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Growth Factor Pathways In Human Cancer

Functional and Therapeutic Implications

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To Ada and Sebastian

ABSTRACT

The multi-step development of tumors involves numerous changes at genomic level such as oncogene activation, loss of function of tumor suppressor genes and translocations resulting in fusion genes that encodes for chimeric proteins with tumorigenic functions etc. However, in the selection leading to cancer in somatic tissues it is likely that the cancer cells make use of the normal extracellular signaling for proliferation and/or antiapoptosis to create growth advantage over the normal cells. These signals are, in part, mediated by the growth factor receptors. This thesis aims to explore the mechanisms involved in expression and function of these receptors with special focus on insulin-like growth factor-1 receptor IGF-1R. The final goal is to identify some "Achilles' heel" in the growth factor pathways as a possible target in cancer therapy.

N-linked glycosylation is crucial for expression of growth factor receptors at the cell surface. In Ewing's sarcoma cells, which carry the EWS-FLI-1 fusion gene, we found that inhibition of N-linked glycoproteins suppressed the EWS-FLI-1 protein leading to growth arrest. Since the fusion protein was demonstrated to not be a glycoprotein, we conclude that some other glycoproteins may be involved in regulation of EWS-FLI-1. Since growth factor receptors are N-linked glycoproteins and most N-linked glycoproteins are confined to the plasma membrane, the possibility of a link between cell surface expression of growth factor receptors and EWS-FLI-1 expression may be raised.

We therefore tested different specific growth factor pathways regarding their potential influence on the EWS-FLI-1 protein. Our data indicate that the basic fibroblast growth factor (bFGF) pathway is important for up-regulation the EWS-FLI-1 protein. Other investigated growth factors pathways (e.g. IGF-1) seemed not to regulate the fusion protein.

We investigated the functional impact of p53 for IGF-1R expression in malignant cells. Using three different system-(1) malignant melanoma cell lines expressing mutant p53, (2) malignant melanoma cell lines overexpressing wild type (wt) p53 and (3) BL41tsp53-2 cells (harboring a temperature-sensitive p53), we could demonstrate that induction of normal wt p53 or down regulation of the mutant type p53 impaired the IGF-1R expression. However, the melanoma cell lines expressing wt p53 also responded with decreased expression of IGF-1R upon p53 inhibition. We hypothesize that p53 may interfere with IGF-1R expression at posttranscriptional levels.

Based on the aforementioned data we investigated the mechanisms underlying the interaction between p53 and functional IGF-1R. Our data provides evidence that inhibition of p53 triggers Mdm-2-dependent ubiquitination and proteasomal dependent degradation of the IGF-1R. In fact we could demonstrate: a physical association of IGF-1R to Mdm-2; that inhibition of p53 expression, with maintained expression of Mdm-2, causes ubiquitination of IGF-1R; that co-inhibition of p53 and Mdm-2 expression rescues the cells from IGF-1R down regulation and subsequent death; and that Mdm-2 ubiquitinates IGF-1R in cell-free systems.

Finally, we identified potent and specific inhibitors of IGF-1R and demonstrated their potency in inhibition of malignant cell growth, both in vivo and in vitro.

Key words: IGF-1R, growth factors, p53, fusion protein, tyrosine kinase inhibitors, ubiquitin

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LIST OF PUBLICATIONS

1. Wang M, Xie Y, **Girnita L**, Nilsson G, Dricu A, Wejde J, Larsson O. Regulatory role of mevalonate and N-linked glycosylation in proliferation and expression of the EWS/FLI-1 fusion protein in Ewing's sarcoma cells.

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2. **Girnita L**, Girnita A, Wang M, Meis-Kindblom JM, Kindblom LG, Larsson O. A link between basic fibroblast growth factor (bFGF) and EWS/FLI-1 in Ewing's sarcoma cells.

Oncogene. 2000 Aug 31;19(37):4298-301.

3. **Girnita L**, Girnita A, Brodin B, Xie Y, Nilsson G, Dricu A, Lundeberg J, Wejde J, Bartolazzi A, Wiman KG, Larsson O. Increased expression of insulin-like growth factor I receptor in malignant cells expressing aberrant p53: functional impact.

Cancer Res. 2000 Sep 15;60(18):5278-83.

4. **Leonard Girnita**, Ada Girnita and Olle Larsson. P53/Mdm-2 dependent ubiquitination of the insulin-like growth factor-1 receptor

Manuscript

5. Ada Girnita*, **Leonard Girnita***, Fabrizio del Prete, Armando Bartolazzi, Olle Larsson and Magnus Axelson. Selective inhibitors of the insulin-like growth factor-1 receptor and malignant cell growth

Submitted

*** Equal contributions**

CONTENTS

LIST OF ABBREVIATIONS

aa	Aminoacids
Akt	Protein kinase B
AS	Antisense
ARF	Alternative reading frame
ATP	Adenosine threephosphate
bFGF	Basic fibroblast growth factor
BL	Burkitt's lymphoma
cDNA	Complementary DNA (DNA copy of mRNA)
DTT	Dithiothreitol
E.g.	Example
EGF	Epidermal growth factor
EGFR	Epidermal growth factor receptor
ES	Ewing's sarcoma
FGFR	Fibroblast growth factor receptor
FLI-1	Friend leukemia virus integration site 1
HLH	Helix loop helix structural domain
HMG	High mobility group
IGF-1	Insulin-like growth factor-1
IGF-1R	Insulin-like growth factor-1 receptor
IRS-1	Insulin-receptor substrate-1
kDa	Kilo Dalton
L	Lipofectin
MAPK	Mitogen-activated protein kinase
MDM-2	Murine double minute-2
mt	mutant type
NES	Nuclear export signal
ODN	Oligonucleotides
PBS	Phosphate-buffered saline
PDGF	Platelet derived growth factor
PI3-K	Phosphatidilinositol-3 kinase
PNET	Primitive neuroectodermal tumour
RT-PCR	Reverse transcription-polymerase chain reaction
SDS-PAGE	Sodium dodecyl sulphate-poliacrylamide gel electrophoresis
Shc	Src homology and collagen
Sos	Son of sevenless
SV40LT	Simian virus 40 large antigen
TM	Tunicamycin
ts	temperature sensitive
wt	wild type

INTRODUCTION

Most forms of cancer arise through an evolutionary process that favors the growth of clones and subclones of cells, less and less responsive to the normal intra- and extracellular growth control mechanisms. The multi-step development of tumors has involved changes at genomic level (oncogene activation, loss of function of tumor suppressor genes, translocations resulting in fusion genes that encodes for chimeric proteins with tumorigenic functions etc) (Bertram 2000). However, in the selection leading to cancer in somatic tissues it is likely that the cancer cells make use of the normal extracellular signaling for proliferation and/or antiapoptosis to create growth advantage over the normal cells. Most of these signals are mediated by growth factors.

Growth factors regulate important cellular activities involving cell proliferation, differentiation, and apoptosis. Emerging evidence suggests that members of the IGF, FGF, PDGF and EGF families play important role in the development and progression of cancer (Aaronson 1991).

Growth factors mediate biological responses by binding and activating cell-surface receptors with intrinsic protein kinase activity (Aaronson 1991). To date, more than 50 receptor tyrosine kinases (RTKs), belonging to at least thirteen different receptor families, have been identified. All RTKs contain a large glycosylated extracellular ligand-binding domain, a single transmembranous region, and a cytoplasmic portion with a conserved protein tyrosine kinase domain. In addition to the catalytic domain, a juxtamembranous region and a carboxyl-terminal tail can be identified in the cytoplasmic portion. RTKs can be defined as membrane-associated kinases with the plasma membrane separating the kinase and ligand binding domain (Ullrich and Schlessinger 1990). Tyrosine phosphorylation represents the way by which the information from extracellular environment is transduced to the intracellular milieu. In most cases, the ligand-induced activation of the kinase domain and its signaling potential are mediated by receptor oligomerization (for reviews (Ullrich and Schlessinger 1990; Heldin 1995; Weiss and Schlessinger 1998)). This event favors interactions between cytoplasmic domains and induces kinase activity. Dimerization can take place between two identical receptors (homodimerization), between different members of the same receptor family, or, in some cases, between a receptor and an accessory protein (heterodimerization) (Carraway and Cantley 1994; Lemmon and

Schlessinger 1994; Heldin 1995; Heldin and Ostman 1996). How ligands bind to receptors and induce oligomerization seems specific for each class of RTKs (reviews (Heldin and Ostman 1996; Schlessinger 1997)). Interestingly, the IR family exists as disulfide-bound homo- or heterodimers of receptor subunits. Thus, ligand binding does not induce receptor dimerization but presumably causes a conformational change in the preformed dimeric receptor, which leads to receptor activation (Heldin 1995). In the unphosphorylated state, the receptor catalytic activity is very low due to the particular inhibitory conformation of a specific domain in the kinase region, which interferes with the ATP-binding and phosphotransfer event. The activation of intrinsic protein kinase activity results in the autophosphorylation of specific tyrosine residues in the cytoplasmic portion of the RTK. Phosphorylation of the kinase domain removes the conformational inhibition, and the catalytic activity is enhanced and persists for some time independently of the presence of the ligand. There is substantial evidence that autophosphorylation occurs in trans by a second receptor tyrosine kinase after dimerization or conformational changes induced by ligand binding. For monomeric receptors, the emerging model is that of FGFR. It has been shown that phosphorylation of a tyrosine in the activation loop (A-loop) in the catalytic domain of the FGF receptor allows rotation of a proline residue in position 663 that normally interferes with the binding of substrate to the kinase domain and, therefore, maintains the kinase in an inactive state (review (Hubbard, Mohammadi et al. 1998)). The kinase activity is at a low basal level in the monomeric state, but this activity is sufficient to induce trans-autophosphorylation, once the dimer has been formed. Autophosphorylation also occurs outside the kinase domain and serves the important function of creating docking sites for downstream signal transduction molecules (see below). While the mechanism of RTK activation seems to be common for different classes of receptors, the tyrosine kinase domain is the most conserved among tyrosine kinase receptors, and an intact protein tyrosine kinase domain is absolutely required for receptor signaling.

The transmembranous domain function is to anchor the receptor in the plane of the plasma membrane, thereby connecting the extracellular environment with internal compartments of the cell. The juxtamembranous sequence, that separates the transmembranous and cytoplasmic domains, is not well conserved between different families of receptors. This region may also participate in signaling, as well as in the receptor internalization

(Castagnino, Biesova et al. 1995). The carboxyl-terminal tail sequences are among the most divergent between known RTKs (Yarden and Ullrich 1988). The carboxyl-terminal domain of the receptor is thought to play an important role in regulating kinase activity. This region typically contains several tyrosine residues, which are phosphorylated by the activated kinase. The evolution of multicellular organisms has involved the development of intercellular signaling network for processes as embryonic development, tissue differentiation, and systemic responses to wounds and infections. These complex signaling networks are in a large part mediated by growth factors, cytokines and hormones. 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 molecules that mediate these responses form the signaling transduction pathways. The transmission of external signals to the nucleus leads to effects on the expression of an array of genes involved in the appropriate responses.

THE IGF FAMILY

The IGF family includes ligands, receptors and IGF-binding proteins (IGFBPs).To date, there are three ligands (insulin, IGF-1 and IGF-2), three cell-surface receptors (IR, IGF-1R and IGF-2R) and at least six IGFBPs modulating the biological activity of the growth factors. Besides these "classic" members, more recent work has identified other proteins as potential members of IGF family: the orphan insulin-receptor-related receptor (IRR), the insulin-IGF-1 hybrid receptor and a growing number of IGFBPs. Additionally, the activity of IGFs is modulated by a number of IGFBP-proteases which cleave the binding proteins, regulating the availability of the ligands (Werner and Le Roith 2000).

IGF-1

The human IGF-1 gene has been mapped to the long arm of chromosome 12 (Sara and Hall 1990). The gene spans more than 90 kb of chromosomal DNA and consists of at least six exons. Transcription of the mammalian IGF-1 gene and processing of its primary transcript are elaborate, with alternative leader exons 1 and 2 encoding mutually exclusive 5′ untranslated regions (UTRs) and distinct N- termini of the signal peptide.

The mature peptide is encoded by exons 3 and 4, and the E peptide sequences by exons 4-6 (Sara and Hall 1990).
Transcription of exon 1 starts from at least four sites dispersed over a 350 bp region (LeRoith, Neuenschwander et al. 1995; LeRoith, Werner et al. 1995). This " diffuse " pattern of initiation appears to be due to the lack of TATA- and CCAAT- elements in the exon 1 promoter. On the other hand, the exon 2 promoter contains these two elements and, as a result, transcription of this exon is initiated from a cluster of sites located 50-70 nucleotides upstream of the 3´end of this exon . The expression of the IGF-1 gene is developmentally regulated, with levels of IGF mRNA in most tissues increasing 10- to 100- fold between birth and adulthood (Roberts, Brown et al. 1986).

IGF-2

The human IGF-2 gene is located on the distal end of the short arm of chromosome 11, contiguous to the insulin gene, and it encompasses ~ 30 kb of chromosomal DNA. The gene includes nine exons, and the coding sequence of the mature peptide is encoded by exons 7-9. Similar to the IGF-1 gene, transcription of IGF-2 is extremely complex, with multiple leader exons controlled by four promoters. Promoter P1 is a TATA- less, GC- rich promoter, which is active in adult liver, where it directs a heterogeneous pattern of transcription initiation. The P2-P4 promoters are active in fetal and most adult non-hepatic tissues and, in general, direct transcription from specific sites. Unlike IGF-1 mRNA, IGF-2 mRNA levels in all tissues are high during late fetal and prenatal periods, and decline thereafter (in humans, however, IGF-2 can be detected in the circulation at adult stages) (Yu and Rohan 2000).

IGF-1 and 2 are produced by the liver, the most important source of circulating IGFs. IGF-1 synthesis is tightly correlated with circulating level of growth hormone (GH). Later studies demonstrated that multiple tissues are able to synthesize IGFs , which can act locally (autocrine, paracrine), in addition to their endocrine modes (D'Ercole, Applewhite et al. 1980). Finally, a recent work has shown that ablation of liver IGF-1 production does not affect growth and development of mice (Yakar, Liu et al. 1999).
Regarding function, whereas insulin mediates mainly metabolic effects, both IGF-1 and 2 were originally identified as potent mitogens and

mediators of growth hormonal actions. It has been recognized by now that, in addition to mitogenic function, these polypeptide growth factors play a crucial role in cell survival, transformation, antiapoptosis and maintenance of the malignant phenotype in many cell and tumor systems (Werner and Le Roith 2000).

The IGF binding proteins

Six members of the IGF binding proteins have been identified (Yu and Rohan 2000). Their function is still ill-defined. The family of six IGFBPs contains regions with strong homology, including cysteine-rich N-terminal

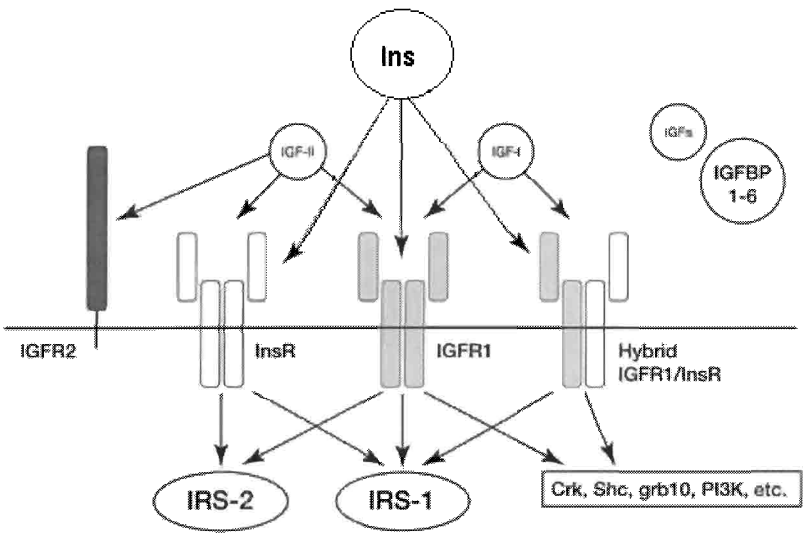

Fig. 1. The IGF family: ligands (insulin, IGF-1 and IGF-II), receptors and IGFBPs

and C-terminal regions (Rajaram, Baylink et al. 1997). In particular, the alignment of 18 cysteines in these regions is highly conserved. Those

cysteines are involved in disulphide bond formation between the N- and C-terminal domains, giving rise to the tertiary structure. IGFBP-1 and –2 also contain an Arg-Gly-Asp sequence at their C-termini that binds to integrin receptors. Some IGFBPs are N-linked glycosylated, whereas others are O-linked. In addition, phosphorylation of serine residues may affect ligand binding (Lee, Giudice et al. 1997).

Receptors

The third component of IGF family is the set of cell surface receptors. Although both IGF-1 and IGF-2 bind weakly to the IR, each of them has its own receptor (Fig. 1.). In fact, all three ligands (insulin, IGF-1 and IGF-2), can bind to each other's receptor in a competitive manner. IGF-1 and IGF-2 bind with high affinity to the IGF-1R, a transmembranous tyrosine kinase, widely expressed across many cell types in fetal and postnatal tissues. There is an ample consensus today, that most of the biological actions of IGFs are mediated by IGF-1R.
IGF-1R is a member of tyrosine kinase receptor (RTK) family and together with IR and IR/IGF-1 hybrid receptor forms a distinct subclass of RTK. Its amino acid sequence is 70% homologous to that of the IR (Ullrich, Gray et al. 1986; LeRoith, Werner et al. 1995). Characteristic for this subclass is that unlike others RTK, IR and IGF-1R exist as preformed dimers.

IGF-1R biosynthesis and molecular organization

The human IGF-1R gene was mapped to chromosome15 q25-26 (Abbott, Bueno et al. 1992). Similarly to the IR gene, the gene for IGF-1R consists of 21 exons, 10 for the alpha chain and 11 for the beta chain, spanning over 100kb of the genomic DNA (Abbott, Bueno et al. 1992).The complementary DNA (cDNA) for human IGF-1R consists of 4989 nucleotides and codes for a 1367 amino-acid precursor. The exon / intron organization of the IGR-1R gene, predicted on the basis of cDNA, is quite similar to that of the IR gene, the main difference being that the IR gene contains an alternatively spliced exon 11 not present in the IGF-1R receptor gene (Ebina, Ellis et al. 1985; Ullrich, Bell et al. 1985; Ullrich, Gray et al. 1986). The IGF-1R is organized into functional domains that reflect the exonic arrangement of the gene: exons 1-3 encode the long 5´UTR (~1 kb), the signal peptide, and the N-terminal non-cysteine-rich

and the cysteine rich domains of the α subunit (ligand-binding domain). The rest of the α- subunit is encoded by exons 4-10. Exon 11 encodes the peptide cleavage site that generates the mature α- and β-subunits from the proreceptor. The exon 12-21 encode the β-subunit, with exon 14 encoding the transmembrane and exon 16-20 encoding the tyrosine kinase domain (Ullrich, Gray et al. 1986).

Transcription from the IGF-1R gene results in a transcript product of 11kb, often together with a minor band of 7kb (Chernausek, Jacobs et al. 1981; Lowe, Adamo et al. 1989). Both subunits, encoded in the same message, are translated in a precursor protein of 1367 amino acids in length, with the structure: NH2-signal peptide, α subunit and β-subunit-COOH. It is customary to count the amino acid residues of the IGF-1R from the first amino acid of the mature peptide (after removal of the signal peptide), up to 1337 (Ullrich, Gray et al. 1986). Following removal of the signal peptide, the pro-receptor is cleaved after residue 706, to form the α- and β-subunit, linked by disulphide bonds. The α-subunit, containing 706 amino acids, is required for ligand binding. It is entirely extracellular, forms a dimer with another α-subunit, and it is in this form that it is active. The α-subunit contains a cysteine-rich domain (aa 148-302), involved in ligand binding, 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). The β subunit spans the plasma membrane and contains 627 amino acid residues. The transmembranous domain is located at position 906-929. The extracellular domain of the β subunit, 196 aa in length, contains all the 5 potential glycosylation sites. The intracellular portion of the β-subunit can be divided in three domains: a juxtamembranous domain, the TK domain, and a C-terminal tail. The juxtamembranous domain contains an NPXY motif, which could be important for receptor internalization (Brown and Goldstein 1986; Backer, Kahn et al. 1990; Hsu, Knudson et al. 1994; Prager, Li et al. 1994). The TK domain is highly homologous to that of IR (84%), the juxtamembranous domain shares 61% of homology with IR, whereas the C-terminal domain shares only 44% (Ullrich, Gray et al. 1986). Similar to other RTKs, the catalytic region of IGF-1R contains the ATP binding motif (Gly-XXX-Gly-XXX-XXX-Gly) at position 976-981, and a catalytic Lys in position 1003, which is critical for the MgATP binding (Hanks, Quinn et al. 1988). Within the TK domain, a cluster of

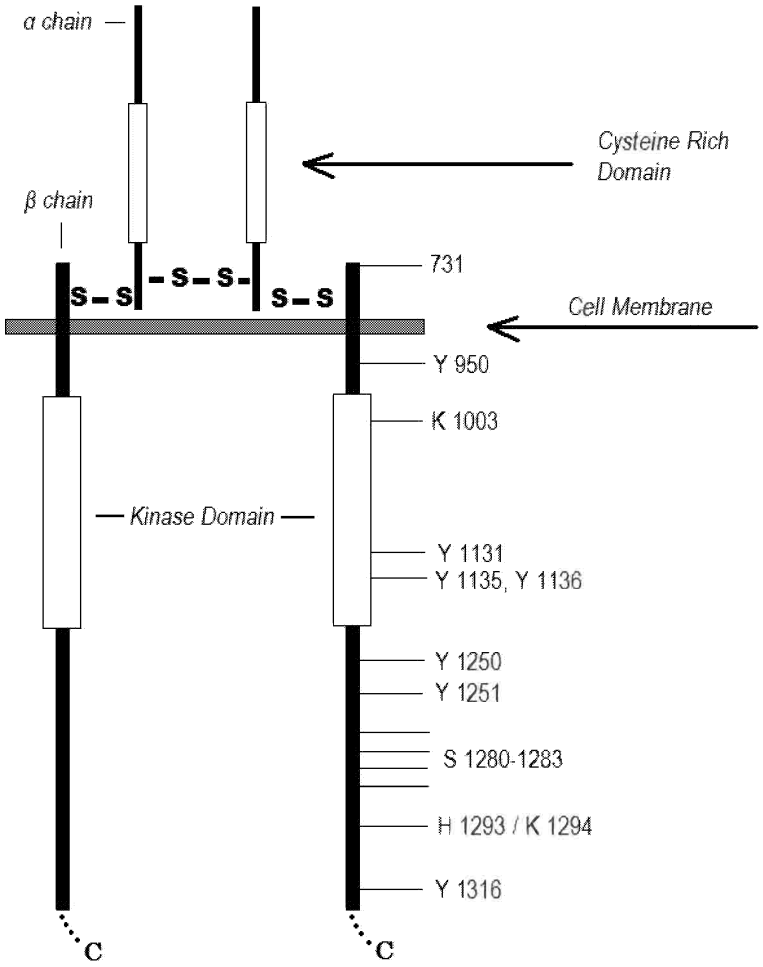

Fig. 2. Cartoon of the IGF-1R showing the distribution of domains across the α and β chains. Important amino acid residues for regulation of tyrosine kinase activity and signal transduction are also indicated.

three tyrosines, located at position 1131, 1135 and 1136, is critical for receptor autophosphorylation (LeRoith, Werner et al. 1995). The IGF-1R, like the IR, undergoes extensive post-translational modification, which include serine and tyrosine phosphorylation, and glycosylation (Ullrich,

Gray et al. 1986). The predicted size, based on the protein sequences is 80,423 kDa for the α- and 70,866 kDa for the β-subunit, but due to heavily glycosylation their molecular weights are 135 kDa and 90 kDa, respectively. The α-subunit contains 11 potential glycosylation sites, whereas β-subunit contains only 5. It has been shown that N-linked glycosylation precedes proteolysis of the immature αβ precursor (Jacobs, Kull et al. 1983). The result is that the mobility of the receptor subunits in gels is substantially slower compared to their amino acid composition. Under normal condition, the processing of the IGF-1R has a half life of approximately 1 hour (Sepp-Lorenzino 1998).
N-linked glycosylation is a co-translational modification that occurs in ER and consists in addition of a sugar chains to the nascent protein. This process is energy consuming (Kornfeld and Kornfeld 1985) and requires a non-sterol isoprenoid product of the mevalonate pathway. Because the sugar chains have limited flexibility, they protect the glycoprotein from coming in contact with other macromolecules (i.e. proteases) (Kobata 1992; Gary and Clarke 1995). Sugar chains also play an important role as signals for cell-surface recognition in multi-cellular organism (Kobata 1992).

IGF-1R activation

After ligand binding autophosphorylation of IGF-1R, and the IR , is initiated at the three tyrosine residues of the A-loop (1131, 1135, and 1136 for IGF-1R; 1158, 1162 and 1163 for the IR) of the kinase domain of the β-subunit (Sepp-Lorenzino 1998). Tyrosine phosphorylation of this triad further increases the intrinsic TK activity towards phosphorylation of other tyrosines in the receptor and subsequently of exogenous substrate proteins. When this triple tyrosine cluster is substituted with phenylalanine, both IR and IGF-1R lose all biological actions (Gronborg, Wulff et al. 1993; Kato, Faria et al. 1994; Li, Ferber et al. 1994; Jiang, Chan et al. 1996; O'Connor, Kauffmann-Zeh et al. 1997). Autophosphorylation is an intramolecular process and the velocity of this reaction is not dependent on IGF-1R concentration (Sasaki, Rees-Jones et al. 1985), but dependent on intact tetramers (Tollefsen, Thompson et al. 1987; Feltz, Swanson et al. 1988).The function of the triple tyrosine cluster in the tyrosine kinase domain of the IR is well characterized (Hernandez-Sanchez, Blakesley et

al. 1995), but much less is known about corresponding tyrosine cluster in the IGF-1R. In case of IR, the second tyrosine in the cluster, 1162, is bound to the active site (1158, 1163) of the same b-subunit, seemingly in that position to be autophosphorylated in the cis position (Hubbard and Till 2000). However, this does not occur because the ATP binding site is not properly positioned (Hubbard and Till 2000). Based on structural studies of the IR kinase domain, it has been suggested that Tyr 1162 is phosphorylated in trans by the neighboring b-chain (Hubbard, Wei et al. 1994; Hubbard 1997). Prior to autophosphorylation, Tyr 1162 competes with the other β-chain for the active site (Wei, Hubbard et al. 1995; Hubbard, Mohammadi et al. 1998). The autoinhibitory role for Tyr 1162 is consistent with the observation that substitution of it with phenylalanine results in an increment of basal kinase activity (Ellis, Clauser et al. 1986; Hubbard, Mohammadi et al. 1998). Less is known about the structure-function relationship of the IGF-1R. Single substitution of the second tyrosine (1135) has relatively small inhibitory effect on receptor autophosphorylation and, unlike IR, does not result in an increase of basal activity (Stannard, Blakesley et al. 1995). The same effect is obtained by modifying the first tyrosine (1131) (Li, Ferber et al. 1994). In contrast, substitution of the Tyr 1136 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). A more recent study, (Favelyukis, Till et al. 2001) using a truncated form of the β-subunit, suggested that the order of tyrosine phosphorylation for the IGF-1R is identical with that of IR (i.e., first 1135 and then 1131 and 1136). The same study demonstrated that the phosphorylation is activated in trans, consistent with the autoinhibitory role of the second tyrosine, as for the IR.

While these three tyrosines have been shown to play a critical role in receptor function, other tyrosines within the carboxyl-terminal and juxtamembranous domains may also be phosphorylated and involved in the regulation of IGF-1R activity and functions. **(Table 1)** This activation of the IGF-1R tyrosine kinase implies the stimulation of diverse intracellular pathways, involving different signaling substrates. A schematic presentation of the cascades activated upon IGF-1R activation is shown in Fig. 3. The best characterized substrates of the IGF-1R are members of IRS (insulin receptor substrate) family (Craparo, O'Neill et al.

1995; Tartare-Deckert, Sawka-Verhelle et al. 1995; He, Craparo et al. 1996) and Shc (Craparo, O'Neill et al. 1995; Tartare-Deckert, Sawka-Verhelle et al. 1995). Thus, activation of the IGF-1R tyrosine kinase results in stimulation of an array of various intracellular signaling cascades, including the Ras/Raf/Map kinase and PI-3 kinase pathways. Induction of a positive signal, by means of these signaling pathways, ultimately results in cell survival and proliferation. Conversely, a negative signal, represented by absence of the ligand or non-functional receptor, will result in a decrease of cell number, apoptosis, and ultimately cell death (Butler, Yakar et al. 1998; Kalebic, Blakesley et al. 1998).

Functional domains for signal specificity

The IRSs constitute a family of structurally related adaptor proteins that can link the IGF-1R to downstream signal transduction mediators regulating cellular growth. Of these, IRS-1 is the most extensively studied (Sun, Rothenberg et al. 1991). This 165–195 kDa molecule does not contain SH2 (Src homology 2) or SH3 domains and may bind to the β-subunit through a PTB (pTyr-binding) domain (Sun, Wang et al. 1995). It contains at least 20 potential tyrosine phosphorylation sites and can act as a multisite "docking" protein associating with multiple downstream signaling proteins including PI-3 kinase (Backer, Myers et al. 1992; Myers, Grammer et al. 1994), SH2 domain-containing tyrosine phosphatase (Syp) (Myers, Grammer et al. 1994), Fyn, Nck, and growth factor receptor-bound protein-2 (Grb2) through their SH2 domains (Lee, Li et al. 1993; Myers, Grammer et al. 1994; Myers, Wang et al. 1994). Stimulation of PI-3K leads to the activation (phosphorylation) of several downstream substrates including protein kinase B (Akt), which can phosphorylate Bad and attenuate its proapoptotic effect and the pp70 S6 kinase (LeRoith, Werner et al. 1995). Grb2 is tightly associated with the guanine nucleotide exchange factor mSOS linking the IGF-1R to the Ras/Raf-1/ mitogen-activated protein kinase (MAPK) signaling pathway, leading to activation of nuclear transcription factors (Butler, Yakar et al. 1998). Like IRS-1, tyrosine phosphorylation of Shc promotes association with Grb2, linking it to the Ras pathway via the Grb2–mSOS complex (Werner and Le Roith 2000).

Table 1. Biologic effects of different point mutations or C terminal truncations of IGF-1R

A = antiapoptotic function, **M** = mitogenic function, **T**= transforming function,

TK = tyrosine kinase activity of the receptor, **?** = unknown, ↓ = decrease.

Modified from O'Connor et al. (O'Connor, Kauffmann-Zeh et al. 1997)

IGF-1R	***A***	***M***	***T***	***TK***	***Reference***
Wild type	+	+	+	+	
K 1003A/R	-	-	-	-	(Kato, Faria et al. 1993; Coppola, Ferber et al. 1994; O'Connor, Kauffmann-Zeh et al. 1997)
Y 1131F	?	+	-	↓	(Li, Ferber et al. 1994; Stannard, Blakesley et al. 1995)
Y1135 F	?	+	-	↓	(Li, Ferber et al. 1994; Jiang, Chan et al. 1996)
Y1136F	?	-	-	-	(Li, Ferber et al. 1994; Jiang, Chan et al. 1996)
Y 1131-35	?	-	-	-	(Hernandez-Sanchez, Blakesley et al. 1995)
Y1131-36	?	-	-	↓	(Hernandez-Sanchez, Blakesley et al. 1995)
Y1135-36	?	-	-	↓	(Hernandez-Sanchez, Blakesley et al. 1995)
Y 1131-35-36	-	-	-	-	(Li, Ferber et al. 1994; Jiang, Chan et al. 1996)
V 922 E	?	-	?		(Takahashi, Yonezawa et al. 1995)
Y 950 F	+	-	-	+	(Miura, Li et al. 1995; Jiang, Chan et al. 1996; Esposito, Blakesley et al. 1997)
W 1173 A	?	-	?	-	(Blakesley, Kato et al. 1995)
Y 1250 F	+	+	+	+	(Miura, Surmacz et al. 1995; Jiang, Chan et al. 1996; O'Connor, Kauffmann-Zeh et al. 1997)
Y 1251 F	-	+	-	+	(Miura, Surmacz et al. 1995; Jiang, Chan et al. 1996; O'Connor, Kauffmann-Zeh et al. 1997)
Y 1250F-51H	-	+/-	-	+	(Miura, Surmacz et al. 1995; O'Connor, Kauffmann-Zeh et al. 1997)
F 1310 Y	?	+	+	+	(Esposito, Blakesley et al. 1997)
Y 1250F-51H F 1310 Y	?	+	-	+	(Esposito, Blakesley et al. 1997)
S 1280-83 A	+	+	-		(O'Connor, Kauffmann-Zeh et al. 1997)
Y 1316 F	+	+	+/-		(Hongo, D'Ambrosio et al. 1996; O'Connor, Kauffmann-Zeh et al. 1997)
Δ952	?	-	-	-	(Prager, Li et al. 1994)
Δ 1229	+	+	-		(O'Connor, Kauffmann-Zeh et al. 1997)
Δ 1245	+	+	-		(Hongo, D'Ambrosio et al. 1996; O'Connor, Kauffmann-Zeh et al. 1997)
Δ1270	?	?	+		(Hongo, D'Ambrosio et al. 1996)
Δ 1289	?	+	?		(Gronborg, Wulff et al. 1993)
Δ 1293	+	+	-		(Miura, Surmacz et al. 1995; O'Connor, Kauffmann-Zeh et al. 1997)
Δ 1310	?	+	-		(Hongo, D'Ambrosio et al. 1996)
H 1293 F K 1294 R	-	+	-		(Hongo, D'Ambrosio et al. 1996)

Preferential phosphorylation of Shc or IRS-1 could depend on the cellular context and may direct IGF-1R signaling preferentially towards cellular proliferation or differentiation (LeRoith, Werner et al. 1995). In fact, IRS and Shc were shown to compete for the same pool of Grb/2/Sos. Although both types of complexes mediate the activation of Ras, the Shc pathways seems to be dominant in this respect (Yamauchi and Pessin 1994).

The relative importance of these pathways in signal transduction by IGF-1R is probably cell context-dependent and remains to be fully elucidated. Mutational analyses have identified in the receptor β-subunit several amino acid residues essential for receptor functions (Table 1). Lysine at position 1003 (the ATP-binding site) and tyrosine 950 (thought to be essential for IRS-1 and Shc binding and phosphorylation) are critical for all receptor functions. Tyrosines 1131, 1135, and 1136 of the a-loop of the

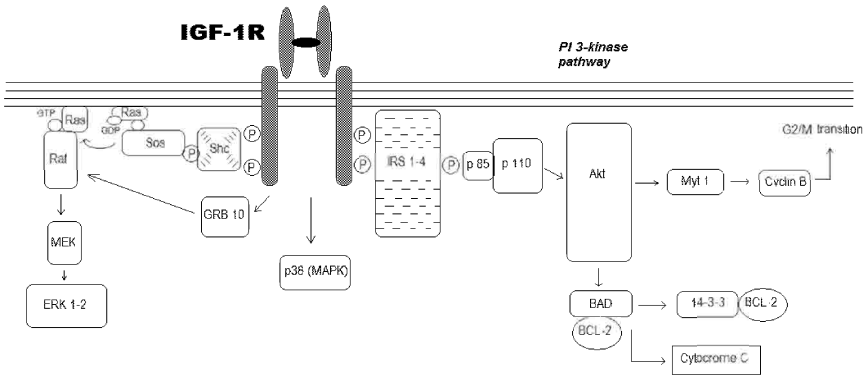

Fig. 3. Signal transduction cascades activated by the IGF-1R. Activation of the IGF-1R induces the binding and subsequent tyrosine phosphorylation of SHC and IRS adapter proteins to the IGF-1R β subunit. SHC and IRS creates binding sites for other proteins in the signal transduction cascade.

kinase domain are essential for the mitogenic and transforming activities of the receptor, but there is some controversy regarding their role in regulating the antiapoptotic effect of the receptor. In contrast, the tyrosine at position 1251, although essential for neither the receptor nor IRS-1 and Shc phosphorylation, is critical for the transforming (anchorage-independent growth) and antiapoptotic effects of the receptor, possibly through involvement in cytoskeletal reorganization. The role of these tyrosines in mitogenesis is controversial. Finally carboxyl terminal serines at position 1280–1283 also appear to play a role in regulating the transforming function of the IGF-1R.
The IGF-1R, like the IR, has anabolic functions, but in terms of growth it has three properties that distinguish it from IR: it is mitogenic; it is necessary for establishment and maintenance of the transformed phenotype; and it protects cells both in vitro and in vivo from apoptosis.

Receptor internalization and signal attenuation

Receptor down regulation 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: signaling attenuation of an activated receptor and signal activation facilitating the interaction between RTK and downstream signaling molecules. 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). Some receptors are internalized constitutively and recycled (transferrin receptor), whereas most of the tyrosine kinase growth factor receptors and G protein-coupled receptors are internalized after the ligand binding (Robinson 1989; Koenig and Edwardson 1997). After binding of the ligand, the activated receptors are targeted to the clathrin-coated membrane invaginations (Ceresa and Schmid 2000), a process mediated by a specific internalization signal

situated within cytoplasmic domain of the receptor (Hicke 1999).To date 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/sorting motifs are usually located within the juxtamembrane region of the receptor (Johnson and Kornfeld 1992; Bremnes, Madsen et al. 1994). For the IR two tyrosine-based motifs (residues 950-953 GPLY and residues 957-960 NPEY) have been reported to be required for rapid endocytosis (Pearse and Robinson 1990; Rajagopalan, Neidigh et al. 1991; Kaburagi, Momomura et al. 1993). The human IGF-1R contains three tyrosine residues in the submembrane region (Prager, Li et al. 1994), two of which being contained in GVLY and NPEY motifs similar to the GPLY and NPXY sequences in the IR (Fig.4). However, contradictory results regarding the role of these tyrosine-based motifs as internalization signals have been reported. (Backer, Kahn et al. 1990; Rajagopalan, Neidigh et al. 1991) demonstrated that NPXY and GPLY motifs are essential for the IR internalization, and mutation of tyrosine to another amino acid impaired receptor internalization. On the other hand, (Kaburagi, Momomura et al. 1993) reported that none of the motifs is required for the receptor internalization. (Prager, Li et al. 1994) demonstrated that NPXY motif from IGF-1R is important for receptor internalization, whereas others reported the contrary. Miura et al (Miura and Baserga 1997) demonstrated that in fact tyrosine 1250 within IGF-1R is the functional tyrosine-based internalization signal. For the IR, a di-leucine based motif (962-987 EKITLL) has been identified as the 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 corresponding sequence (EKITMS). In addition, it has been demonstrated that intracellular itineraries of insulin/IR and IGF-1/IGF-1R are quite different: endocytic rate constant is three times higher for insulin than for IGF-1; insulin dissociates from its receptor more rapidly than IGF-1; and ligand degradation is 3-fold higher for insulin (Zapf, Hsu et al. 1994). Ligand/receptor retroendocytosis was found to be 53% for IGF-1 in contrast with 28% for insulin. It might be speculated that the di-leucine motif present in the IR could increase its internalization and 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). Instead EKITMS

motif is not as efficient as EKITLL to target the protein to lysosomes. Almost 80% of chimeric proteins carrying the EKITLL motifs were internalized and targeted to lysosomes, whereas chimeric proteins expressing EKITMS motif were internalized more slowly and delivered to endosomes and the Golgi network in a very high proportion (more than 80%). Only a small fraction (less than 10%) was targeted to lysosomes. The substitution of MetSer for LeuLeu alone does not explain the difference in the rates at which receptors for insulin and IGF-1 undergo endocytosis. It is likely that other structural differences between the two receptors explain the dissimilarity of the endocytic traffic between IR and IGF-1R (Haft, De La Luz Sierra et al. 1998).

Recent 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 polypeptide, 76 amino acids in length, playing many cellular functions (Hicke 1997). Ubiquitination of proteins requires the action of three enzymes: 1. ubiquitin activating enzyme (E1) that bound ubiquitin to generate a high energy E1-ubiquitin intermediate; 2. ubiquitin-conjugating enzyme (E2), an ubiquitin carrier protein; and 3. an ubiquitin ligase that transfer the ubiquitin to the target protein (Bonifacino and Weissman 1998; Glickman and Ciechanover 2002). The ubiquitin molecule is generally transferred to a Lys residue of the substrate, but in some cases ubiquitin is conjugated to the amino terminal group of the substrate. 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, and therefore E3, the substrate or both must be switched on by posttranslational modifications. In proteasome degradation ubiquitin serves as a tag that target 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 trigger the plasma membrane proteins into the endocytic pathway with 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).

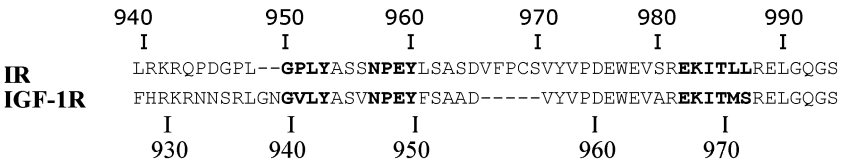

Fig. 4. Juxtamembrane domains of the IR and IGF-1R. The potential tyrosine-based internalization motifs and di-leucine motif are highlighted.

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)

Mechanism of ubiquitin induced internalization

Similar to cytosolic proteins that undergo ubiquitination and degradation, plasma membrane protein ubiquitination is positively regulated by phosphorylation in response to ligation. 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 factors receptors undergo ligand-induced ubiquitination. The mechanism of ubiquitin-mediated internalization has not been defined. 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 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 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.
The function of ubiquitination as an internalization and endocytic sorting signal is clearly demonstrated. 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).

Control of IGF-1R expression

Control of gene expression
Physiological and pathological regulation

The IGF-1R gene is constitutively expressed in most cells. The IGF-1R promoter exhibits a high basal transcriptional activity, and is under physiological control of nutritional factors (Lowe, Adamo et al. 1989; Olchovsky, Song et al. 1993; Matsumura, Domeki et al. 1996), hormonal stimulation (GH, follicle stimulating hormone, glucocorticoids, estrogens and thyroid hormones) (Adashi, Resnick et al. 1988; Lin, Blaisdell et al. 1988; Goldfine, Papa et al. 1992; Hernandez 1995; Clarke, Howell et al. 1997) and the developmental stage (Werner, Woloschak et al. 1989; Bondy, Werner et al. 1992). Its expression is altered in certain diseases,

including cancer (Baserga 1995; LeRoith, Baserga et al. 1995; Baserga, Hongo et al. 1997) and diabetes (Werner, Shen-Orr et al. 1990; Werner, Shalita-Chesner et al. 2000) as well as infectious diseases like B virus hepatitis (Werner, Shalita-Chesner et al. 2000).

The promoter of IGF-1R is CG-rich and lacks TATA and CCAAT elements (LeRoith, Werner et al. 1995), but has elements found in housekeeping genes, containing regulatory elements characteristic for highly regulated genes (Werner, Stannard et al. 1990; Werner, Stannard et al. 1991). Some of the transcription factors regulating the expression of the IGF-1R gene have been identified. These are sequences within long 5' UTR and promoter containing sites for binding transcriptional regulators, like Sp1, E2F and members of early growth response family (LeRoith, Werner et al. 1995). Sp1, a ubiquitous nuclear protein which activates GC-rich promoters, plays a critical role in IGF-1R expression, and no IGF-1R activity was detected in cells lacking Sp1 promoter (Werner, Bach et al. 1992; Beitner-Johnson, Werner et al. 1995). One physiological factor upregulating IGF-1R expression is nutritional status which seems to be related to decreased levels of the circulating IGF-1. Similarly, decreased levels of circulating IGF-1 in growth disorders cause an increase of IGF-1R mRNA levels. In fact, one of the most important regulators of IGF-1R expression is IGF-1. Generally, increasing IGF-1 concentration causes a decrease in receptor number, by translocation of cell-surface receptors to an intracellular pool. This process is thought to be a key event in the regulation of receptor bioavailability and activity (Brodt, Samani et al. 2000). Other growth factors generally increase the IGF-1R expression, e.g. bFGF, EGF and PDGF (Rubin and Baserga 1995; Werner and LeRoith 1996). Basic FGF has been shown to increase receptor binding and mRNA levels, whereas PDGF increased the activity of the IGF-1R promoter through a promoter fragment located immediately upstream to the transcription start site. This region has a canonical c-Myc binding site. Since PDGF induces c-myc the effect of PDGF on IGF-1R expression may be mediated by c-Myc. Steroid hormones also upregulate the expression of IGF-1R gene, the action of which is induced by peptide growth factors (Clarke, Howell et al. 1997). This indicates that estradiol stimulates cellular proliferation by increasing the number of IGF-1R.

Interplay between oncogenes and tumor suppressors in control of IGF-1R gene expression

The IGF-1R promoter is also targeted by multiple oncogenes. Constitutive overexpression of the proto-oncogenes c-myb in Balb: c-3T3 cells have been shown to abrogate the requirement for IGF-1 in the growing media (Reiss, Ferber et al. 1991; Travali, Reiss et al. 1991; Kim, Park et al. 1996). The hepatitis B virus X (HBx) protein is another oncogene known to stimulate IGF-1R promoter activity (Kim, Park et al. 1996). Therefore HBx may play a role in the etiology of hepatocellular carcinoma by stimulating the expression of the IGF-1R gene. The oncogenes increasing IGF-1R promoter activity can also affect IGF-1R action by nontranscriptional mechanisms. For instance, transformation of human cells by the src oncogene of the Rous sarcoma virus results in constitutive phosphorylation of the receptor β-subunit, whereas addition of IGF-1 further increases the level of phosphorylation (Werner and Le Roith 2000). Interestingly, v-src is the only single oncogene that is able to transform IGF-1R negative (R-) cells (Valentinis, Morrione et al. 1997). The explanation could be that v-src activates pathways downstream of the IGF-1R, thereby bypassing it. Consistently, src activates both the PI3-kinase and MAPK pathways (Penuel and Martin 1999), which are major pathways activated in IGF-1-mediated mitogenesis.

Tumor suppressors, a family of negative growth regulators, are involved in a wide variety of human cancers (Marshall 1991; Knudson 1993). It has been postulated that a potential mechanism by which the postmitotic, fully differentiated, cell is kept in G_0 may involve the constitutive inhibition of the IGF-1R gene by wild-type tumor suppressors (Werner 1998). The IGF-1R gene contains several binding sites for members of EGR family of transcription factors. WT1, a member of this family, is a tumor suppressor gene and its product has been shown to suppress the activity of promoters containing WT1 binding sites. These target promoters include IGF-1R, IGF-2, PDGF-A, and CSF-1. WT1has been shown to bind to IGF-1R promoter region, and to suppress its activity as well as the endogenous levels of IGF-1R mRNA (Werner, Re et al. 1993; Werner, Shen-Orr et al. 1995). Loss of WT1 activity in Wilms' tumor and related malignancies may result in transcriptional de-repression of the IGF-1R gene (Ladanyi and Gerald 1994; Gerald, Rosai et al. 1995). Pathologic fusion of EWS to WT1 has been shown to abrogate the tumor suppressor function of WT1 and to generate an oncogenic chimeric protein capable of binding and

activating the IGF-1R promoter (Karnieli, Werner et al. 1996). Another tumor suppressor gene product, p53 can interact in vitro with both Sp1 and WT1 proteins. In addition, IGF-1R gene promoter contains several potential binding sites for this transcription factor.
Likewise, p53, which is the most frequently mutated tumor suppressor, is capable of suppressing the activity of the IGF-1R promoter as well as lowering the endogenous levels of IGF-1R mRNA. In addition, other components of the IGF system are regulated by p53: transcription of the IGF-2 gene is similarly reduced by wild-type p53 (Zhang, Kashanchi et al. 1996), whereas IGFBP3 is stimulated by normal p53 (Buckbinder, Talbott et al. 1995). In contrast, tumor-derived, mutant versions of p53 significantly stimulated promoter activity (Werner, Karnieli et al. 1996; Ohlsson, Kley et al. 1998). These data therefore suggest that upregulation of IGF-1R due to loss-of-function of p53 may facilitate selection of a malignant population of cells.
In conclusion, p53 controls the IGF signaling system at different levels, i.e., regulation of ligands, receptors and binding proteins. The interplay between wild-type and mutant forms of these transcription factors are very complicated and may involve additional mechanisms. It is likely that interactions between these stimulatory and inhibitory factors control the level of the IGF-1R expression. Revealing these mechanisms could be important for targeting IGF-1 or p53 pathways in a therapeutic approach

Role of IGF-1 family in malignancy

Several lines of evidence implicate IGF-1 and IGF-1R in malignant transformation (Baserga 1999; Werner and Le Roith 2000; Yu and Rohan 2000). Increased expression of IGF-1, IGF-1R or both has been documented in many human malignancies including carcinomas of the lung, breast, thyroid, gastrointestinal tract and prostate, as well as glioblastoma, neuroblastoma, melanomas rhabdomyosarcoma, and leukemias (Belfiore, Pandini et al. 1999; Hakam, Yeatman et al. 1999; Xie, Skytting et al. 1999; All-Ericsson, Girnita et al. 2002). Epidemiological prospective studies identified high plasma levels of IGF-1 as potential risk factor for several malignancies (Mantzoros, Tzonou et al. 1997; Hankinson, Willett et al. 1998). In addition, the IGFs are a potent mitogen for a wide range of tumor cell types in vitro (Baserga 1994; Valentinis, Porcu et al. 1994; Baserga 1995; Werner and LeRoith 1996).

Furthermore, several oncogenes have now been shown to affect IGF-1 and IGF-1R expression (Baserga 1994; Werner, Shalita-Chesner et al. 2000). Treatments interfering with IGF-1R expression or function suppressed tumor cell growth (Baserga 1999).
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 positive regulator of the invasive/metastatic phenotype and IGF-1 as a paracrine growth-promoting factor for liver metastasis (All-Ericsson, Girnita et al. 2002). The transforming function of IGF-1R depends on its mitogenic and antiapoptotic activities.

Mitogenic function of IGF-1R

The mitogenic function of IGF-1 was initially proposed based on the results of experiments using specific anti-IGF-1 antibodies (Russell et al., 1984). 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 'nonmitogenic' 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).

Antiapoptotic function of IGF-1R

The IGF-1R exhibits a potent antiapoptotic activity. The antiapoptotic function, in addition to mitogenic one, 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). The capacity of the IGF-1R to protect cells from programmed death has been demonstrated in many different systems, in fibroblasts, neuroderived cells, hematopoietic cells, (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 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 sensitized tumor cells to apoptosis and reversed tumor cell phenotype.

Targeting the IGF-1 pathways in cancer therapy: blocking transformation and inducing apoptosis

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The fact that interference with the function of the IGF-1R results in tumor cell death, inhibition of tumorigenesis and prevention of metastases, may appear to not be especially remarkable since many agents and modalities can do the same. But there is something unique about the IGF-1R. Interference with the function of the IGF-1R:
1. Causes massive apoptosis of tumor cells in vivo;
2. Inhibits tumorigenesis
3. Elicits a host response leading to the eradication of surviving malignant cells
4. Has only a moderate effect on normal cells.
Thus, IGF-1R appears to be a promising cancer target. Indeed, a variety of approaches aimed at targeting IGF-1R has been utilized to prove the concept, or are being developed for potential anticancer therapies. These include targeting functional IGF-1R on cell surface, targeting ligand/receptor interaction, targeting receptor expression and functions. Strategies aimed to block the ligand-receptor interaction involve receptor

neutralizing antibodies (Rohlik, Adams et al. 1987; Kalebic, Tsokos et al. 1994), IGF-1 peptide analogues (Ren, Ezzat et al. 1992; Pietrzkowski, Mulholland et al. 1993) or upregulation of the expression of IGFBP (Ren, Ezzat et al. 1992). Antibody blockade of IGFR1 has been attempted in breast cancer model systems. In a xenograft model of human breast cancer, infusion of αIR3, a mouse monoclonal antibody blocking IGF action, has been shown to inhibit the growth of MDA-231 cells. In contrast, MCF-7 xenograft tumors were not inhibited by this strategy. The explanation could be that αIR-3 is not a completely inert agent and can cause serine/threonine phosphorylation of IGF-1R. In cells overexpressing IGF-1R, αIR-3 takes on the undesirable property of a full receptor agonist, presumably by causing receptor cross-linking. In another attempt to inhibit the IGF-1R signaling, dominant negative mutants of the IGF-1R have been successfully used by several laboratories (Burgaud, Resnicoff et al. 1995; Reiss, D'Ambrosio et al. 1998). Cells transfected with IGF-1R cDNA, in which the codons for the three tyrosines 1131, 1135 and 1136 were substituted to phenylalanine, have lost their invasive and metastatic potential (Brodt, Samani et al. 2000).

A direct strategy to interfere with IGF-1R activity is to induce selective inhibition of its tyrosine kinase. Recently we showed that the anti-estrogen tamoxifen at relatively high concentrations could reduce tyrosine autophosphorylation of IGF-1R in melanoma cell lines (Kanter-Lewensohn, Girnita et al. 2000). This effect was correlated with growth arrest and decreased cell survival. An ideal way to inhibit IGF action would be to develop a small-molecule inhibitor of IGF-1R function. Several compounds have been formulated for inhibition of EGFR family members and are now in clinical trials. Indeed, small-molecule inhibitors of IGF-1R have been reported, but they also caused substantial inhibition of IR. An alternative approach to suppress IGF-1R signaling is to target the post ligand binding events that regulate receptor turnover. This process is thought to be a key event in the regulation of receptor bioavailability and activity. Recent reports suggest that cysteine proteinase inhibitors can impair tumor cell growth and IGF-1R signaling interfering with receptor traffic (Navab, Chevet et al. 2001). A similar approach has been used by Sepp-Lorenzino (Sepp-Lorenzino, Ma et al. 1995), who showed that Herbimycin A decreased IGF-1R expression through enhanced receptors degradation. Furthermore this increased receptor degradation was prevented by the proteasome inhibitors but not by lysosomal inhibitors. Drugs that interfere with IGF-1R expression at the cell surface (TM and

lovastatin) were also used to block IGF-1R function. Manipulation of gene expression by antisense oligonucleotides, plasmids expressing IGF-1R antisense cDNA or triple helix-forming oligodeoxynucleotides, has been proven to be effective in downreglation of IGF-1R expression (Adams, Epa et al. 2000).
Targeting IGF-1 pathways only affects cycling cells. Therefore it is expected to have minimal harmful effects on the majority of the normal cells (Adams, Epa et al. 2000).

AN OVERVIEW OF THE P53 TRANSCRIPTION FACTOR: STRUCTURE AND FUNCTION

The p53 transcription factor is a critical mediator of the cellular response to diverse cellular aggressions, and coordinates adaptation to the insult (Strous and Schantl 2001). Structural analyses have shown that p53 is a transcription factor with a sequence-specific DNA binding domain in its central region, and a transcription activation domain at its N-terminus (Prives and Hall 1999). Binding to DNA requires a tetrameric state, as a consequence of interactions of four separate p53 molecules via the tetramerization domain (Jeffrey, Gorina et al. 1995). The C-terminal region is predominantly composed of basic residues and forms a key regulatory region. This region is modified by acetylation, phosphorylation, O-glycosylation, but the physiological significance of these post-translational modifications remains uncertain. The acidic N-terminal transcriptional activation domain allows p53 interaction with the basal transcriptional machinery and modulation of target genes expression. This region is also involved in regulating the stability and activity of p53 protein via interactions with proteins such as Mdm2, (Haupt, Maya et al. 1997; Kubbutat, Jones et al. 1997), which allows targeting of p53 for the ubiquitin-proteasome machinery. Mdm2 binding also blocks the ability of p53 to interact with the transcriptional apparatus.

Normal p53 function

p53 is a transcription factor capable of regulating the expression (either by activation or by repression) of a range of downstream genes. The number

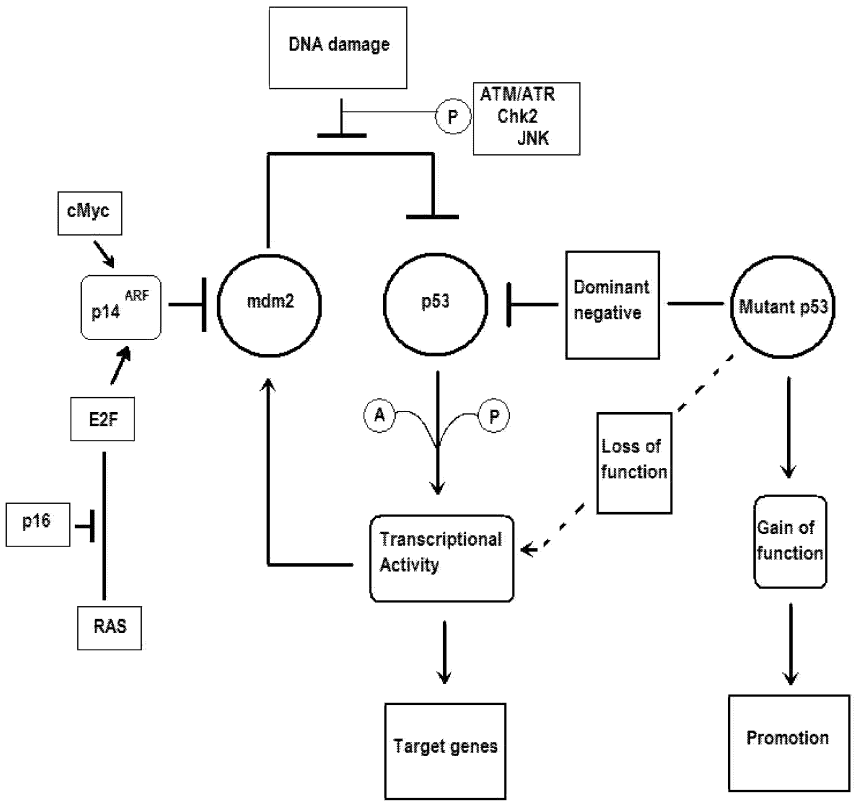

Fig. 5. The p53-Mdm-2 is the central molecular unit for various types of selection pressure during neoplastic transformation. The different pathways mediate p53 stabilization and accumulation.

of proposed target genes, which are transactivated by p53, is constantly growing (Oren, Damalas et al. 2002). In addition, p53 can also repress several genes by a mechanism not fully elucidated so far. The main function is to coordinate the cell response to insults by controlling genes of which products facilitate adaptive and protective activities, including apoptosis and growth arrest (Prives and Hall 1999). p53 activation blocks the cell cycle by binding directly to genomic p53 response elements and stimulating the expression of p21WAF1/CIP1, an inhibitor of cyclin-dependent kinases (CDKs) (Prives and Hall 1999). It has been reported that p21WAF1 is mainly responsible for p53-induced G1 arrest (Prives and Hall 1999). GADD45, another target of p53, may participate in the coupling between chromatin assembly and DNA repair (Kastan, Zhan et al. 1992).

The accepted model is that p53 is turned on by DNA damage, hypoxia, nucleotide imbalance, oxidative damage, and various forms of oncogene activation (Guimaraes and Hainaut 2002). All these forms of stress induce p53 post-translational modification, leading to stabilization and activation of the p53 protein. The adaptive responses include, but are not restricted to, growth arrest and apoptosis, as well as viable cell cycle exit. However, in this pathway the major player is the level of p53 protein that is greatly increased in cells. The level of p53 is dependent on a balance between protein synthesis and degradation and it is clear that the absolute level of p53 has the potential for causing different effects (Chen, Ko et al. 1996; Lassus, Ferlin et al. 1996).

P53/Mdm2 regulatory circuit

As p53 is such a potent inhibitor of cell growth, its function must be tightly controlled to allow normal growth development. There are diverse levels of p53 synthesis control (transcription, stability, translation) and of its degradation by ubiquitin-mediated proteolysis. Additional levels of control include diverse protein–protein interactions, post-translational modifications (phosphorylation, RNA binding, and glycosylation) and sub-cellular localization (Woods and Vousden 2001).

Mdm2 and control of p53 expression and stability

The p53 level is principally regulated by its interaction with Mdm2 in a regulatory feedback loop: p53 stimulates Mdm2 synthesis at

transcriptional level and, in turn, Mdm2 protein binds p53, inactivating its transcriptional activity and also targeting p53 for ubiquitin-mediated degradation. Mdm2 ubiquitinates both p53 and itself, contributing to the rapid turnover of both proteins (Woods and Vousden 2001).
Mdm2 causes degradation of p53 in transient assays, and mutations of p53 or Mdm2 preventing the interaction between them lead to greatly stabilized p53. Mdm2-targeted degradation of p53 is blocked by proteasome inhibitors and disruption of the p53–Mdm2 interaction in vivo by a peptide leads to increased quantities of p53 (Bottger, Bottger et al. 1997). Honda et al.(Honda, Tanaka et al. 1997) have provided evidence that Mdm2 may serve as a ring-finger E3 ubiquitin ligase.
Mechanisms that regulate the Mdm2-induced degradation of p53 include: direct repression of Mdm2 expression, post-translational modification of p53 and MDM2, (Woods and Vousden 2001), expression of proteins that inhibit Mdm2 function (see below) and regulation of the subcellular localization of p53 or Mdm2.

Regulation of p53 protein localization

Transcriptional function of p53 depends on nuclear localization. p53 contains both a nuclear export signal (in fact there are 2 NES, one localized within the amino-terminal domain and, the other one at the carboxyl terminal domain) and a nuclear import signal within its carboxyl terminus (Gottifredi and Prives 2001), but efficient export of p53 to the cytoplasm depends on Mdm2 function. Like p53, Mdm2 shuttles from the nucleus to the cytoplasm (Roth, Dobbelstein et al. 1998), and the shuttling of Mdm2 may be important for p53 export in some cells. The ubiquitin ligase activity of Mdm2 is critical for the export of p53 from the nucleus (Boyd, Tsai et al. 2000). In a number of tumor types that retain wild-type p53, loss of p53 activity is associated with failure of the protein to accumulate in the nucleus. This cytoplasmic sequestration could be associated with excess Mdm2 activity (Lu, Pochampally et al. 2000).

The p53 and Mdm2 interaction can be blocked, through covalent modification of the proteins or through the induction and action of another protein, ARF. For example, DNA-damage phosphorylates a site within the amino terminus of p53, that inhibits the interaction of p53 with MDM2, and so prevents the degradation of the p53 protein (Hirao, Kong et al. 2000). p53 activation in response to oncogenic stimuli (e.g. Ras activation) is not dependent on p53 phosphorylation. In this case, activation of the

ARF protein, result in binding and inhibition of Mdm2 and subsequent p53 activation (Sherr and Weber 2000). Overall, it seems to be many pathways that allow stress-induced stabilization of p53, and defects in those pathways have been identified in cancers retaining wild-type p53.
Altogether, this data demonstrates that the major, if not exclusive, cause of p53 turnover is mediated by its interaction with Mdm2.

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P53 and cancer

Along with the huge diversity of genetic modifications involved in cancer pathways, p53 abnormalities are probably the most prevalent molecular defects in human cancer (Prives and Hall 1999). For this reason p53 is one of the most extensively studied proteins in cell biology. In order to survive, malignant cells have to suppress the normal p53 functions (Hanahan and Weinberg 2000) and this could explain why p53 pathway is altered in the majority, if not all, of the malignant tumors. Alterations typically include loss of alleles, point mutations and inactivation of the protein. The roles played by p53 in cancer can be greatly simplified in three main categories: 1. dominant negative effect of mt p53; 2. loss of normal p53 functions and 3. gain of function-the ability of mutant p53 to acquire new functions (Blagosklonny 2000; Cadwell and Zambetti 2001).
The generally accepted mechanism of mutant p53 dominant-negative suppression is the shutdown of wild-type p53 function due to heteromerization with mutant p53. Wild-type p53 forms a tetramer to perform its tumor suppressor activity, and that oligomerization is mediated by the oligomerization region (residues 319–360). This region remains fully functional in core domain mutants (Cadwell and Zambetti 2001). It appears that the mutant has the ability to drive wild-type p53 into a mutant or perhaps inactive conformation. The loss-of-function is mainly related to the p53 role as a transcription factor and is acquired by mutations. Most of the identified mutations cluster in the DNA-binding domain and support the idea that this region is essential for the capacity of p53 to act as a tumor suppressor gene (Hainaut and Hollstein 2000). p53 differs from other suppressors by the fact that over 75% of all mutations are missense mutation, resulting in a substitution of a single amino-acid. It has been suggested that mutant p53 may possess novel functions not seen in the wild type p53, described as gain-of-function.

One of the most intriguing observations on p53 mutations is that most of them do not result in the loss of protein. Quite the opposite, cancer cells accumulate the mutant (even wild type) protein (Oren, Damalas et al. 2002). This property cannot be accounted by only the fact that the mutation inactivates the protein function. It has been postulated since more than 10 years ago that mutant p53, from tumor suppressor gene, is converted into an oncogene (Lane and Benchimol 1990). The mechanisms of gain-of-functions are still poor understood (van Oijen and Slootweg 2000).

P53-IGF-1 axis

Previous studies have established a relationship between the IGF-1 pathways and p53. The actions of these two pathways are distinctly opposite: IGF-1 through its receptor promotes mitogenic and antiapoptotic signals, whereas p53 induces cell cycle arrest and apoptosis. It has been shown that IGF-1 is able to block p53 apoptotic function and the anti-apoptotic effect of IGF-1 in myocytes is mediated by its ability to depress p53 transcriptional activity (Leri, Liu et al. 1999). IGF-1-induced growth stimulation in MCF-7 cells was accompanied by tyrosine phosphorylation and nuclear exclusion of p53. A role for IGF-1R activation in DNA repair in response to 4-NQO-induced damage (UV-mimetic-treated cells) in NIH3T3 fibroblast cells was also demonstrated (Mayo and Donner 2001; Heron-Milhavet and LeRoith 2002). In this study it has been shown that p53, Mdm2 and p19ARF are parts of the pathway used by IGF-1 to promote DNA repair in damaged cells. In the context of 4-NQO-induced DNA damage, subsequent IGF-1 treatment induced an increase in mdm2 mRNA levels with decrease in p53 protein levels and p53 functional activity (as shown by reduced levels of p21 protein). IGF-1 was shown to regulate MDM2 activity by inhibiting the association between ARF and Mdm2 in a p38 MAPK-dependent manner. Thus, when IGF-1 was used to rescue the cells from UV-mimetic induced DNA damage, the p53 protein was degraded through the ARF/MDM2-mediated pathway.
Others studies indicate that both pAkt expression and serum treatment increase Mdm2 ubiquitination of p53 (Mayo and Donner 2001). The serum-induced increase in p53 ubiquitination was blocked by LY294002, a PI3K inhibitor. Those results suggest that Akt enhances the ubiquitination-promoting function of Mdm2, determining reduction of the p53 protein.

Collectively, these results clearly demonstrate that in normal cells, as long as IGF-1 signaling pathways are active, p53 cannot promote its growth arrest and proapoptotic function. Therefore, it is not surprising that wild type p53 represses IGF-1R transcription and activates IGFBP3 synthesis at transcriptional level. It is surprising that cooperation between IGF-1 and mutant p53 enables cultured cells to grow in the absence of serum (Gai, Rizzo et al. 1988), as well the ability of mt p53 to transactivate the promoter of IGF-1R. This suggests that there is a p53/IGF axis, whereby increases in wild type p53 by DNA damage may induce apoptosis, in part by down-regulating the antiapoptotic effects of IGF-1 and a possible p53 gain-of-function mediated by IGF-1R.

EWING'S SARCOMA

Ewing's sarcoma (ES) is a high-grade malignant sarcoma that mostly affects children and adolescents. This tumour has a specific chromosomal translocation, t (11; 22) (q24; q12). This translocation results in fusion of the EWS gene (on chromosome 22) with the FLI1 gene (on chromosome 11) forming the fusion gene EWS-FLI1, which encodes a chimeric protein. The fusion protein is believed to play a causative role in the tumor transformation of ES. EWS-FLI1 is suggested to be a transcription factor, but little is known about its target genes. Furthermore, the regulation of EWS-FLI1 is poorly understood.

ES is a neuroectodermal tumor with limited neural differentiation that affects mostly children or adolescents, and is the second most common primary pediatric bone tumor (Lizard-Nacol, Lizard et al. 1989; Noguera, Triche et al. 1992). Morphological, ES displays small, round, undifferentiated cells, with no structural, ultrastructural, or enzymatic specificity.

Studies of chromosomal translocations and fusion genes in tumors have revealed much about the molecular biology of cancer. Chromosomal translocations have been well studied in hematopoietic malignancies. The first consistent chromosomal aberration observed in human neoplasia was that of the Philadelphia chromosome in chronic myeloid leukemia (CML), created by the translocation between the long arms of chromosome 9 and 22. In solid tumours consistent chromosomal translocations occur mainly in sarcomas (Table 2).

The creation of fusion molecules through gene translocations is probably an early and necessary event in tumor formation in malignancies in which

such chromosomal abnormalities occur. Fusion genes and proteins can give rise one of two following effects: 1. they lead to deregulation caused by overexpression of the oncogene by juxtapositioning it to an enhancer or promoter sequence that is active. This can be exemplified by an inversion between the long and short arms of chromosome eleven in parathyroid adenomas. The result of this being the juxtapositioning of the parathyroid hormone regulatory elements and PRAD 1 putative oncogene causing overexpression of PRAD1; 2. Formation of a translocated gene coding for a chimeric protein, whose transforming abilities are drawn from both partners. For example, in CML the BCR -ABL fusion occurs where BCR normally encodes a GTPase activating protein GAP for P21, and ABL encodes a tyrosine kinase whose activity is unmasked by the BCR sequence.

The involvement of transcription factors at translocation breakpoints is a recurring theme. For example, in pre-B acute lymphatic leukemia (ALL) a fusion protein results from the translocation t(1;19)(q23;p13). This fuses the E2A on chromosome 19, encoding the Ig enhancer binding proteins E12 and E47, with the homeobox PBX gene on chromosome 1. This fusion switches the DNA binding domain of E2A with that of PBX, thus placing those genes usually regulated by PBX under the trans-activational control of E2A. Furthermore, because PBX is not normally transcribed in pre-B cells, the translocation results in ectopic expression of the PBX DNA binding domain.

Over the last ten years several Ewing's sarcoma-specific translocations have been discovered. Ninety percent of the cases carries the translocation t(11;22) (q24;q12) (EWS/FLI1) (Turc-Carel, Aurias et al. 1988; Zucman, Delattre et al. 1992; Downing, Head et al. 1993). The resulting EWS/ETS chimeric protein is oncogenic (May, Lessnick et al. 1993) and functions as an aberrant transcription factor (May, Gishizky et al. 1993; May, Lessnick et al. 1993). This transcription factor transactivates promoters containing binding sites for FLI1. Specific inhibition of EWS-FLI1 in ES cell lines resulted in decreased cell proliferation and loss of tumorigenicity (Kovar, Aryee et al. 1996; Tanaka, Iwakuma et al. 1997).

Although this malignancy is well characterized, relatively little is known about the signaling pathways that govern the growth and survival of ES cells. Recently, Silvany et al. (Silvany, Eliazer et al. 2000) have found a correlation between ERK1/ERK2 activation and EWS-FLI1-dependent transformation. Several potential autocrine growth factor loops have also been described. IGF-1R appears to be essential for transformation by

EWS/ETS proteins and this receptor may be important in the survival and/or mitogenic signaling in Ewing tumors (Scotlandi, Benini et al. 1996; Toretsky, Kalebic et al. 1997; Lawlor, Scheel et al. 2002). Recently, Lawlor et. Al. (Lawlor, Scheel et al. 2002), using different cell culture models demonstrated serum-dependent phosphorylation of ERK1/2 and Akt and constitutively high serum-independent cyclin D1 protein expression for cells cultured in monolayer. When cells were placed in suspension there was a serum-independent activation of ERK1/2 and Akt, whereas the cyclin D1 protein expression was completely blocked.
The signal transduction cascades initiated in ES cells in response to growth factors and to EWS/ETS fusion proteins themselves remain to be elucidated. The RAS-RAF1-MEK-ERK1/2 mitogen-activated protein kinase (MAPK) pathway and the phosphatidyl inositide-3-kinase (PI3K) - AKT pathway are possible candidates given the reported essential roles of these cascades in malignant transformation.

Table 2
Gene fusions in sarcomas

Affected gene	**Re-arrangements**	**Disease**	**Protein type**
FLI1,EWS	t(11:22) (q24:q12)	Ewing's sarcoma	Ets transcription factor family
ERG,EWS	t(21:22) (q22:q12)	Ewing's sarcoma	Ets transcription factor family
ATV1,EWS	t(7:21) (q22:q12)	Ewing's sarcoma	Ets transcription factor family
ATF1,EWS	t(12:22) (q13:q12)	Soft-tissue clear cell sarcoma	Transcription factor
CHN,EWS	t(9:22) (q22 31:q12)	Myxoid chondrosarcoma	Steroid receptor family
WT1,EWS	t(11:22) (p13:q12)	Desmoplastic small round cell tumor	Wilms' tumor gene
SSX1,SSX2,SYT	t(X:18) (p11.2:q11.2)	Synovial sarcoma	HLH domain
PAX7,FKHR	t(1:13) (q36:q14)	Rhabdomyosarcoma	Homeobox homologue
CHOP,TLS	t(12:16) (q13:p11)	Myxoid liposarcoma	Transcription factor
var,HMG1-C	t(var:12) (var:q13-15)	Lipomas	HMG DNA-binding protein

AIMS OF STUDY

To elucidate in malignant cells:
1) Mechanisms involved in expression of growth factor receptors, with special focus on IGF-1R;
2) Role of growth factors receptors expression in tumor transformation and maintenance of malignant phenotype;
3) Possible use of growth factor receptors as a therapeutic target.

MATERIALS AND METHODS

MATERIALS

Antibodies

A mouse monoclonal antibody against the human IGF-1R (αIR3) was purchased from Oncogene Science, NY, USA. A rabbit polyclonal IGF-1R antibody (N-20), that recognized the α subunit, mouse monoclonal antibodies against human p53 (DO1), a mouse monoclonal antibody to Mdm-2 (including the p53-Mdm-2 complex), a monoclonal antibody to phosphotyrosine (PY99) and an antibody to actin (H-196) were purchased from Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA. From the same company we bought EGF-R and PDGFR antibodies as well as the antibody against FLI-1, EWS and goat anti-rabbit IgG-HRP. A pan-CD44 (IM-7) monoclonal antibody was from American Type Culture Collection (ATCC), Rockville, MD. An antibody against Bcl-2 and protein G plus/protein-A were obtained from Oncogene Science, NY. The anti-IRS-1 agarose conjugate antibody was obtained from UBI (Lake Placid, NY).

Reagents

The proteasome inhibitors lactacystin and MG132 were from Calbiochem (Darmstadt, Germany). Compounds PPT and DPPT (99.97 % purity) and PPT-4, 6-O-benzylidene-b-D-glucopyranoside (AS 3738) were kind gifts from Dr. K. Leander, Analytecon Inc., Switzerland. PPP and DPPP were prepared from the former two lignans, respectively, by incubation with sodium acetate in aqueous methanol 26 and were then purified by HPLC. Other phytoestrogens were those used in previous studies or from Sigma (St. Louis, MO). Lovastatin was obtained from Merck, Sharp, and Dohme. Chemicals for protein gel electrophoresis were obtained from Bio-Rad. Hyperfilm-ECL, Western blotting detection reagents, nitrocellulose membranes, [3H]glucosamine, [3H]thymidine, and [35S]methionine were bought from Amersham, UK. Cell culture media were obtained from Gibco-BRL (Life Technologies). Scintillation fluid was from Canberra Packard. All other chemicals, unless not stated otherwise, were from Sigma Chemicals (St. Louis, MO).

Cell lines

The human melanoma cell lines SK-MEL-5, SK-MEL-28 the Ewing's sarcoma cell lines (RD-ES, ES-1) and the human p53-negative cell lines Saos-2 and HL-60 were obtained from ATCC. The human diploid fibroblasts (HDF) (GM08333) were obtained from Coriell Institute of Medical Research (USA). BE cells, established from a lymph node metastasis specimen from a patient with advanced malignant melanoma, as well as melanoma cell lines DFB, DFW, C8161, AA and FM 55 cells were kindly provided by Professor Rolf Kiessling, CCK, R8:01, Karolinska Hospital, Stockholm. BL41-tsp53-2 is an EBV-negative Burkitt lymphoma cell line carrying mutant p53 (codon 248) transfected with ts p53 mutant (p53-Val135) with mutant conformation at 37°C and wt conformation at 32°C (13–15). Hep G2 (hepatoma), PC3 (prostatic carcinoma) and MCF7 (breast carcinoma) cell lines were from American Tissue Culture Collection (Rockville, MD). The R- and P6 mouse cell lines were gifts from Professor R. Baserga, Thomas Jefferson University, Philadelphia, PA. The R- fibroblasts are IGF-1R negative, derived from an IGF-1R knockout mouse embryo. The P6 line is a 3T3 derivative over-expressing the human IGF-1R.

METHODS

Cell culture

SK-MEL-5, SK-MEL-28, BE and GM 08333 cells were cultured in Minimum Essential Medium supplemented with 10% fetal calf serum (FCS), HL-60 in Iscove's modified Eagle's medium with 15% FCS, Saos-2 in McCoy's medium with 20% FCS, RD-ES in RPMI-1640 medium with 10% FCS, and BL-41cells in RPMI-1640 medium with 10% FCS. Hep G2 (hepatoma), PC3 (prostatic carcinoma) and MCF7 (breast carcinoma) cell lines were cultured in RPMI supplemented with 10% FCS. P6 and R- were cultured in DMEM with 5% (P6) or 10% fetal bovine serum (FBS). P6 and R- cell lines were cultured in the presence of G-418 (Promega).
The cells were grown in tissue culture flasks maintained at 95% air/5% CO2 atmosphere at 37°C in a humidified incubator.

Isolation of plasma membranes

Preparation of plasma membranes was performed essentially as described elsewhere. In brief, cells were harvested and homogenized in a buffer containing 0.32 M sucrose, 1 mM taurodeoxycholic acid, 2 mM MgCl2, 1 mM EDTA, 25 mM benzamidine, 1 mg/ml bacitracin, 2 mM phenylmethylsulfonyl fluoride, 10 mg/ml aprotinin, 10 mg/ml soybean trypsin inhibitor, and 10 mg/ml leupeptin. After a 10-min centrifugation at 600g (4°C), the pellet (containing unbroken cells, nuclei, and cytoskeleton) was discarded. The supernatant was then centrifuged at 17,300g for 30 min. The resulting pellet, containing plasma membrane, was then used for isolation of plasma membrane proteins.

Immunoprecipitation

The isolated cells were lyzed as described elsewhere (Kanter-Lewensohn, Dricu et al. 2000). 15 µl Protein G Plus-A/G agarose and 1µg antibody were added to 1-ml protein material. After overnight incubation at 4°C on a rocker platform, the immunoprecipitates were collected by centrifugation in a microcentrifuge at 2,500 rpm for 15 min. The supernatant was discarded whereupon the pellet was washed. The material was then dissolved in sample buffer for SDS-PAGE

Immunoprecipitation of EWS/FLI-1 fusion protein. The prepared cell compartments were lysed in 1 ml of ice-cold PBSTDS solution (containing PBS, Triton X-100, sodium deoxycholate, and SDS) containing the afore-mentioned protease inhibitors. To 1 ml of material was added 15 ml of Protein G Plus-Agarose and 1 mg of the FLI-1 antibody (Sc-356). After overnight incubation at 4°C on a rocker platform, the immunoprecipitates were collected by centrifugation at 2500 rpm for 15 min. The pellet was washed four times with 1 ml of PBDTDS.

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).

Metabolic labeling of cells with [35 S] methionine

In order to determinate the IGF-1R synthesis and degradation the cells were labeled with [35S] methionine. After indicated experimental procedures cells were transferred to methionine–free DMEM (Gibco, UK) supplemented with 10% FBS and 100μCi/ml L-[35S] methionine (specific activity >1000Ci/mM, Amersham UK) for a 2h incubation. For determination of IGF-1R protein synthesis, the cells were quickly washed twice with ice-cold PBS and lyzed in RIPA buffer (RIPA, 1x phosphate-buffered saline, 1% Triton-X-100, 0.5% sodium deoxycholate, 0.1% SDS, supplemented with protease inhibitor tablet). An equal amount of protein from each sample was immunoprecipitated with a polyclonal anti-β IGF-1R antibody (C-20), collected by protein A Sepharose (CL-4B, Amersham), resolved by SDS-PAGE, and visualized by autoradiography. IGF-1R protein degradation was determined by pulse-chase experiments. The cells were, after the 12h labeling with [35S]methionine (see above), carefully washed twice with DMEM and transferred to radioactive-free DMEM containing 10% FBS for the indicated time periods. Cells were then harvested for detection of radioactive IGF-1R as described above.

RNA isolation

Total RNA was isolated from the cells using Qiaquick Rneasy (Quiagen, Hilden, Germany).The cells were lysed in the presence of denaturing guanidium isothiocyanate-containing buffer to inactivates Rnases and to insure isolation of intact RNA. Ethanol was added to provide appropriate binding conditions and the sample was applied to Rneasy mini spin column. The total RNA bound to the membrane and contaminants were

washed away. RNA was eluted with RNA-free water, and the RNA concentration was measured by a spectrophotometer.

Reverse Transcription-Polymerase Chain reaction (RT-PCR)

Total RNA was first transcribed to a complementary DNA strand (cDNA) by a reverse transcriptase, using random primers PdN6 (Amersham). The reaction was performed in a 20 µl solution containing 1µM primer, 1mM dNTP DNA polymerization mix (Perkin Elmer), 20 U Rnase inhibitor (Boehringer Mannheim, Mannheim, Germany), 10 µg bovine serum albumin, 2 µM DTT (Gibco), 4 µl 5x first strand buffer (Gibco), and 200 U Superscript^{TM} (Gibco). The reaction was first incubated at 65^{o} C for 5 min, following 42^{o}C for 1H, and finally 95^{o}C for 5 min to inactivate the transcriptase activity. The resulting cDNA was amplified by PCR. The primers used are presented in the **Table 3**. The PCR reaction solution contained 0.8 mM dNTP DNA polymerization mix, 5 µl 10x PCR buffer (Perkin Elmer), 0.2 µM for each primer, 2.5 mM MgCl2 (Perkin Elmer) and 1 U AmpliTaq DNA polymerase (Perkin Elmer) in o volume reaction of 50 µl. Amplification was performed in a PTC-200 DNA engine (MJ Research, Watertown, MA, USA).The reaction conditions: 94^{o}C for 2 min to activate the polymerase, denaturation at 94^{o} C for 30 sec, annealing 60-68^{o} C for 30 sec, and extension 72^{o}C for 30 sec. The cycle was repeated 30-35 times, and finally elongated for 10 min. A control without reverse transcriptase was included at the RT step (as a control for DNA contamination). A negative control without template was also included in every step. The PCR products were detected by ethidium bromide staining on 1 or 2% agarose gel.

Table 3

Primer	Sequence 5'-3'
IGF-1R forward	GCC CGA AGG TCT GTG AGG AAG AA
IGF-1R reverse	GGT ACC GGT GCC AGG TTA TGA
β-actin forward	CAC GGA GTA CTT GCG CTC AGG AGG
β-actin reverse	CAC GGA GTA CTT GCG CTC AGG AGG
p53 forward	CCGAGTGGAAGGAAATTTGCGTGTGGAGTA
p53 reverse	CAAGGCCTCATTCAGCTCTCGGAACATCTC

Semi-quantitative RT-PCR

To compare the targeted mRNA level in different cell samples, a semi-quantitative RT-PCR was performed. This is based on the PCR kinetic study using different range of cycles (20-30).As an internal control, representing the amount of mRNA template, β-actin was used. The relative amount of amplified genes could be determined by normalizing the signal for β-actin (Noonan, Beck et al. 1990).

Antisense experiments

Antisense phosphorothiolate oligonucleotides (AS-ODN) (5'-CCC TGC TCC CCC CTG GCT CC-3') and sense (S-ODN) (5'-GGA GCC AGG GGG GAG CAG GG-3') to p53 were purchased from Pharmacia Biotech and Interactiva. AS-ODN is complementary to position 1071-1090 of exon 10 of the p53 mRNA. Lipofectin (Life Technologies) was used to deliver antisense oligonucleotides to cultured cells. Since AS-ODN induces RNAse H cleavage and further degradation of target mRNA, we tested the specificity of the p53 AS-ODN using semi quantitative RT-PCR. RNA was isolated from SK-MEL-5 and SK-MEL-28, which had been treated with p53AS-ODN for 24 h. The p53 transcript was dramatically decreased after treatment with AS-ODN. In contrast, no decrease was observed in the lipofectin control or after treatment with antisense plus sense (S-ODN).
EWS/FLI-1 antisense oligonucleotides. Antisense and sense oligonucleotides were synthesized by Applied Biosystems Model 381A DNA synthesizer by the use of standard phosphoamidite chemistry. Antisense and sense oligonucleotides were directed against the sequences including ATG initiation of the EWS/FLI-1 mRNA (nucleotides 34–58). The sequence of antisense was ATC CGT GGA CGC CAT TTT TTT TCCT and that of sense was AGG AGA GAA AAT GGC GTC CAC GGAT.
Mdm-2 AS-ODN and S-ODN (human Mdm-2 position 696) were previously described (Goetz, van der Kuip et al. 2001). The S-ODN sequences are for human cells: (5'- CCT TGA AGG TGG GAGTGA TC-3'); for murine cells: (5'-CCT GAA GGT GGG AGT GAT C-3') or AS-ODN for human cells: (5'-GAT CAC TCC CAC CTT CAA GG-3'); for murine cells: (5'-GAT CAC TCC CAC CTT CAG G-3').

Technical considerations

There are several critical steps in the use of antisense strategy: design of AS-ODN, transfection method, transfection conditions and control of transfection. In the design of AS-ODN several principles should be consider: (1) Length: the length of 15-25 is optimal to ensure both specificity and a good affinity to bind the target; (2) Backbone modification: phosphodiester backbone modification increase ODN stability by preventing its rapid degradation by ubiquitous nucleases. Transfection conditions to be optimized are: (1) cell density: optimal cell density is 50-60% confluence for adherent cells; (2) serum concentration: optimal transfection, require low serum concentration and should be empirical determined; the serum concentration may be increase by using Transfectin instead Lipofectin. In order to confirm that biological effects of AS-ODN are specific, several control conditions should be included. Generally, the control ODNs may comprise scrambled, sense or mismatch ODNs. The mismatch ODNs provide most stringent controls than sense or scrambled ODNs (Myers and Dean 2000).

Sequence analysis

In order to analyse the p53 sequence, the DNA was isolated by standard methods. Exon 2-10 of the human p53 was amplified from cellular DNA using a multiplex/nested PCR protocol. PCR products were directly sequenced by cycle sequencing with dye-labeled terminators (BigDye Terminators, Perkin Elmer, Norwalk, Connecticut, USA), and analyzed on a DNA sequencer ABI PRISM 377XL (PE Applied Biosystems, Foster City, California, USA). The sequences obtained were identified and aligned together with the wt sequence (from BLASTN, NCBI) using the DNA analyzer program sequencer (Gene Codes).

Assay of cell growth and survival

Proliferation of the cell lines, with the exception of HDFs and BL-41tsp53-2, was measured by determining the number of cells attached to the plastic surface of duplicate 35-mm dishes. This was performed by microscopic counting of cells in several ink-marked areas on the dish bottom. By repeating the counting after specified time intervals, changes in

the number of attached cells could be followed. In HDFs, cell growth was assayed by determining changes in cellular protein content, and in BL-41tsp53-2 cells, cell growth was assayed by counting cells in a Burker chamber.
We also performed a colorimetric assay of cell viability using the Cell proliferation kit II (Roche Inc, Indianapolis, IN), which is based on colorimetric change of the yellow tetrazolium salt XTT in orange formazan dye by the respiratory chain of viable cells. All standards and experiments were performed in triplicates.

Determination of DNA-synthesis

Cells cultured in 35-mm dishes were, after the experimental conditions, labeled with [3H]thymidine (1 mCi/ml, 5 Ci/mmol) for 1 h. The acid-precipitable material was then taken for scintillation counting.
[35S]methionine labeling of the EWS/FLI-1 fusion protein. RD-ES cells, cultured in 35-mm dishes, were labeled with 45 mCi/ml [35S]methionine for 4 h in methionine-free medium. In some dishes TM had been added 2 h before the start of labeling and was also present during the whole labeling period. Thereafter the cells were washed twice with cold PBS and cultured in methionine-containing medium for 0, 4, and 24 h. After the chase periods, nuclear and cytoplasmic fractions were isolated. Proteins in these two fractions were then subjected to immunoprecipitation using the antibody against FLI-1. Ten percent of the samples was used directly for measuring the radioactivity of the labeled immunoprecipitates, and the remaining 90% of the samples was loaded on SDS–PAGE gels. Following SDS–PAGE, the proteins were fixed and stained in a solution containing 45% methanol, 10% acetic acid, and 2.5% brilliant blue. The radioactivity was measured using fluorography with hyper-Film-MP after impregnating the gel with Amplify (Amersham), according to the description of the manufacturer. The signals on the fluorography were quantified by a transmittance/reflectance densitometer.

Determination of N-linked glycosylation of EWS/FLI-1.

RD-ES cells in 35-mm dishes were, after the experimental conditions, labeled with 10 mCi/ml [3H]glucosamine (50 Ci/mmol) for 2 h,

whereupon cellular proteins were subjected to immunoprecipitation using Sc-356. SDS–PAGE and fluorography was performed as described above.

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.

Assay of tyrosine phosphorylation of receptors in intact cells.

We transferred to the cells, cultured to subconfluency in 6-cm plates, fresh medium containing 10% FBS and the desired compounds for 1 h. The cells were then lysed and subjected to immunoprecipitation using appropriate antibodies, as described. We separated the immunoprecipitates by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE), and then we made the transfer to nitro-cellulose membranes (Hybond, Amersham, UK) and incubated with anti-phosphotyrosine antibody. We used antibodies to actin (in cell extract) or to IGF-1R β-subunit as loading controls. We scanned the films and the numerical data are means and standard errors of the mean of triplicate determinations.

In vitro tyrosine kinase assays

IGF-1R-catalyzed substrate phosphorylation of Poly Tyr Glu (pTG), using a 96-well plate tyrosine kinase assay kit (Sigma), was performed essentially as described elsewhere. We used recombinant EGFR (Sigma), immunoprecipitated IR from HepG2, immunoprecipitated IGF-1R from P6 cell, and immunodepleted supernatant from P6 (representing "non-IGF-1R tyrosine kinases"). After 30-min treatment of the receptors with the desired compounds in the kinase buffer (50 mM HEPES buffer pH 7.4, 20mM MgCl2, 0.1 MnCl2 and 0.2 Na3VO4), the kinase reaction was activated by addition of ATP. The phosphorylated polymer substrate was probed with a purified phosphotyrosine specific monoclonal antibody conjugated to horseradish peroxidase (HRP), clone PT-66. Color was developed with HRP chromogenic substrate O-phenylenediamine dihydrochloride and quantitated by spectrophotometry (ELISA reader).
IGF-1R tyrosine autophosphorylation was analyzed by a sandwich ELISA assay. Briefly, 96-well plates (Immunolon, Nunc) were coated overnight at

4° C with 1µg/well of the monoclonal antibody Ab-5 (LabVision) to the IGF-1R b-subunit. The plates were blocked with 1 % BSA in PBS Tween for 1 h, and 80 µg/well of total protein lysate from the P6 cell line was added. As a negative control we used total protein lysate from R- cell line. The investigated compounds were added in tyrosine kinase buffer without ATP at room temperature for 30 min, prior to kinase activation with ATP. Kinase assay was performed using the Sigma kit (see above). After spectrophotometry the IC50 values of inhibitors were determined using the REGRESSION function of Statistica program.

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 was expressed in E. coli and purified using glutathione-Sepharose (Pierce). IGF-1R was isolated from P6 cell lines by immunoprecipitation with a polyclonal rabbit antibody directed against beta subunit (clone H60, Santa Cruz) and protein G-Sepharose (Amersham). IGF-1R Sepharose beads were mixed with or without MDM2-GST, rabbit E1 (Calbiochem), E2 bacterial recombinant UbcH5B (Calbiochem) and His6-Ubiquitin (Calbiochem) in a 30 µl reaction. After 1 hour incubation at 30^0C reaction was stopped by addition of SDS sample buffer. Reaction products were loaded on a 7,5 % polyacrylamide gel, transfer to nitrocellulose membrane and detected using either antibody against IGF-1R (clone C20, Santa Cruz), anti-ubiquitin antibody or anti His-tag antibody.

IGF-1R/MDM2 interaction in a cell free system

MDM glutathione sepharose beads were mixed with total proteins extracts from P6 or R- cells. After 60 min incubation at room temperature the beads were washed 3 times with PBS, dissolved in SDS sample buffer and loaded on 7,5 % gel and visualized after transfer on nitrocellulose membrane with an anti IGF-1R antibody (C20).

In vivo experiments

Four to five-week old pathogen-free nude mice (nu/nu) were used and housed within plastic isolators in a sterile facility. ES-1 and BE cells (both proved to express IGF-1R) were injected subcutaneously at 10^7 cells/mice in a 0.2 ml volume of sterile saline solution. Experimental treatments with PPP (2 mg/kg) were performed by daily intraperitoneal injections of the

compound in 10 µl volume of DMSO. Control mice were treated with the solvent. Three animals were treated in each group. Animals were monitored three times a week for signs of disease and tumor growth. Tumor mass was estimated by measuring the tumor volumes (in mm3). The mice were carefully observed for presence of side effects and were sacrificed at the end of the experiments for histological analysis of the lesions. A separate experiment on PPP-treated (systemically and locally) tumor-free mice, including histological analysis of various organs, confirmed previous observations that PPP is non-toxic. In another experiment the plasma concentrations of PPP after intraperitoneal injections were measured by gas chromatography-mass spectrometry. All experiments were performed according to the ethical guidelines for laboratory animal use, provided by institutional ethical committee.

RESULTS AND DISCUSSION

PAPER I

Inhibition of N-linked protein glycosylation decrease the expression of the EWS-FLI-1 fusion protein

We have shown that N-linked glycosylation is crucial for expression of growth factor receptors at the cell surface. In ES cells, which carry the EWS/FLI-1 fusion gene, we found that suppression of overall N-linked protein glycosylation decreased the EWS/FLI-1 protein and cell growth. HMG-CoA (3-hydroxy-3-methylglutaryl coenzyme A) reductase catalyses the conversion of HMG-CoA to mevalonate (MVA), which is a precursor of e.g. cholesterol and dolichyl phosphate. Dolichyl phosphate, which is a carrier of oligosaccharides, is necessary for N-linked glycosylation. The ES cell line RD-ES, which carries the EWS/FLI-1 fusion gene, responded to Lovastatin (an HMG-CoA reductase inhibitor) with growth arrest. Replenishment of MVA restored cell growth. When TM (a specific inhibitor of N-linked glycosylation,) was added together with MVA the cells remained arrested, indicating that N-linked glycosylation is of importance for growth of ES cells. Treatment with Lovastatin or TM also drastically decreased the expression of EWS/FLI-1 which was localized in the cell nuclei. The decreased expression of the fusion protein was found to be due to a lowered stability of de novo-synthesized protein. Inhibition of the biosynthesis of EWS/FLI-1 fusion protein by treatment with antisense oligonucleotides also arrested the cells, suggesting that this protein is of importance for cell growth. We investigated whether N-linked glycosylation could be directly involved in expression of the EWS/FLI-1 fusion protein, which in fact contains four potential sites for N-linked glycosylation. The fusion protein was demonstrated to not be a glycoprotein. This seems reasonable since the fusion protein probably acts as a transcription factor (Zoubek, Pfleiderer et al. 1994) and in this case its destination should be the nucleoplasm. Glycosylated nuclear proteins are located at the nuclear envelope or perinuclear cistern. Therefore, we conclude that some other glycoproteins may be involved in regulation of EWS-FLI-1. Since growth factor receptors are N-linked glycoproteins and most N-linked glycoproteins are confined to the plasma membrane, the possibility of a link between cell surface expression of growth factor

receptors and EWS-FLI-1 expression may be raised. It has previously been demonstrated that growth factor receptors require an adequate N-linked glycosylation to be translocated to the cell surface. One potential growth factor receptor could be IGF-1R. It has been demonstrated that IGF-1 pathway is necessary for transformation of fibroblast transfected with EWS/FLI-1 (Toretsky, Kalebic et al. 1997). The tumorigenic and antiapoptotic role of IGF-1R factor for ES is now well established (Scotlandi, Benini et al. 1996) and treatments that interferes with IGF-1R expression or function is ES induces massive cell death (Girnita, Wang et al. 2000; Scotlandi, Avnet et al. 2002; Scotlandi, Maini et al. 2002).

PAPER II

Basic FGF pathway is involved in regulation EWS-FLI-1 fusion protein.

Based on results of paper I we investigated whether growth factor receptors or growth factor pathways may be involved in regulation of the EWS-FLI-1 protein. The effect of EGF (epidermal growth factor), bFGF (basic fibroblast growth factor), IGF-1 and PDGF (platelet–derived growth factor) on the fusion protein expression and growth of ES cells was studied. After a 24-h serum deprivation of ES cells, there was almost no EWS-FLI-1 protein detectable. When bFGF was added, the expression of EWS/FLI-protein maintained at a normal level. In contrast, the three other growth factors, including IGF-1, had no effects. Consistently, bFGF neutralization by specific antibodies resulted in down-regulation of the fusion protein. Our data suggest an important role of bFGF and FGF receptors in ES.

Relatively little is known about the signaling pathways that controls the growth and survival of ES cells and about the place of EWS/FLI-1 fusion protein in these pathways. The results reported seems to be contradictory: Sturla et al. (Sturla, Westwood et al. 2000) reported that bFGF induce cell death in ES cells, an unexpected result since bFGF is a mitogenic factor, and increased expression of this growth factor and its receptors has been implicated in transformation and malignant progression. Another study coming from the same lab reported that bFGF-induced cell death in ES is mediated through a caspase-mediated receptor pathway (Westwood, Dibling et al. 2002). In this study it was been demonstrated that apoptotic effect is secondary to the activation of the Ras-MAPK pathway induced by bFGF-FGFR. However, this signaling pathway was activated within

minutes whereas the apoptotic effect was seen first after 48-72 hours. As other tyrosine kinase receptors FGFR signals through the main pathways: Ras-Raf-MEK-ERK1/2 and PI3K-Akt pathway. Silvany et. al (Silvany, Eliazer et al. 2000) have been found a constitutive activation of MAPK pathway in ES cell lines. In addition, interference with ERK activation by specific MEK inhibitors or a dominant negative Ras mutant impaired the ability of EWS/FLI-1 to transform murine fibroblast. Thus, Ras signaling pathway seems to promote cell proliferation and not apoptosis. Moreover, inhibition of PI3K-Akt in ES cells significantly reduced ES cell proliferation. One possible explanation for these contradictory results is that different cell lines respond in different ways to FGF. However, the bFGF-induced effects we reported were obtained in serum-depleted conditions and reflect the cellular changes after a 24h bFGF treatment. The apoptotic effects were obtained mostly in the serum-enriched conditions after long time (72h) exposure to bFGF. Recently Lawlor et al. (Lawlor, Scheel et al. 2002) suggested that signal transduction pathways in ES cells are dependent on the cell culture system. Monolayer cells demonstrated serum-dependent phosphorylation of ERK1/2 and Akt and serum-independent cyclin D1 expression. In suspensions, ERK1/2 and AKT were activated serum-independently, but cyclin D1protein expression was completely blocked until stable cellular spheroids had been formed, indicating the role of cell-cell adhesion in proliferation of ES cells.

PAPER III

Role of p53 in regulation of IGF-1R in malignant cells.

We investigated the functional impact of p53 for IGF-1R expression in malignant cells using p53 antisense oligonucleotides (AS-ODN) and a cell line (BL-41tsp53-2), carrying temperature-sensitive (ts) p53 and endogenous mutant p53 (codon 248).
Specifically, we aimed to evaluate the effect of aberrant p53 on IGF-1R expression. Using the three different system-(1) malignant melanoma cell lines expressing mutant p53, (2) malignant melanoma cell lines overexpressing wt p53 and (3) BL-41tsp53-2, we could demonstrate that induction of normal wt p53 or down regulation of the mutant type p53 impaired the IGF-1R expression at the cell surface. Our results are in line with the study of Werner et al (Werner, Karnieli et al. 1996) showing that wt p53 repress the transcription of the IGF-1R gene. They showed that

mutant p53, in contrast to wt p53, stimulates the transcriptional activity of the IGF-1R gene. Thus, expression of IGF-1R in malignant cells carrying mutant type p53 could be explained by both p53 loss-of-function (mutant p53 is not longer able to trans-repress IGF-1R gene) and p53 gain-of-function (mutant p53 stimulate directly the transcriptional activity of IGF-1R promoter). However, in our study the melanoma cell lines expressing wt p53 responded with decreased expression of IGF-1R upon p53 inhibition. This means that p53 does not need to be mutated to increase IGF-1R expression. In turn, our findings suggests that p53 may interfere with IGF-1R expression at posttranscriptional levels.

PAPER IV

Mdm-2 is involved in ubiquitination and degradation of IGF-1R

Based of the results from paper III we aimed to investigate the mechanisms underlying the interaction between p53 and functional IGF-1R in malignant cells. Using two different systems, (1) melanoma cell lines expressing mutant type p53 and (2) melanoma cell lines expressing wt p53 we could first confirm that inhibition of p53 by p53 AS-ODN impaired expression of IGF-1R in all 4 cell lines. However, Mdm-2 inhibition also drastically decreased IGF-1R, but only in cells harboring wild type p53. Unexpectedly, combined treatment with p53 and Mdm-2 AS-ODNs substantially decreased the effect of p53 inhibition on IGF-1R, although the protein levels of p53 and Mdm-2 were adequately decreased. Consistent with our previous study (Girnita, Girnita et al. 2000) disruption of p53 dependent regulation of IGF-1R inhibited cell growth in all cell lines. In contrast, Mdm-2 inhibition impaired cell growth and survival only in cell lines harboring wt p53. We investigated the effects of p53AS-ODNs on degradation of IGF-1R using pulse-chase experiments with [35S] methionine. We found that the control cells exhibited 18 and 30% degradation after 12 and 24h, respectively whereas in cells treated with p53 AS-ODN degradation fraction was 31 and 79%. Upon inhibition of Mdm-2 or both Mdm-2 and p53 there were no significant effects (compared to the control). Since Mdm-2 is an E3 ubiquitin ligase (not only for p53), we investigated whether IGF-1R down-regulation following p53 AS-ODN treatment could involve ubiquitin-mediated degradation. We found that ubiquitin is bound to the IGF-1R, and provided evidence that

proteasome pathway is involved in IGF-1R degradation. We investigated whether Mdm-2 is physically associated with the IGF-1R and demonstrated that Mdm-2 co-immunoprecipitated with IGF-1R. Consistent results were also obtained in a cell free/antibody free system, using recombinant Mdm-2 beads to extract IGF-1R from total protein lysate. Finally, we demonstrated that Mdm-2 could induce ubiquitination of IGF-1R in vitro.

The principal function of the ubiquitin system is to target proteins to selective degradation. The specificity of ubiquitination depends largely on the enzymes that recognize the substrates, the class of ubiquitin ligases called E3s. In only three cases of mammalian membrane proteins have E3 enzymes been identified: the Ring domain-containing adaptor protein c-Cbl is the E3 ligase mediating EGFR ubiquitination and sorting into multivesicular bodies, the HECT domain-containing E3 Nedd 4function as E3 ligase for the control of sodium channel ENaC internalization and the third E3, Mdm-2 member of the RING domain family involved in regulating the G protein-coupled receptors.

Our present study provides strong evidence that the oncoprotein Mdm-2 serves as a ligase (ligase E3) in ubiquitination of IGF-1R. Firstly, we could demonstrate a physical association of IGF-1R to Mdm-2; secondly addition of Mdm-2 together with IGF-1R in an in vitro ubiquitin assay, resulted in ubiquitination of IGF-1R; thirdly we were able to detect ubiquitinated IGF-1R in a cell system, and we demonstrated that IGF-1R ubiquitination was Mdm-2 dependent. The IGF-1R ubiquitination might be involved in receptor internalization and in receptor degradation. A comparable scenario is the one involving the EGFR and its probably E3 ubiquitin ligase c-Cbl. Both c-Cbl and Mdm-2 are Ring containing domain E3 ligases, and both are involved in ubiquitination of plasma membrane receptors. Ligand-induced down-regulation of two EGF receptors, correlates with differential ability to recruit c-Cbl, whose invertebrate orthologs are negative regulators of ErbB. The ligand-binding induced degradation of internalized ErbB-1, mediated by transient mobilization of a minor fraction of c-Cbl into ErbB-1-containing endosomes. The alternative fate is recycling of internalized ErbBs to the cell surface. Cbl-mediated receptor sorting involves covalent attachment of ubiquitin molecules, and subsequent lysosomal and proteasomal degradation. The oncogenic viral form of Cbl inhibits down-regulation by shunting endocytosed receptors to the recycling pathway. These results reveal an endosomal sorting machinery capable of controlling the fate, and, hence,

signaling potency, of growth factor receptors (Levkowitz, Waterman et al. 1998). In the case of IGF-1R, Mdm-2-dependent ubiquitination could direct the receptor to the degradation pathways instead to the recycling pathways.

A key question is: why the ubiquitin system is required for regulation of IGF-1R? The likely answer is that ubiquitination may coordinate critical functions. The essential role for Mdm-2 in controlling normal p53 activities has to be supported by coordination of the signaling activity of key surface receptors (Strous and Schantl 2001) especially those with opposing functions to p53. Is tempting to speculate that Mdm-2 may control the switch between growth arrest and apoptotic signals of p53 and cell cycle progression and antiapoptotic signals of IGF-1R. This switch might be dependent on the Mdm-2 locations. Probably, the major function of nuclear Mdm-2 is to control the p53 activities, a function that depends strictly on protein-protein interactions (not affected by most of p53 mutations found in human cancers). Followed detachment from p53, Mdm-2 might shuttle back to the cytosol, where it can be degraded by the proteasome or be available for other tasks. Alternatively, mutant type p53 can sequester Mdm2 into the cytoplasm. However, distribution between the nucleus and cytosol is strictly controlled, so the cytosol concentration is low compared to the nucleus. The “free” Mdm-2 concentration in the cytosol depends on its synthesis/degradation rate, PI3K-Akt pathway and on its interaction with p53.The balance among these processes determines how much Mdm-2 is the cytoplasm available eventually for IGF-1R. A massive p53 expression would deplete the cytoplasmic Mdm-2 pool increasing recycled IGF-1R rate. Furthermore, PI3K-Akt activation by increased IGF-1R expression would accentuate nuclear Mdm2 localization. For the wt p53 expressing cells this process would affect p53 activity.The versatile function of Mdm2 connects the nuclear activity with extra cellular signals. Our data provide evidence that inhibition of p53 triggers Mdm-2-dependent ubiquitination and increased proteasomal dependent degradation of the IGF-1R, whereas co-inhibition of p53 and Mdm-2 expression rescues the cells from IGF-1R down regulation and subsequent death.

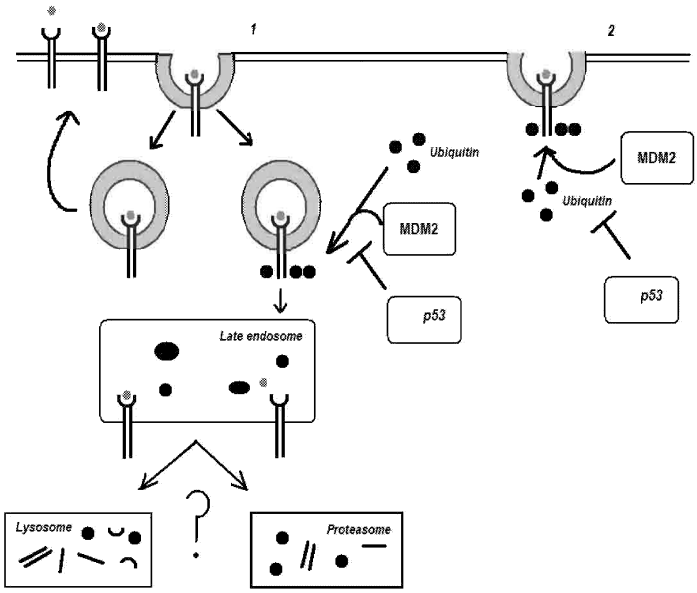

Fig. 6 A scenario for IGF-1R endocytosis and down regulation:
(1) Ligand binding induces receptor phosphorylation and subsequent internalization. Differential endocytic trafficking of *internalized* receptor is mediated by Mdm-2 dependent ubiquitination.
(2) Formation of the IGF-1R/Mdm-2 complex and IGF-1R ubiquitination as a mechanism for receptor internalization. In this pathway, ubiquitin function as an internalization signal.

PAPER V

Selective inhibitors of IGF-1R

Recently we showed that relatively high doses of the anti-estrogen tamoxifen, could reduce tyrosine autophosphorylation of IGF-1R in melanoma cell lines (Kanter-Lewensohn, Girnita et al. 2000). In order to identify selective and highly potent IGF-1R inhibitors, we have therefore investigated the properties of various tamoxifen-like compounds.

We first investigated the effects of twelve phytoestrogens and related compounds, including flavones, isoflavones and lignans, on tyrosine phosphorylation of IGF-1R in intact melanoma cells. Genistein and quercetin, representing biologically active isoflavones and flavones, respectively, had only little or no inhibitory effect on IGF-1R phosphorylation. In contrast, the cyclolignan PPT almost completely blocked IGF-1R phosphorylation of intact cells with an IC50 value for IGF-1R phosphorylation of 0.04 μM in the intact cells. PPT significantly reduced IGF-1R phosphorylation in malignant cell lines of various origins, as well as in mouse fibroblasts over-expressing the human IGF-1R (line P6).

In order to determine structural requirements for inhibitors of IGF-1R phosphorylation, we tested a number of PPT analogues and we found that the cyclolignans PPP, deoxy-PPT (DPPT) and deoxy-PPP (DPPP) were all potent inhibitors of IGF-1R phosphorylation. We determined the specificity of PPT and PPP as IGF-1R inhibitors by investigating their effects on tyrosine phosphorylation of other growth factor receptors both in intact cells and in vitro after isolation of the receptors. Phosphorylation of the FGFR, PDGFR, EGFR and, notably, the insulin receptor was not affected by PPT in intact cells even at such a high concentration as 15 μM.

To investigate the mechanism of action, we isolated the receptor and determined the effects of PPT and PPP on both IGF-1R-catalyzed substrate tyrosine phosphorylation and IGF-1R autophosphorylation in cell-free systems. PPT efficiently decreased the poly Tyr Glu (pTG) substrate phosphorylation but it did not inhibit the substrate phosphorylation of EGFR and IR tyrosine kinases. To investigate whether this type of cyclolignans interfere with tyrosine autophosphorylation at the ATP level or at the substrate level, various concentrations of ATP (19-300 μM) were added to the reaction buffer during the assay. ATP concentration did not alter the IC50 value of them. We also studied the effects of PPT

and PPP and their deoxy derivatives on viability of different types of tumor cells. The results showed a dose-dependent decrease in cell survival up to about 0.05-0.1 μM. In contrast, the mouse fibroblast cell line R-, being IGF-1R negative, did not show any response to PPT or PPP. Finally, we investigated the effects of the nontoxic PPP on tumor growth in vivo. For this purpose ES-1 (Ewing's sarcoma cells) and BE (melanoma cells) xenografts were established in nude mice. Our results demonstrate that PPP induced a drastic inhibition of tumor growth in xenografted mice. In contrast to PPP, PPT caused extensive side effects.

Recently a class of drugs named tyrphostins has been identified as a potent IGF-1R tyrosine kinase inhibitor. However, the application of these drugs is not possible due to lack of specificity (cross inhibition of IR). Co-inhibition of the IR would lead to a diabetogenic response in-vivo and this side effect cannot be overcome by any treatment. In contrast, as shown in our study PPP does not interfere with the IR tyrosine kinase.

Our discoveries open the possibility to utilize PPP or related non-toxic cyclolignans as selective IGF-1R inhibitors in treatment of cancer.

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