In addition, the transcription of the IGF-2 gene is similarly reduced by wild-type p53 (Zhang, Kashanchi et al. 1996). In contrast, tumor-derived, mutant versions of p53 significantly stimulated promoter activity (Werner, Karnieli et al. 1996). These data therefore suggest that upregulation of IGF-1R may facilitate selection of a malignant population of cells in the presence of mutant p53. However, the role of p53 in regulation of IGF-1R seems to be more complex and probably also involves post-transcriptional mechanisms (Girnita, Girnita et al. 2000; Lee, Han et al. 2003). This can be exemplified by malignant melanoma cells, most often harbouring wild-type p53 (Hussein 2004), which exhibit overexpression of IGF-1R. Upon inhibition of wild-type p53 in these cells, they surprisingly responded with a drastic IGF-1R downregulation and cell death (Girnita, Girnita et al. 2000). Similar results have been obtained in other studies (Lee, Han et al. 2003). These observations points to the action of other mechanisms in the p53-dependent control of IGF-1R expression.

Post-Ligand Binding Receptor Processing

Role of phosphatases

Ligand binding stimulates the phosphorylation of the membrane scaffolding protein Src homology 2 domain-containing protein tyrosine phosphatase substrate-1 (SHPS-1). Phosphorylated SHPS-1 then recruits the Src homology 2 domain tyrosine phosphatase (SHP-2) to the phosphorylated IGF-1R (Maile and Clemmons 2002) SHP-2 can have both positive and negative effects on IGF-1R signaling. The dephosphorylation of IGF-1R by SHP-2 attenuates PI3K-mediated IGF-1R signaling, as has also been shown for the IR (Myers, Mendez et al. 1998). In contrast, data suggest that MAPK signaling may actually be enhanced by SHPS-1 dephosphorylation and SHP-2 recruitment through Shc/Grb2 (Ling, Maile et al. 2005)

Src can associate with SHP-2 and the p85 subunit of PI3K is the receptor for activated C kinases (RACK1), a homolog of the β-subunit of heterotrimeric G proteins. It has been shown that RACK1 also interacts with IGF-1R (Hermanto, Zong et al. 2002) in a tyrosine kinase activity- and receptor autophosphorylation-independent manner and that this requires an intact serine 1248 in the C terminus of the receptor. Interestingly, overexpression of RACK1 has a negative effect on the activation of the PI-3K pathway, but a positive effect on the activation of the MAPK and c-Jun N-terminal kinase (JNK) pathways, as was also shown for IGF-1R-associated SHP-2 (Kiely, Sant et al. 2002).

Protein tyrosine phosphatase 1B (PTP-1B), a 50-kDa non-transmembrane tyrosine phosphatase, is localized predominantly in the endoplasmic reticulum with its phosphatase domain oriented toward the cytoplasm (Frangioni, Beahm et al. 1992). PTP-1B can dephosphorylate both IGF-1R and IRS1. In MEFs derived from PTP-1B-deficient mice, IGF-1-induced IGF-1R autophosphorylation and kinase activity were higher than in controls (Buckley, Cheng et al. 2002). IGF-1R may, in turn, inhibit PTP-1B activity, perhaps through a negative feedback loop.

Role of integrin αvβ3

An important role in IGF-1R signaling and its biological functions plays the integrin αvβ3 (Clemmons and Maile 2003). The αvβ3 signaling changes the subcellular localization of SHP-2 in a way that decreases its access to phosphorylated IGF-1R, thereby prolonging IGF-1R signaling. Ling *et. al*(Ling, Maile et al. 2003) identified β3 as the subunit that recruits SHP-2 and prevents its association with IGF-1R. In smooth muscle cells, echistatin, a disintegrin that blocks αvβ3 ligand binding, reduced receptor phosphorylation, cellular migration and DNA synthesis in response to IGF-1(Maile and Clemmons 2002). In some cells, IGF-1 can induce a potent JNK response (Monno, Newman et al. 2000). JNK, in turn, can mediate serine phosphorylation of IRS1 and thereby attenuate IGF-1R signaling (Mamay, Mingo-Sion et al. 2003).

Role of IGFBP-3

IGF-1 up-regulates IGFBP-3 at transcriptional and/or posttranscriptional levels (Bale and Conover 1992). The cell context determines the final effect of IGFBP-3 on the cell and whether it acts as an inhibitor or potentiator of IGF functions. In normal and transformed mammary epithelial cells, IGFBP-3 potentiates the mitogenic effects of IGF-1 (Cohick, Wang et al. 2000). On the other hand, in other cancer cells such as the human breast carcinoma cell line MCF-7, IGFBP-3 activates a phosphotyrosine phosphatase that dephosphorylates IGF-1R, thereby disrupting the signaling. This inhibitory effect is independent of IGF-1 binding (Ricort and Binoux 2002). In several non-small cell lung cancer cell lines, IGFBP-3 has been shown to act as a potent inhibitor of IGF-1R signaling by interfering with both the PI3K/Akt and MAPK signaling pathways, causing growth arrest and inducing apoptosis (Lee, Chun et al. 2002).

1.8 INTERNALIZATION, DEGRADATION / UBIQUITINATION AND RECIRCULATION

The most prominent regulator of IGF-1R signal termination is downregulation – a term used to denote the desensitization of receptors by the removal of activated receptors from the cell surface by accelerated endocytosis.

Internalization of IGF-1R

Receptor downregulation allows the cells to return to an unstimulated basal state. This process is initiated by internalization of the phosphorylated receptors (Sepp-Lorenzino 1998). Similar to other signal transducing receptors, ion channels and transporters located at the plasma membrane, the activity of the IGF-1R is regulated by controlling the level of the protein present at the cell surface. To reduce the receptor activity, the protein is internalized through a process called endocytosis. Ligand-mediated endocytosis plays at least two functions, these are signaling attenuation of an activated receptor and signal activation (facilitating the interaction between RTK and downstream signaling molecules).

Some receptors are internalized constitutively and recycled (e.g. transferrin receptor), whereas most of the tyrosine kinase growth factor receptors and G protein-coupled receptors are internalized after ligand binding (Robinson 1989; Koenig and Edwardson 1997). Internalized receptors can either be transported to the lysosomes, where they are degraded or are recycled to the plasma membrane(Hicke 1999).The fate of an internalized receptor is decided within early endosomes.

Many cell surface receptors undergo endocytosis, being incorporated in clathrin-coated vesicles (Pearse and Robinson 1990). After binding of the ligand, the activated receptors are targeted to the clathrin-coated membrane invaginations (Ceresa and Schmid 2000). This is a process mediated by a specific **internalization signal** situated within cytoplasmic domain of the receptor (Hicke 1999). Two types of internalization signals have been described, a tyrosine-based motif and a di-leucine based motif (Goldstein, Brown et al. 1985; Davis, van Driel et al. 1987; Letourneur and Klausner 1992). These internalization motifs are usually located within the juxtamembrane region of the receptor (Johnson and Kornfeld 1992; Bremnes, Madsen et al. 1994).

Emerging evidence suggests that IGF-1R internalization and signaling are regulated by mechanisms both similar to, and distinct from, those of other receptor kinases. The mitogenic responses of IGF-1R were shown to be regulated by adaptor protein-2 (AP-2)-dependent endocytosis and two potential "linkers" (Lin, Daaka et al. 1998; Rotem-Yehudar, Galperin et al. 2001). In addition, ligand-induced IGF-1R recruitment into lipid raft caveolae has also been documented (Maggi, Biedi et al. 2002). The human IGF-1R contains three tyrosine residues in the submembrane region (Prager, Li et al. 1994) that may be involved in internalization. Prager and collaborators (Prager, Li et al. 1994) demonstrated that NPXY motif in IGF-1R is important for receptor internalization, whereas others reported the contrary results (Kaburagi, Momomura et al. 1993). Miura *et al* (Miura and Baserga 1997) demonstrated that residue 1250 is the functional tyrosine-based internalization signal.

Backer's studies (Backer, Kahn et al. 1990; Rajagopalan, Neidigh et al. 1991) demonstrated that NPXY and GPLY motifs are essential for the IR internalization and that mutation of these tyrosines impaired receptor internalization. In IR a di-leucine based motif (962-987 EKITLL) has been identified as mediator of efficient receptor internalization (Haft, De La Luz Sierra et al. 1998). Interestingly, the di-leucine motif found in the juxtamembrane domain of the IR is not conserved in the IGF-1R. In addition, it has been demonstrated that intracellular itineraries of insulin/IR and IGF-1/IGF-1R are quite different. The endocytic rate constant is three times higher for

insulin than for IGF-1. Insulin dissociates from its receptor more rapidly compared to IGF-1 and degradation is 3-fold higher for insulin (Zapf, Hsu et al. 1994). Ligand/receptor retro-endocytosis was found to be 53% for IGF-1, compared to 28% for insulin. It might be speculated that the di-leucine motif present in the IR could increase its internalization/degradation and decrease the rate of recycled receptors. Accordingly, the substitution of di-leucine motif of the IR with corresponding sequence of IGF-1R did not impair endocytosis of the mutant receptor (Haft, De La Luz Sierra et al. 1998). It is likely that other structural differences explain the dissimilarity of the endocytic traffic between IR and IGF-1R (Haft, De La Luz Sierra et al. 1998).

Degradation/Ubiquitination

Numerous experimental data has identified the ubiquitin proteasome pathways as a regulatory system for endocytosis (Hicke 1997; Hicke 1999; Shih, Sloper-Mould et al. 2000; Hicke 2001). Ubiquitin is a small (76-residue) protein whose sequence is highly conserved between single-cell eukaryotes and mammalian cells. One ubiquitin molecule is initially linked via its C-terminal glycine to the ε-amino side chain of a lysine present in a target protein (in some cases ubiquitin is conjugated to the amino terminal group of the substrate). A second ubiquitin molecule is then linked to lysine-48 of the first ubiquitin, and the process is repeated a number of times, yielding a polyubiquitin chain. A protein tagged in this fashion makes its way to a proteasome, in which it is degraded.

Ubiquitination (or ubiquitylation) of proteins requires the action of three enzymes. The first one is ubiquitin-activating enzyme (E1) that binds ubiquitin to generate a high energy E1-ubiquitin intermediate. The second one constitutes ubiquitin-conjugating enzyme (E2), acting as a carrier protein. The third one being ubiquitin ligase (E3) that transfers the ubiquitin to the target protein (Bonifacino and Weissman 1998; Glickman and Ciechanover 2002). E3 plays a key role in the ubiquitin-mediated pathways since it serves as the specific recognition factor. In most cases substrates are not recognized in a constitutive manner by the E3. Therefore, either E3 or the substrate or both must be switched on by posttranslational modifications. In proteasome degradation ubiquitin serves as a tag targeting the proteins for the proteasome (multi-subunit proteolytic enzymes). Old or damaged cytosolic proteins are labeled with a poly-ubiquitin chain, which is recognized by the proteasome.

In addition to the degradation of cytosolic proteins, ubiquitin has recently been implicated in the internalization and degradation of plasma membrane proteins. The function of plasma membrane protein ubiquitination is still unclear, but for several yeast proteins the role of ubiquitin has been defined. Ubiquitination triggers the plasma membrane proteins into the endocytic pathway for vacuolar (yeast lysosome equivalent) degradation. In mammalian cells a number of membrane proteins, which are ubiquitinated, are degraded through both the proteasome and lysosomal pathways (Hicke 1999).

First evidence for a role for ubiquitin in the regulation of the plasma membrane proteins were obtained for PDGFR beta and the growth hormone receptor (Bonifacino

and Weissman 1998). Today, ubiquitination of several multi-subunit receptors of the immune system and RTKs has been demonstrated (Bonifacino and Weissman 1998).

Similar to cytosolic proteins that undergo ubiquitination and degradation, plasma membrane RTK ubiquitination is positively regulated by phosphorylation in response to ligand binding. PDGFR beta dimerizes in the presence of ligand with increase in tyrosine kinase activity followed by internalization and lysosomal degradation. Receptor phosphorylation is also accompanied by ubiquitination of its intracellular region. Similar to PDGFR beta other growth factor receptors undergo ligand-induced ubiquitination.

The mechanism of ubiquitin-mediated internalization has not been totally clarified. The simplest explanation is that an ubiquitinated plasma membrane protein is recognized by an adaptor protein that links ubiquitinated receptors to the endocytic machinery (Hicke 1999). One example is β-arrestin, the protein recognizing activated β-adrenergic receptor and promoting clathrin-mediated internalization (Shenoy, McDonald et al. 2001).

In addition to its role as an internalization signal, ubiquitin is involved in the endosomal sorting of the internalized receptors. Cbl is a 120 kDa ring finger E3 well characterized as a negative regulator of several tyrosine kinase receptors (including PDGFR, and EGFR). Levkowitz *et al.* (Levkowitz, Waterman et al. 1998) found that Cbl-dependent ubiquitination of EGFR (ErbB1) targets the receptor to lysosomal degradation, whereas in the absence of Cbl the receptors were recycled. Results on other cell surface receptors suggest a general role for ubiquitin in regulating endocytic trafficking (Strous and Govers 1999).

A key question regarding ligand-induced ubiquitination of plasma membrane receptors is whether this modification induces lysosomal versus proteasomal degradation. Degradation of several mammalian receptors, known to be ubiquitinated, is impaired by inhibitors of proteasome as well as by agents blocking the lysosomal degradation (Bonifacino and Weissman 1998; Glickman and Ciechanover 2002). It is possible that a fraction of these receptors is degraded by the proteasome, whereas another fraction is degraded by the lysosome. Alternatively, the proteasome and lysosome might destroy different parts of the receptor. A third possibility is that the proteasome mediates degradation of another protein, which in turn is required for an efficient targeting of the receptor to the lysosome. However, ubiquitination might also play diverse roles in targeting the plasma membrane proteins to the lysosomes and the proteasome. The molecular mechanism of clathrin-mediated endocytosis determines to a great extent the presence of membranous proteins at the cell surface (Strous and Govers 1999).

Recent studies with cultured cells have implicated Nedd4 in IGF-1R ubiquitination and processing. Nedd4 binds IGF-1R through the adaptor protein Grb10. Studies in MEFs overexpressing Nedd4 and IGF-1R have shown that the Grb10/Nedd4/IGF-1R complex drives ligand-dependent ubiquitination of the internalized IGF-1R. In these cells, ubiquitination was shown to occur at the plasma membrane, probably before the formation of endocytic vesicles (Vecchione, Marchese et al. 2003). Interestingly, mice with a disruption in the maternal Grb10 allele had embryo and placental overgrowth and were 30% overweight compared to wild-type controls, identifying Grb10 as a potent growth inhibitor (Charalambous, Smith et al. 2003).

Another member of the E3 ligase family involved in IGF-1R degradation is the MDM2 proto-oncoprotein, a RING finger ubiquitin ligase that is transcriptionally regulated by IGF-1 (Heron-Milhavet and LeRoith 2002). The MDM2 protein binds to the IGF-1R β-subunit through the adaptor β-arrestin (Girnita, Shenoy et al. 2005) thereby recruiting ubiquitin to the IGF-1R and initiating its degradation (Girnita, Girnita et al. 2003).

MDM2 and IGF-1R

The murine double minute 2 (*MDM2*) gene was initially identified as one of three genes (*mdm1, 2,* and *3*) which were overexpressed by amplification in a spontaneously transformed mouse BALB/c cell line (3T3-DM). The *mdm* genes were located on small, acentromeric, extrachromosomal nuclear bodies, called double minutes, which were retained in cells only if they provided a growth advantage. The product of the *MDM2* gene was later shown to be responsible for transformation of cells when overexpressed (Cahilly-Snyder, Yang-Feng et al. 1987; Fakharzadeh, Trusko et al. 1991). Soon after identification of the *MDM2* gene, it was discovered that degradation of p53 in normal, unperturbed cells is regulated by MDM2 in mouse cells and by Hdm2 in human cells. This protein recognizes p53 as a target that should be ubiquitinated shortly after its synthesis and therefore marked for destruction (Momand, Zambetti et al. 1992). Biochemically, MDM2 functions as the E3 ligase to ubiquitinate p53 at several lysine residues (Nakamura, Roth et al. 2000; Rodriguez, Desterro et al. 2000). It also has the ability to ubiquitinate itself (Honda and Yasuda 2000; Rodriguez, Desterro et al. 2000). The RING motif is common in E3 ligases and is responsible for the E3 ligase activity of MDM2. Both the *MDM2* gene and its human counterpart, *HDM2*, consist of 12 exons.
The 90 kDa MDM2 protein contains different domains. The p53 interaction domain is encoded by the amino terminal 100 amino acids of MDM2. This domain binds the amino terminal transactivation domain of p53.

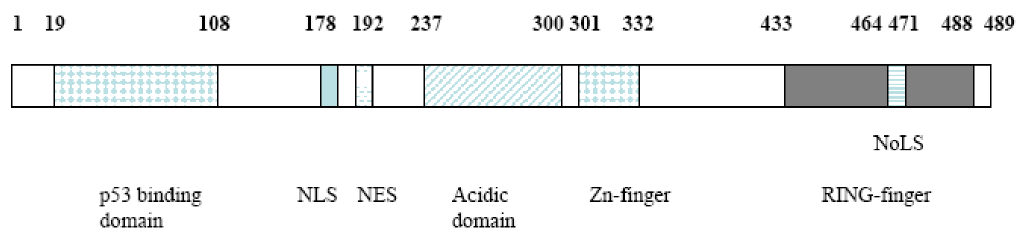

Figure 4. Structure of MDM2 protein. NLS, nuclear localization signal; NES, nuclear export signal; Zn-finger, zinc-finger domain; NoLS, nucleolar localization signal; RING-finger, ring-finger domain; The numbers above drawing represent amino acid numbers.

Thus, even if MDM2 cannot degrade p53, it interferes with the ability of p53 to interact with the transcription machinery. Other motifs include a nuclear localization signal and a nuclear export signal which shuttle MDM2 back and forth between the cytoplasm and the nucleus and provide yet another means by which p53 activity is tightly regulated (Roth, Dobbelstein et al. 1998). Amino acids 464–471 can function as a nucleolar localization signal (Lohrum, Ashcroft et al. 2000), although the biological significance of this regulation is unclear. The central acidic domain of MDM2 is necessary for interaction with the ribosomal protein L5, and with p300/CBP (CREB-binding protein). Recently, this domain was found to contribute to p53 degradation because an MDM2 mutant lacking part of this domain ubiquitinated p53 well but failed to degrade p53 (Argentini, Barboule et al. 2001; Zhu, Yao et al. 2001). Downstream of the acidic domain is a zinc finger domain of unknown function followed by the RING domain, responsible for ligase activity. P53 transcriptionally activates many target genes, one of which is the *MDM2* gene. P53 binds to the *MDM2* P2 promoter and transcriptionally upregulates *MDM2* expression. Because MDM2 inhibits p53 activity, this forms a negative feedback loop that tightly regulates p53 function. In turn, decreased p53 activity results in decreased MDM2 to constitutive levels. MDM2 can also ubiquitinate itself and induce its own degradation. Upon DNA damage, p53 is posttranslationally modified to inhibit interactions with MDM2. Several kinases also phosphorylate MDM2 and modulate interactions with p53.

This ability of p53 to regulate *MDM2* provides a feedback loop with an important role in regulating cell cycle progression and apoptosis (Deb 2003). Several other proteins have been identified that also interact with MDM2. One of the first proteins discovered to interact with MDM2 is ARF. The interaction of ARF with MDM2 blocks MDM2 shuttling between the nucleus and cytoplasm via the nucleolus. Nuclear export of p53 by MDM2 is required for efficient degradation. Sequestration of MDM2 in the nucleolus thus results in activation of p53 (Ganguli and Wasylyk 2003). Like ARF, the ribosomal protein L11 binds MDM2 and can sequester it in the nucleolus, resulting in stabilization of p53 levels (Lohrum, Ludwig et al. 2003).

Similarly, hypoxia-inducible factor 1a (HIF-1a) also interacts with MDM2 and enhances p53 function (Chen, Li et al. 2003). This interaction (examined only in transfection experiments) prevents nuclear export of p53, but provides another example of a protein that may physically prevent MDM2 from binding to p53.The ATM kinase phosphorylates MDM2 at serine 395 and impairs the degradation and nuclear export of p53 (Khosravi, Maya et al. 1999). PI3K and its downstream target Akt/PKB appear to bind and phosphorylate MDM2 at serines 166 and 186 following mitogen-induced activation also (Zhou, Liao et al. 2001). Phosphorylation on these sites is necessary for translocation of MDM2 from the cytoplasm into the nucleus. Expression of constitutively active Akt promotes nuclear entry of MDM2, diminishes cellular levels of p53 and decreases p53 transcriptional activity. The effect of MDM2 on p53 has been verified in numerous studies. But many other proteins that interact with MDM2 also appear to be regulated by MDM2. MDM2 was identified as an Rb binding protein. Rb is a potent tumor suppressor that is mutated in different kinds of cancers. MDM2 inhibits the ability of Rb to inhibit E2F1 function, thus inhibiting arrest of the cell cycle in G1 (Xiao, Chen et al. 1995). However, there is no evidence that MDM2 ubiquitinates or degrades Rb. Martin *et al*.(Martin, Trouche et al. 1995) showed that MDM2 contacts E2F1 and DP1 in immunoprecipitation experiments on NIH3T3 cells. The MDM2/E2F1/DP1 complex stimulates transcription. Additional reports indicate

that MDM2 stimulates the growth-promoting activity of E2F1 similar to the first set of experiments. Additionally, MDM2 blocks the apoptotic activity of E2F1. These experiments indicate that MDM2 promotes cell proliferation by regulating other important components of the cell cycle in addition to regulating p53.

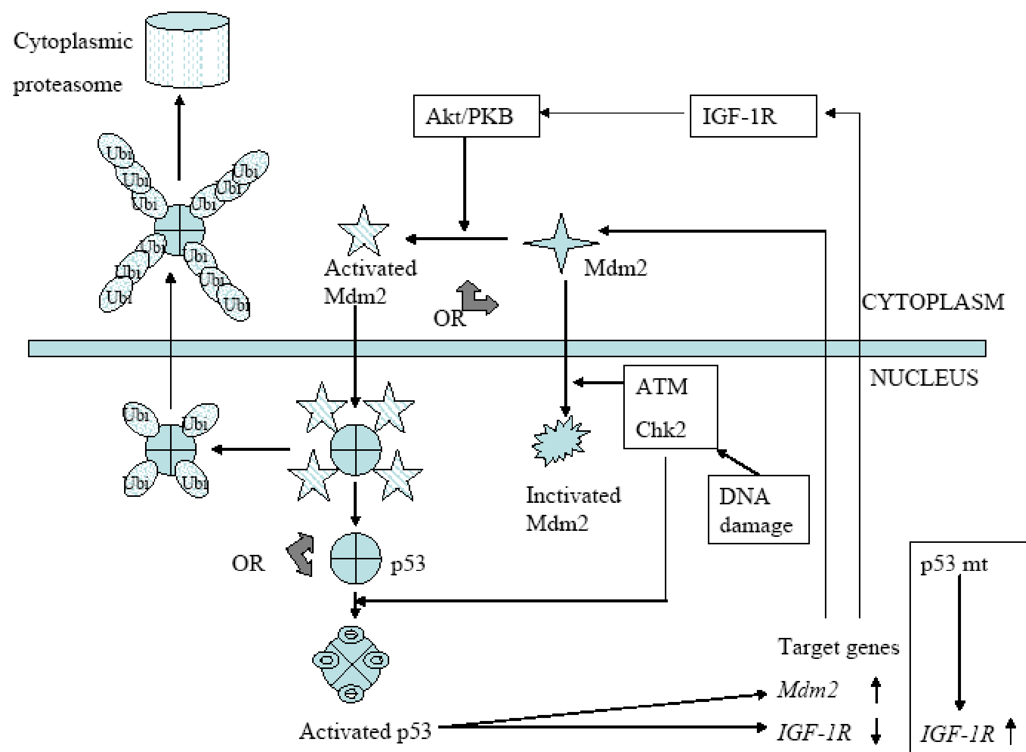

Figure 3. Interplay between p53, Mdm2 and IGF-1R

Interestingly, MDM2 has recently also been found to associate with certain cell surface receptors and regulate their functions (Shenoy, McDonald et al. 2001). Recently, it was shown that under conditions when p53 was inhibited, MDM2 was redistributed and bound to the IGF-1R (Girnita, Girnita et al. 2003). MDM2 was proven to ubiquitinate the IGF-1R and degrade it in a proteasome-dependent manner , eventually leading to cell death(Girnita, Girnita et al. 2003). This action of MDM2 explains earlier results that inhibition of wild-type p53 unexpectedly leads to downregulation of the IGF-1R (Girnita, Girnita et al. 2000). These data are in consistent with several other studies reporting apoptotic effects due to overexpressed MDM2 (Vousden and Prives 2005). On the other hand, an increased distribution of MDM2 to the cell nucleus to interact with p53 may indirectly increase the expression of IGF-1R since lesser cytoplasmic MDM2 will be available to ubiquitinate and degrade the receptor. Reciprocally, the IGF-1 system has been shown to influence the activity of MDM2. IGF-1 was demonstrated to regulate MDM2 activity by inhibiting the association between p19ARF and MDM2 in a p38 MAPK-dependent manner (Heron-Milhavet and LeRoith 2002). Thus, when IGF-1 was used to rescue the cells from UV-induced DNA damage, the p53 protein was degraded through the MDM2-mediated pathway. Other studies indicate that expression of phosphorylated Akt increases MDM2-mediated ubiquitination of p53 (Mayo and Donner 2001). The serum-induced increase in p53 ubiquitination was blocked by a PI3K inhibitor, suggesting that phosphorylated Akt enhances the ubiquitination-promoting function of MDM2, determining reduction of the p53 protein.

In conclusion, there seems to exist a p53/MDM2/IGF-1R axis, in which signals are propagated in either direction. Changes leading to increased distribution of MDM2 to the cell nucleus to inactivate p53 may contribute with a growth advantage for the tumor cells by upregulating the IGF-1R. This could be due to a derepressed transcription of the IGF-1R gene as well as a decreased ubiquitination and degradation of the receptor.

Recirculation

A part of internalized receptors are sorted for recycling to the cell surface. In activated T lymphocytes, internalization of the IGF-1R from the cell membrane was initially accompanied by a reduction in its mRNA. This was followed by re-expression of IGF-1R on the cell surface and an increase in IGF-1R mRNA levels in the cytoplasm, reaching levels higher than those initially recorded. However, a slower increase in the mRNA levels was observed suggesting that the earlier recovery of IGF-1R results from receptor recycling, followed by *de novo* synthesis. This down- and upregulation suggests that restoration of IGF-1R would be the result of both receptor recycling and *de novo* synthesis (Segretin, Galeano et al. 2003).

1.9 β-ARRESTINS

β-arrestins were discovered in the late 1980s from the observation that progressively more pure β-adrenergic receptor kinase lost the ability to desensitize G protein activation (Benovic, Kuhn et al. 1987). This loss of desensitization could be counteracted by addition of high molar excesses of visual arrestin (Pfister, Chabre et al. 1985), suggesting that a homologous protein could exist in nonretinal tissues (Benovic, Kuhn et al. 1987). Molecular cloning confirmed two isoforms of the hypothesized protein, termed β-arrestin 1 and 2 (Lohse, Benovic et al. 1990; Attramadal, Arriza et al. 1992). The two β-arrestin isoforms are 78% identical and most of the coding differences appear in the C-termini. Knockout studies show that mice lacking either β-arrestin 1 or 2 are viable (Conner, Mathier et al. 1997; Bohn, Lefkowitz et al. 1999), whereas the double-knockout phenotype is embryonic lethal (Kohout, Lin et al. 2001). This suggests that each β-arrestin isoform functionally substitutes for the other isoform to some degree. However, not all β-arrestin-mediated functions are redundant. Internalization of some GPCRs is mediated primarily by one isoform, like β2AR via β-arrestin2 and protease-activated receptor 1 (PAR1) through β-arrestin1 (Paing, Stutts et al. 2002). For others, like the angiotensin II type 1A receptor ($AT1_AR$), both β-arrestin isoforms are equally capable (Oakley, Laporte et al. 2000; Kohout, Lin et al. 2001).

β-arrestin functions

Adapters for Internalization

β-Arrestins are expressed ubiquitously in all cells and tissues and function in desensitization of most GPCRs. Initially they were analyzed for their role in the termination of signaling. However, later research demonstrated that β-arrestins serve also in receptor internalization. β-arrestins bring activated receptors to clathrin-coated pits by acting as adapters for AP2 and clathrin (Goodman, Krupnick et al. 1996; Krupnick, Goodman et al. 1997). β-arrestins also bind to various other proteins implicated in endocytosis, a critical process for receptor recycling and degradation.

Arrestin Regulation of Ubiquitination and degradation

Ubiquitin modification was originally discovered to function as a protein tag for destruction by the cellular proteasomal machinery. Agonist stimulation of the β2AR leads to β-arrestin ubiquitination, which is mediated by MDM2. β-arrestin ubiquitination is required for rapid receptor internalization (Shenoy and Lefkowitz 2003). Stimulation of Class A GPCRs, such as the β2AR, leads to transient ubiquitination of β-arrestin (Shenoy and Lefkowitz 2003; Perroy, Pontier et al. 2004). In contrast, stimulation of Class B receptors, which recruit β-arrestin to the plasma membrane and subsequently internalize as receptor-arrestin complexes into endosomes, results in sustained β-arrestin ubiquitination (Shenoy and Lefkowitz 2003; Perroy, Pontier et al. 2004).

Agonist stimulation of β2AR and the V2R also leads to the ubiquitination of the receptors themselves. β-arrestin 2 but not β-arrestin1 is required for this process (Martin, Lefkowitz et al. 2003; Shenoy and Lefkowitz 2003). β-arrestin 2 likely acts as an adapter to bring one or more E3 ubiquitin ligases to the activated receptors. For the above GPCRs, the specific E3 ligases that collaborate with β-arrestin remain to be elucidated. In contrast, the E3 ligases AIP4 and c-Cbl reportedly mediate the ubiquitination of CXCR4 and PAR2 (Marchese and Benovic 2001; Marchese, Raiborg et al. 2003). The exact role of β-arrestin in these systems remains to be determined.

β-Arrestin and MAPK Signaling

During the last years, a novel function of β-arrestins has been revealed. β-arrestins functions as scaffolds for several signaling networks. Among them, most prominent is MAPKs.

The first evidence that β-arrestins could act to facilitate signal transduction from GPCRs came from studies of receptor internalization–defective systems. These studies demonstrated that a defective β-arrestin 1 (Daaka, Luttrell et al. 1998) diminish ERK1/2 signaling. Luttrell et al. (Luttrell, Ferguson et al. 1999) discovered that β-arrestin 1 can recruit c-Src, a nonreceptor tyrosine kinase family member, to GPCRs, with subsequent ERK activation. DeFea et al. (DeFea, Zalevsky et al. 2000) also demonstrated that Src recruitment by β-arrestin is necessary for the prevention of apoptosis and propagation of mitogenic signals.

Parallel to the discoveries of the participation of β-arrestin in various signaling pathways, exciting findings that β-arrestin can bind receptors that are structurally unrelated to GPCRs were reported. Thus, β-arrestins regulate signaling and/or endocytosis of IGF-1R, Frizzled, smoothened, TGFβRIII, LDLR, Na^+/H^+ exchanger NHE5, Toll-like receptor, Interleukin1 receptor, and *Drosophila* Notch (Lin, Daaka et al. 1998; Chen, Hu et al. 2001; Chen, Kirkbride et al. 2003; Shenoy and Lefkowitz 2003; Mukherjee, Veraksa et al. 2005).

The biological roles of β-arrestin in signal transduction are likely much broader than we currently appreciate. β-arrestin signaling represents a new paradigm in cell biology and potentially a new therapeutic target for diseases

1.10 IGF-1R AS A TARGET FOR CANCER THERAPY

In order to increase apoptosis and to prevent tumor cell growth, different strategies to block the IGF-1R have been developed. The rationale for targeting the IGF-1R in the therapy of human tumors is based on two crucial findings: first, in experimental animals tumor cells undergo apoptosis when the IGF-1R is downregulated, whereas normal cells are only partially affected; and second, cells with IGF-1R deletion are refractory to transformation by viral and cellular oncogenes (Baserga 2005). Numerous attempts to directly inhibit IGF-1R functions caused massive apoptosis of tumor cells *in vitro* and *in vivo*, inhibition of tumorigenesis (Arteaga 1992; Trojan, Blossey et al. 1992; Kalebic, Tsokos et al. 1994; Resnicoff, Coppola et al. 1994; Resnicoff, Sell et al. 1994; Shapiro, Jones et al. 1994; Reiss, D'Ambrosio et al. 1998) and metastases (Long, Rubin et al. 1995; Dunn, Ehrlich et al. 1998). Overall, strategies leading to downregulation of the receptor, and not only

inhibition of its TK activity, have been associated with the strongest antitumor efficacies (Baserga, Peruzzi et al. 2003). This may be due to downregulation of IGF-1R being necessary to produce a complete inhibition of its function (Larsson, Girnita et al. 2005).

However, targeting of IGF-1R to block its signaling may be obtained by (1) blocking the ligand-receptor interaction (receptor neutralising antibodies, downregulation of IGF-1R ligands, IGF-1 mimetic peptides), (2) targeting IGF-1R synthesis (antisense strategies, siRNA, triple-helix strategy), (3) interfering with function of IGF-1R (dominant negative mutants, modulators of tyrosine kinase activity, e.g. ATP antagonists and non-ATP antagonists), (4) modulators of IGF-1R internalisation and recycling and (5) inhibitors of N-linked glycosylation.

A very attractive way to target the cellular molecules involved in signaling pathways is to use the low-molecular-weight compounds as anti-cancer drugs. These drugs are, in general, synthesized far more readily than molecules of higher molecular weight, and small molecules are more likely to penetrate into the interstices of a tumor, thereby exerting therapeutic effects on all of its component cells. Recently, have been demonstrated that the low-molecular-weight compound cyclolignan picropodophyllin (**PPP**) is an inhibitor of the IGF-1R tyrosine phosphorylation (Girnita, Girnita et al. 2004). It did not inhibit the highly homologous insulin receptor (IR) or tyrosine kinases of other major cancer relevant growth factor receptors (Girnita, Girnita et al. 2004). PPP did not interfere with the IGF-1R tyrosine kinase at the level of ATP binding site (Girnita, Girnita et al. 2004), suggesting other mechanisms of action (e.g. inhibition at the level of receptor substrate). Consistently, treatment with PPP reduced phosphorylation of Akt in IGF-1 stimulated cells (Girnita, Girnita et al. 2004). *In vivo* PPP rapidly caused complete regression of xenografts derived from IGF-1R positive cells and did not affect the tumors derived form IGF-1R negative cells (Girnita, Girnita et al. 2004). The accurate mechanism by which PPP inhibits IGF-1R activity is still under investigation.

2 AIMS

1. To investigate potential role of β-arrestins in the MDM2-dependent ubiquitination and expression of IGF-1R

2. To investigate whether MDM2 and β-arrestins are involved in IGF-1R induced signaling, with special focus on ERK activation.

3. To explore the effects of the cyclolignan PPP on expression level of IGF-1R and to approach possibly involved mechanisms of action.

4. To characterize the effects of PPP on IGF-1R induced ERK phosphorylation.

3 MATHERIALS AND METHODS

3.1 REAGENTS

PPP was synthesized (Buchardt, Jensen et al. 1986) and following recrystallization its purity was 99.7%. For experimental purposes, PPP was dissolved in saline (5 mM) or DMSO (0.5mM) before addition to cell cultures. Protein G Sepharose was from Amersham Pharmacia Biotech (Uppsala, Sweden). Human recombinant IGF-1, human recombinant insulin and phosphatase inhibitor cocktail were purchased from Sigma (St Louis, MO, USA). Chemically synthesized, double strand siRNAs, with 19-nucleotide duplex RNA and 2-nucleotide 3'-dTdT overhangs targeting human β-arrestin 1 and β-arrestin 2 were purchased from Xeragon (Germantown, MD) and the siRNA targeting the human IGF-1R and the non-silencing RNA duplex were purchased from Dharmacon (Lafayette, Colorado).

3.2 ANTIBODIES

A monoclonal antibody (mAb) against phosphotyrosine (PY99), polyclonal antibodies to the β-subunit of IGF-1R (C-20 and H-60), polyclonal antibody to the β-subunit of insulin receptor (C-19) and monoclonal antibodies to MDM2 (SMP14) and to ubiquitin (p4D1) were from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, USA). Rabbit polyclonal antibodies against β-arrestin 1/2 were generated in the Lefkowitz laboratory. A mouse monoclonal antibody against the human IGF-1R, a mouse monoclonal antibody to MDM2 used in the paper 1, and the proteasome inhibitor MG 132 were from Calbiochem. Antibodies against IGF-1R, pAkt (S473), Akt, pERK1/2 and ERK1/2 were from Cell Signaling Technology, Danvers, MA. A mouse monoclonal antibody to EGF receptor was from CIMAB SA (Havanna, Cuba). A mouse monoclonal antibody directed to the human Kit (CD 117) and a polyclonal swine anti-rabbit FITC linked antibody was purchased from Dako (Carpinteria, CA). A monoclonal antibody to β-actin (clone AC-15) was purchased from Sigma (St Louis, MO, USA).

3.3 CELL CULTURES

BE cells, established from a lymph node metastasis specimen from a patient with advanced malignant melanoma, as well as melanoma cell lines DFB, were kindly provided by Professor Rolf Kiessling (CCK, Karolinska Institute, Stockholm). The cells were cultured in monolayers in standard media RPMI supplemented with 10% fetal bovine serum (FBS) and 1% penicillin-streptomycin. The human glioblastoma cell line U343MG was given to us by Dr. Monica Nistér (CCK, Karolinska Institute Stockholm). The R-, 46, 56, 96, R+ and P6 mouse cell lines are obtained from R. Baserga (Thomas Jefferson University, Philadelphia, PA). Wild type, MDM 2 knock-out (KO) and β-arrestin1, 2 KO mouse embryonic fibroblast (MEFs) are cultured in DMEM supplemented with 10% fetal calf serum. The R- fibroblasts are IGF-1R negative, derived from BALB/3T3 mouse embryo with a targeted disruption of the type 1 receptor for the insulin like growth factors (Rubini, Hongo et al. 1997). The P6 and R+ cell lines is a 3T3 derivative overexpressing the human IGF-1R (Rubini, Hongo et al. 1997). 46,56 and 96 are R- cells stably transfected with different IGF-1R

constructs possessing a mutation in the substrate binding site (SBS) (Y950F) (46) with a truncated C-terminal domain construct (56) or with an IGF-1R construct expressing both SBS mutation (Y950F) and truncated C terminus (96). The cells are cultured in monolayers in standard media supplemented with 5% (P6) or 10% fetal bovine serum (R-, 46, 56, 96, R+) in the presence of G-418 (Promega). R- v-src (IGF-1R negative mouse cells transformed with v-src) cell line are cultured in monolayers in standard media DMEM supplemented with 10% fetal bovine serum (FBS) in the presence of G-418 (Promega). The cells were grown in tissue culture flasks maintained at 95% air/5% CO_2 atmosphere at 37°C in a humidified incubator.

3.4 SMALL INTERFERING RNAS (SIRNAS)

Chemically synthesized, doublestrand siRNAs, with 19-nucleotide duplex RNA and 2-nucleotide 3'-dTdT overhangs were purchased from Xeragon (Germantown, MD) in deprotected and desalted form (Paper1,2). The siRNA sequences targeting human β-arrestin 1 and β-arrestin 2 have been reported previously (Ahn, Nelson et al. 2003) and were used to deplete endogenous β-arrestin levels in BE and DFB cell lines. The siRNA sequences targeting mouse β-arrestin 1 and 2 are:

5'-AAAGCCUUCUGUGCUGAGAAC-3' and
5'-AAGGACCGGAAAGUGUUCGUG-3'.

A non-silencing RNA duplex (5'-AAUUCUCCGAACGUGUCACGU-3'), as the manufacturer indicated, was used as a control for both human and mouse cells.

Small interfering RNAs (siRNAs) expressed from short hairpin RNAs (shRNAs) were used to mediate gene specific RNA interference (RNAi) in the paper 3. MISSION shRNA plasmids were from Sigma (St Louis, MO). SiRNA targeting human β-arrestin1 (NM_004041) was 5'-GCCAGTAGATACCAATCTCAT-3. The non-target shRNA control vector with the sequence ´5'-CAACAAGATGAAGAGCACCAA-3' was used as a negative control.

The siRNA targeting the human IGF-1R sequence 5'-GCAGACACCUACAAC AUCAUU-3' was used to deplete endogenous IGF-1R levels in BE cell line (Paper4). The cells were transfected using Dharmafect siRNA Transfection reagent 1 according to manufacturer's protocol. MDM2 expression was lowered using siRNA targeting human MDM2 mRNA (5′-AAG CCA UUG CUU UUG AAG UUA-3′) supplied by Dharmacon (Lafayette, CO). SiRNA, 200 pmol, was transfected into cells using oligofectamine reagent (Invitrogen, Carlsbad, CA) according to the instructions of the manufacturer.

3.5 TRANSFECTIONS

Transfections: 40–50% confluent cells in 25-cm2 flasks, split 24 h before transfection, were transfected with siRNA targeting the human β-arrestin 1 or 2 using the Lipofectamine 2000 (Invitrogen) according to the modified manufacturer's instructions. Briefly, 10 µl of transfection reagent was added to 300 µl of serum-free medium, while RNA mixtures containing 12 µl of 20µM (3.5 µg) RNA, and 188 µl of medium were prepared. Both solutions were allowed to stand 5–10 min at room

temperature and mixed by inversion. After a 10–20-min incubation at room temperature, the entire transfection mixture was added to cells in a flask containing 3–4 ml of fresh, serum-free medium. After cells were incubated for 24 h at 37 °C, the medium was replaced with normal (serum-containing) growth medium. After additional incubation for 24 h, cells were divided into two flasks or 6-well plates for further experiments. All assays were performed at least 2 days after siRNA transfection. Transfections with pcDNA3 β-arrestin 1 and 2 and MDM mutants were performed as described elsewhere (Brodt, Fallavollita et al. 2001). The cells, plated at subconfluent density in 6-cm dishes, were transiently transfected with 2 mg/ml DNA using Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA), After 24 h the transfected cells were splited into six-well plates and cultured for an additional 24 h in the presence of normal (serum-containing) growth medium. During the last 12 h, cells were starved and then either stimulated with 50 ng/ml IGF-1 or treated with different concentration of PPP. Protein extracts were prepared for immunoprecipitation or Western blot analyses.

3.6 IMMUNOPRECIPITATION .

For determination of the receptor ubiquitination or for the colocalization experiments, immunoprecipitation of IGF-1R, ubiquitin or MDM2 protein was made. The prepared cells compartments were lysed in 500µl of ice-cold PBS-TDS solution (containing PBS, Triton X-100, sodium deoxycholate, and SDS) containing the protease inhibitors and subjected to immunoprecipitation by adding 20 µl of resuspended volume of the sepharose conjugate (Protein G Sepharose) and 1 µg anti-antibody. After overnight incubation at 4°C on a rocker platform, the immunoprecipitates were collected by centrifugation at 6000 rpm for 1 min. The pellet was washed four times with 1 ml of PBD-TDS. Immunoprecipitates were analysed by Western blotting, the material being dissolved in sample buffer for SDS-PAGE.

3.7 SDS-PAGE AND WESTERN BLOTTING

Protein samples were dissolved in a sample buffer containing 0.0625 M Tris-HCl (pH 6.8), 20% glycerol, 2% SDS, bromophenolblue and 100mM dithiothreitol (DTT). Samples corresponding to 50-100 µg cell protein were analyzed by SDS-PAGE with a 4% stacking gel and 7.5% or 10% separation gel essentially according to the protocol of Laemmli. Molecular weight markers (BioRad, Sweden) were run simultaneously. Following SDS-PAGE the proteins were transferred overnight to nitrocellulose membranes (Hybond, Amersham) and then blocked for 1 h at room temperature in a solution of 5% (w/v) skimmed milk powder and 0.02% (w/v) Tween 20 in PBS, pH 7.5. Incubation with appropriate primary antibody was performed for 1-2 h at room temperature. This was followed by washes with PBS and incubation with a biotinylated secondary antibody (Amersham) for 1 h. After incubation with streptavidin-labeled horse peroxidase, detection was made (Hyperfilm-ECL, Amersham). The films were scanned by Fluor-S (BioRad).

3.8 DETERMINATION OF PROTEIN CONTENT

Protein content of cell lysates was determined by a dye-binding assay (Bradford 1976), with a reagent purchased from Bio-Rad. Bovine serum albumin was used as a standard.

3.9 RT–PCR FOR DETECTION OF IGF-1R

Total RNA was isolated from adherent cells using RNeasy kit (Qiagen, Hilden, Germany). For the RT–PCR 500 ng total RNA was reverse transcribed to cDNA using random primers (Promega, Madison, WI, USA) in a 20 µl reaction containing 500 mM dNTP (each) (Invitrogen, Carlsbad, CA, USA) and using SuperScript II Reverse Transcriptase (Invitrogen). The primers for IGF-1R were:

Forward: 5'-GCC CGA AGG TCT GTG AGG AAG AA-3
Reverse: 5'-GGT ACC GGT GCC AGG TTA TGA-3'; (Girnita, Girnita et al. 2000)

Amplification was performed at 94°C for 30 s, 60°C for 30 s and 72°C for 1 min for 29/31 cycles, and a final elongation at 70°C for 7min. Parallel amplification of GAPDH was used as an internal control and as a reference standard for semiquantitative assessment.

The PCR products were detected by ethidium bromide staining on a 1% agarose gel and visualized in a Fluor-S MultiImager System (BioRad, Hercules, CA, USA) (Girnita, Girnita et al. 2000).

3.10 IN VITRO UBIQUITINATION

In vitro ubiquitination of IGF-1R was performed essentially as described (Fang, Jensen et al. 2000). Recombinant glutathione S-transferase (GST)-MDM2 and -β-arrestin 1 (pGEX-4T1) constructs and His6-β-arrestin 2 were expressed in Escherichia coli and purified using glutathione-Sepharose (Pierce). IGF-1R was isolated from P6 cells by immunoprecipitation with a polyclonal rabbit antibody directed against β-subunit (H60) and protein G-Sepharose (Amersham Biosciences). IGF-1R-Sepharose beads were mixed with or without GST-MDM2, rabbit E1 (Calbiochem), E2 bacterial recombinant UbcH5B (Calbiochem), His6-ubiquitin (Calbiochem), and with or without β-arrestins, in a 30-µl reaction. After one hour incubation at 37 °C the reaction was stopped by the addition of SDS sample buffer. Reaction products were loaded on a 7.5% polyacrylamide gel, transferred to nitrocellulose membrane, and detected using either antibody against IGF-1R (C20) or an anti-ubiquitin antibody (Santa Cruz).

3.11 PULSE-CHASE ANALYSIS AND IMMUNOPRECIPITATION

For the analysis of IGF-1R degradation, after the indicated experimental procedures, cells were transferred to methionine-free medium supplemented with 10% fetal bovine serum and 100 µCi/ml L-[35S]methionine (specific activity >1,000 Ci/mM, Amersham Biosciences) for 24 h. The cells were carefully washed and

transferred to radioactive-free methionine-containing medium supplemented with 10% fetal bovine serum for the indicated time periods. Cells were then quickly washed twice with ice-cold phosphate-buffered saline and lysed in radioimmune precipitation assay buffer (1% phosphate-buffered saline, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS) containing dissolved protease inhibitor tablets (Roche Diagnostics). An equal amount of protein from each sample was immunoprecipitated with antibodies for the IGF-1R β-subunit (H-60) collected by protein A-Sepharose, resolved by SDSPAGE and visualized by autoradiography.

3.12 CELL VIABILITY ASSAY

Cell viability was assessed in triplicates by the Cell Proliferation kit II (XTT) (Roche, Mannheim, Germany) which is based on colorimetric change of the yellow 2,3-bis[2-methoxy-4-nitro-5-sulfophenyl]- 2H-tetrazolium-5-carboxanilide inner salt in orange formazan dye by the respiratory chain of viable cells (Roehm, Rodgers et al. 1991). In brief, cells were cultured in 96-wells plates in 100 µl medium. After the incubation periods, 50 µl XTT labeling mixture was added to each well and incubated for additional 1 h. Spectrophotometric absorbance was measured at 492nm using an ELISA reader.

3.13 IMMUNOFLUORESCENCE CONFOCAL MICROSCOPY

The cells were plated on collagen-coated 35- mm glass bottom plates. After experimental conditions, cells were fixed with 5% formaldehyde diluted in phosphate-buffered saline containing calcium and magnesium before confocal analyses. For immunostaining endogenously expressed β-arrestins, polyclonal β-arrestin1/2 antibody and antirabbit ALEXA 594 (Molecular Probes) were used as primary and secondary antibodies, respectively. For immunostaining of phospho- ERK1/2, an anti-pERK1/2 antibody (Cell Signaling Technology) was used.

3.14 CELL CYCLE AND PROLIFERATION ANALYSIS

Cell cycle analysis was performed with a FACS Calibur machine from Becton Dickinson. Following indicated experimental conditions cells were detached with Non-enzymatic Cell dissociation solution from Sigma (St. Louis, MO), and centrifuged for 4 min at 4°C at 1000 rpm. After two washes with PBS, cells were incubated for 30 min with 70% ice-cold ethanol on ice. After another 2 washes cells were centrifuged and pellet stained with propidium iodide 50µg/ml (Sigma) with added RNAse A 20 µg/ml (Sigma) and analyzed by flow cytometry using CellQuest® program.

4 RESULTS AND DISCUSSION

Insulin-like growth factor-1 receptor (IGF-1R) is a broadly expressed transmembrane receptor that plays a key role in supporting malignant cell growth and differentiation. Under normal cellular conditions IGF-1R signaling network is tightly regulated. The most prominent regulator of IGF-1R signal termination is desensitization by the removal of activated receptors from the cell surface by accelerated endocytosis. Recently, it was demonstrated that the IGF-1R undergoes ubiquitination following ligand stimulation (Girnita, Girnita et al. 2003). The proto-oncogene MDM2 was unexpectedly found be an E3 ligase in IGF-1R ubiquitination (Girnita, Girnita et al. 2003).

Studies on events involved in IGF-1R downregulation and intracellular signaling constitute the subject of the present thesis.

4.1 PAPER I

Beta-Arrestin is crucial for ubiquitination and down-regulation of the insulin-like growth factor-1 receptor by acting as adaptor for the MDM2 E3 ligase.

β-arrestins are ubiquitously expressed cytosolic adaptor proteins generally known to be involved in the regulation of endocytosis and signaling elicited by GCPRs. Here we provide evidence that the two widely co-expressed isoforms of β-arrestin (termed β-arrestin 1 and 2) bind to the IGF-1R and by serving as adaptor proteins bring the oncoprotein E3 ligase MDM2 to the IGF-1R and thereby, promote ubiquitination and degradation of the receptor. Furthermore, we show that in contrast to GCPRs where the β-arrestin 2 is involved in ubiquitination and degradation, β-arrestin 1 is more potent then isoform 2 in binding and inducing ubiquitination of the IGF-1R. We could also demonstrate that β-arrestins are an absolute requirement for interaction between E3 ligase MDM2 and IGF-1R, which indicate their relevancy for cell growth and cancer.

MDM2 is widely known as the ubiquitin ligase (E3) involved in ubiquitination of p53 has been shown to be involved in ubiquitination and degradation of IGF-1R (Girnita, Girnita et al. 2003). MDM2-facilitated protein ubiquitination targets the substrate protein to degradation by the ubiquitin-26 S proteasome system (UPS).

In paper1 we could demonstrate that MDM2 ubiquitin-ligase activity is facilitated by β-arrestin. The involvement of β-arrestin is first relieved by the observation that the presence or the absence of the binding site for β-arrestin affects MDM2 dependent IGF-1R ubiquitination. Using transfection with C-terminal truncated variants $MDM2_{1–400}$ and $MDM2_{1–161}$ we noticed that the presence of the mutant $MDM2_{1–161}$ lacking both β-arrestin binding and ligase domains does not affect IGF-1R basal ubiquitination level, IGF-1R expression and IGF-1R-MDM2 association. In contrast, in cells transfected with the $MDM2_{1–400}$ construct, expressing the β-arrestin binding site but lacking the ligase domain, ubiquitination of IGF-1R and IGF-1R-MDM2 association was completely abrogated while the expression of the receptor increased. These observations, taken together with the findings that expression of IGF-1R was reduced and IGF-1R ubiquitination increased in cells transfected with the full-length construct $MDM2_{1–491}$, suggests that only full-length MDM2 associates with,

ubiquitinates and degrades the IGF-1R. Further we could confirm the association of β-arrestins 1 and 2 in complex with IGF-1R and MDM2 using an *in vitro* ubiquitination assay or by co-immnoprecipitation experiments. The requirement of β-arrestins for IGF-1R ubiquitination in cultured cell was also shown by assessing the effect of overexpression or downregulation of β-arrestin-1 or 2 on IGF-1R ubiquitination and expression level. Downregulation of β-arrestin 1 or 2 decreased IGF-1R ubiquitination and stabilized IGF-1R expression. On the other hand β-arrestin 1 and 2 transfections decreased the IGF-1R expression, suggesting that overexpression of β-arrestin accelerates ubiquitination and degradation of the receptor. In basal conditions IGF-1R is poliubiquitinated both in melanoma cell lines and P6 mouse embryonic fibroblasts. The poliubiquitinated IGF-1R is targeted to degradation.

We also showed that a short stimulation with IGF-1 can induce ubiquitination of IGF-1R and that β-arrestins are needed in this respect. β-arrestin 1 was several fold more efficient than β-arrestin 2. Furthermore, β-arrestin 1 was more potent in regulating down-regulation of the IGF-1R, both under basal and IGF-stimulated conditions. Overall, in contrast to GPCRs, in which β-arrestin 2 is involved in receptor ubiquitination and degradation, it is evident β-arrestin 1 is the superior adaptor for MDM2 ligase E3 in ubiquitination of the IGF-1R.

In conclusion this study, demonstrating a strict requirement for β-arrestins for the interaction between the oncoprotein MDM2 and the IGF-1R, suggests a role of this adaptor protein in both physiological and malignant cell growth.

4.2 PAPER II

Beta-arrestin and MDM2 mediate IGF-1 receptor-stimulated ERK activation and cell cycle progression.

In this paper we investigated further the biological role of β-arrestin 1 and MDM2 as regulators of IGF-1R. Here we demonstrate that both MDM2 and β-arrestin 1 are necessary for IGF-1 stimulated phosphorylation of ERK1/2 but not Akt. Furthermore, the modulating effect of MDM2 and β-arrestin 1 on ERK affects cell cycle progression. In conclusion, MDM2 and β-arrestin 1 do not only induce ubiquitination and degradation of IGF-1R but also influence IGF-1R dependent signaling and cell cycle progression.

Upon GPCR activation, β-arrestins translocate to the cell membrane and bind to the agonist-occupied receptors. Following their recruitment β-arrestins are ubiquitinated by MDM2. This uncouples these receptors from G proteins and promotes their internalization into endosomal vesicles, thus causing desensitization.

However, accumulating evidence links GPCRs to intracellular signaling pathways such as MAPK cascades. In the context of these observations and our previous finding that β-arrestin associates with the IGF-1R we explored whether β-arrestin and its ubiquitination play roles in IGF-1R signaling, with a focus on activation of ERKs..

We first investigated the requirement of β-arrestin1 in IGF-1 activated ERK1/2 phosphorylation. Both in β-arrestin1 knockout (KO) mouse embryonic fibroblasts (MEFs) and in melanoma cell line transfected with siRNA targeting β-arrestin1 IGF-1

was no longer able to induce ERK1/2 activation. These data provide evidence that β-arrestin1 is necessary for IGF-1 induced ERK activation. In contrast, that β-arrestin1 did not affect IGF-1R dependent Akt activation.

Using wild type (WT) MEFs and those with MDM2 knockout (KO) we found that β-arrestins are monoubiquitinated or oligoubiquitinated and that the MDM2 E3 ligase is responsible for ubiquitination of β-arrestin1 in response to IGF-1 stimulation. By immunofluorescence confocal microscopy we analyzed cellular distribution of β-arrestins following IGF-1 stimulation in cells depleted of β-arrestins or in MDM2 KO MEF transfected with MDM2. We can conclude that IGF-1 stimulation results in recruitment of β-arrestin into intracellular vesicles, which is enhanced by MDM2, perhaps by regulating β-arrestin ubiquitination status.

MDM2 is involved in IGF-1 induced ERK1/2 phosphorylation since the dominant negative (DN) MDM2 completely blocked IGF-1 stimulated ERK activation, whereas ectopic expression of WT MDM2 in melanoma cells resulted in increased ERK phosphorylation.

We next analyzed whether IGF-1R tyrosine kinase activity was required for the IGF-1-stimulated ERK pathway making use of three different IGF-1R mutants. One construct possessing a mutation in the substrate binding site (SBS) (Y950F), which does not recruit and activate Shc and IRS1. Onother IGF-1R construct has a truncated C-terminal domain and the third one possesses both a SBS mutation (Y950F) and a truncated C-terminus. The data suggest that the C-terminal domain of IGF-1R is important for ERK activation upon IGF-1R stimulation. We also demonstrate that the C-terminal domain of IGF-1R is important for β-arrestin1 recruitment and ubiquitination, processes that can take place independent of the IGF-1R tyrosine kinase signaling. We also studied the effects of MDM2 and β-arrestin 1 on cell cycle progression. By manipulating the levels of MDM2 and β-arrestin 1 we could show that these molecules are important for G1 progression, as well as their effects seem to be specific for the IGF-1R since similar manipulation of MDM2 and β-arrestin 1 did not affect cell cycle progression of the IGF-1R null R- cells.

Besides the classical IGF-1R activation of MAPK pathway, the emerging data of this paper suggest a new scenario for IGF-1R dependent ERK1/2 activation. To transduce IGF-1 stimulated signaling, β-arrestin1 has to bind to the C-terminus of the IGF-1R and become ubiquitinated by the MDM2 E3 ligase. Even though maximal ERK phosphorylation appears to require an intact receptor, but our data indicates that a component of ERK activation by IGF-1R can occur under conditions of impaired tyrosine kinase signaling. Our data further suggest that β-arrestin1 may stabilize pERK leading to a prolonged activity after ligand stimulation. Sustained ERK activation is critical for ensuring G1-S phase progression. Therefore, β-arrestin 1 and MDM2 may play an important role in normal and malignant cell growth.

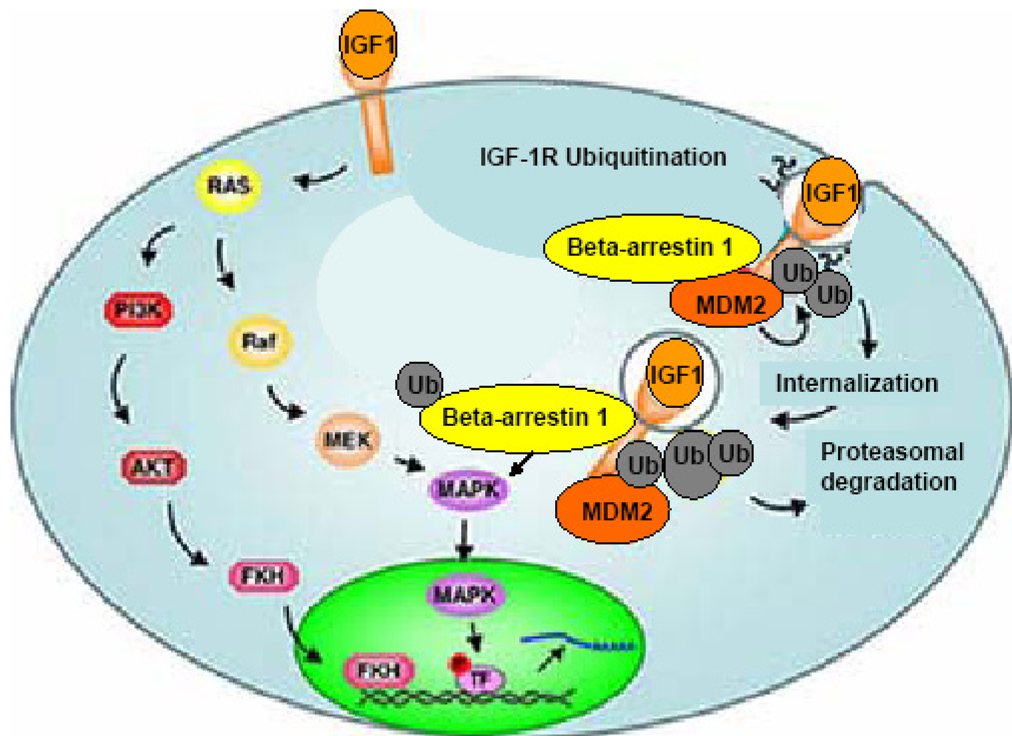

Figure 5. Scenario of beta-arrestin1 and MDM2 involvement in IGF-1R internalization and subsequent ERK activation

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4.3 PAPER III

Picropodophyllin induces downregulation of the insulin-like growth factor 1 receptor. Potential mechanistic involvement of MDM2 and β-arrestin1.

Experience on targeting IGF-1R as an anti-tumor approach has suggested that strategies leading to downregulation of the receptor, and not only to inhibition of its TK activity, are associated with the strongest antitumor efficacies However, since small molecular weight compounds have good biological availabilities and are easier to administer to patients compared to antibodies and antisense oligonucleotides or dominant negative constructs, it would be desirable to develop small molecules inducing degradation of the IGF-1R. The cyclolignan PPP, developed in our lab, was recently demonstrated to inhibit the activity of IGF-1R, while it did not affect the highly homologous insulin receptor or its signaling (Girnita, Girnita et al. 2004). Furthermore, PPP induces apoptosis and reduce cell survival in IGF-1R positive cells (Girnita, Girnita et al. 2004; Vasilcanu, Girnita et al. 2004) as well as it causes regression of tumor xenografts derived from IGF-1R positive cells but not from those derived form IGF-1R negative cells.

This latter result is surprising if assuming that PPP only would block the activity of IGF-1R. In tpaper 3 we demonstrate that PPP also causes downregulation of IGF-1R, whereas other tyrosine kinase receptors like IR, PDGFR, EGFR, c-Kit and VEGFR are not affected. The PPP mediated IGF-1R downregulation was comparable to that one induced by IGF-1 stimulation (30-50%) after 12h treatment of cultured cells and 50% *in vivo* in P12 tumors allografts. In these experiments, a moderate dose of 0.5 μM was used. Pulse-chase analysis of IGF-1R together with the assessment of IGF-1R transcripts suggest that the PPP-induced downregulation of IGF-1R is mediated by an accelerated protein degradation.

Furthermore, the PPP-induced downregulation of IGF-1R required the expression of wild type MDM2 E3 ligase, indicating that MDM2-dependent ubiquitination of IGF-1R may be an important mechanism in this respect**.**

Since we previously demonstrated that β-arrestin 1 acts as an adaptor for the MDM2 E3 ligase and therewith playing a key role in ubiquitination and down-regulation of IGF-1R, we also investigated the involvement of β-arrestin 1 in PPP-induced IGF-1R downregulation. Knockdown of β-arrestin 1 by siRNA was found to decrease the receptor downregulation induced by both PPP and IGF-1, Furthermore, inhibition of β-arrestin 1 significantly reduced PPP-induced cell death.

The present study adds a new aspect on the mechanism of action of PPP, demonstrating that besides inhibiting IGF-1R phosphorylation it induces ligand-independent degradation of the receptor. Both inhibition of phosphorylation and receptor degradation may result in a higher antitumor efficacy. This may be due to that a downregulation of IGF-1R is necessary to produce a complete inhibition of its function (Larsson, Girnita et al. 2005).

4.4 PAPER IV

Insulin-like growth factor 1 receptor (IGF-1R) dependent phosphorylation of ERK1/2 but not Akt (PKB) can be induced without receptor autophosphorylation.

The initial consequence upon ligand binding is autophosphorylation of several tyrosine residues within the IGF-1R kinase domain and activation of the intracellular signaling cascades mainly PI3K and MAPK pathways. We previously showed that the cyclolignan PPP has a superior inhibitory effect on the PI3K/Akt pathway but a weaker and delayed effect on the MAPK pathway. These responses may possibly be explained by a preferential inhibition of phosphorylation of one of the three tyrosine residues within IGF-1R activation loop, Y1136 (Vasilcanu, Girnita et al. 2004).

In paper 4 we demonstrate that inhibition of IGF-1R phosphorylation by PPP temporarily stimulates the intracellular ERK signalling. Studying the mechanism behind this intriguing effect of PPP, we could demonstrate that PPP induces IGF-1R ubiquitination and in turn activates ERK. This effect is IGF-1R-specific since PPP is not able to induce ERK phosphorylation in IGF-1R negative cells. The stimulatory effect on ERK activity occurs after short treatments with PPP.

We showed that 5 to 10 min PPP treatments can induce IGF-1R ubiquitination. Probably this response precedes the ERK activation. In Paper 1 we showed that MDM2 represents an E3 ligase for ubiquitination and down-regulation of the IGF-1R. Besides these roles, MDM2 operates as a transducers of IGF-1R dependent ERK1/2 activation independent of tyrosine kinase signaling (paper 2) (Girnita, Shenoy et al. 2007). Taking into account these findings together with the observed PPP-induced IGF-1R ubiquitination, we investigated whether MDM2 is involved in PPP-induced ERK signaling.

In the absence of MDM2 after siRNA targeting of MDM2 or after transfection with an MDM2 construct with dominant negative effect, phosphorylation of ERK did not occur as a response to neither PPP nor IGF-1. We next investigated the IGF-1R domains required for the PPP-induced ERK signaling. For this purpose we used the three different IGF-1R mutants decribed in paper 2. In cells expressing IGF-1R with truncated C-terminal PPP was not longer able to induce ERK activation, suggesting importance of the C-terminal domain in this respect.

Our data suggest that PPP, apart from inhibiting the receptor kinase, could activate the IGF-1R ubiquitination and stimulate ERK in an MDM2-dependent manner. Thus, in case of IGF-1R ubiquitination and ERK activation PPP exhibits similar effects as ligand-dependent activation of the receptor do. On the other hand, PPP does not activate Akt, but instead inhibits it. The dual effects of PPP on the two main signalling pathway may explain the strong apoptotic effect of PPP (Girnita, Girnita et al. 2004; Vasilcanu, Girnita et al. 2004; Girnita, All-Ericsson et al. 2006). The stimulatory effect on ERK activity helps the cells passing G1. Since cycling cells are more prone to apoptotic cell death compared to G1 arrested ones (Baserga 1994), an agent attenuating Akt phosphorylation but more or less preserving the ERK activity should increase apoptotic cell death.

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