Receptor Binding Interactions of Herstatin and Its Intron-Encoded Domain

by

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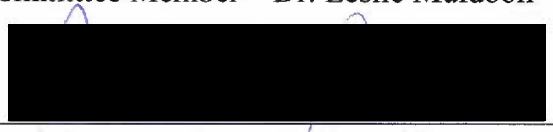

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LIST OF ABBREVIATIONS

ADAM ..... a disintegrin and metalloprotease
AP1 ..... activator protein 1
AR ..... amphiregulin
B_{max} ..... maximum bound
Bad ..... Bcl-2 agonist of cell death
Bcl-2 ..... B-cell CLL/lymphoma-2
Bcl-X ..... B-cell CLL/lymphoma-X
BTC ..... betacellulin
Cbl ..... cysB-like
CKI ..... cyclin-dependent kinase inhibitor
CR ..... cysteine rich
DMEM ..... Dulbecco's modified Eagle's medium
E2F ..... E2 promotor binding factor
ECD ..... extracellular domain
EGF ..... Epidermal Growth Factor
EGFR ..... Epidermal Growth Factor Receptor
ELISA ..... enzyme-linked immunoassay
Elk-1 ..... ets-containing acute leukemia virus protein 1
EPR ..... epiregulin
ESTs ..... expressed sequence tags
FBS ..... fetal bovine serum
FGFR-3 ..... Fibroblast Growth Factor Receptor-3
Gab1 ..... Grb2-associated binder-1
Grb2 ..... growth factor receptor-binding protein 2
HB-EGF ..... heparin bound-Epidermal Growth Factor
HER-1/2/3/4 ..... human Epidermal Growth Factor Receptor-1/2/3/4
HRG ..... heregulin
HRP ..... horseradish peroxidase
Hst ..... herstatin
IGF-I ..... Insulin-Like Growth Factor-I
IGF-IR ..... Insulin-Like Growth Factor-I Receptor
Ig-G ..... immunoglobulin
Int8 ..... intron 8-encoded peptide

IR ..... Insulin Receptor
IRR ..... Insulin-Related Receptor
IRS 1/2 ..... Insulin Receptor Substrate 1/2
JAK ..... Janus kinase
K_d ..... dissociation constant
Ma ..... *Mega annum*, million years ago
MAPK ..... mitogen activated protein kinase
MMP ..... matrixmetalloprotease
MWCO ..... molecular weight cut off
NRG ..... neuregulin
NSCLC ..... non-small cell lung carcinoma
OMM ..... outer mitochondrial membrane
PBS ..... phosphate-buffered saline
PCR ..... polymerase chain reaction
PI3K ..... phosphoinositide-3 kinase
PTB ..... phosphotyrosine binding
RBD ..... receptor binding domain
RTK ..... receptor tyrosine kinase
SH-2 ..... src homolgy-2
Shc ..... src homology-2 domain-containing transforming protein
Sp1 ..... SV40 early promotor specific transcription factor
Src ..... transforming proto-oncogene of *Rous sarcoma* virus
STAT ..... signal transducer and activator of transcription
TGF-α ..... transforming growth factor-α
TKI ..... tyrosine kinase inhibitor

ACKNOWLEDGMENTS

"Escapades, absentmindedness, or irreverence are the stuff out of which scientific folklore is made. It is a half-truism that ideosyncrasy may be the price paid for originality, and most university communities have become accustomed to tolerating a certain amount of eccentricity on the part of their most brilliant scientists."

-Evelyn Fox Keller

A Feeling for the Organism

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ABSTRACT

Herstatin is produced from an alternatively spliced HER-2 mRNA. Retention and read-through of intron 8 leads to the production of the inhibitory secreted ligand, which contains a novel receptor binding domain (RBD) encoded by the intron (Doherty, Bond et al. 1999). This thesis examines the binding interactions of herstatin with the Epidermal Growth Factor Receptor (EGFR) and Insulin-like Growth Factor –I Receptor (IGF-IR) families. Previous studies have characterized the inhibitory effects of herstatin on EGF receptor family signaling (Doherty, Bond et al. 1999; Azios, Romero et al. 2001; Justman and Clinton 2002; Jhabvala-Romero, Evans et al. 2003; Staverosky, Muldoon et al. 2005). Here, I examine the effects of herstatin expression on IGF-1-induced signaling in MCF7 breast carcinoma cells. Both herstatin and its intron-encoded RBD bind to all four members of the EGF receptor family as well as to ΔEGFR, a mutant EGF receptor missing subdomains I and II of its extracellular domain (ECD). Two germline mutations found in the intron-encoded domain lower the binding affinity of herstatin to the EGF and HER-2 receptors by two-to-three fold. Herstatin also binds to both the IGF-IR and the Insulin Receptor (IR), albeit with approximately 10-fold reduced affinity compared to EGFR and HER-2. Here, I present a model for herstatin binding to both the EGF and IGF-IR families. I propose that the intron-encoded domain of herstatin binds within subdomain III of the receptor ECD. Subdomains I and II of herstatin may then be juxtaposed with subdomains I and II of the EGF receptor family allowing for dimer-like interactions via the dimerization arm in subdomain II. This dimerization arm is lacking in the IGF-IR

and IR ECDs, thus possibly explaining the reduced binding affinity of herstatin. Examination of signaling in parental MCF7 cells and MCF7 cells stably transfected with herstatin (MCF7Hst) revealed that herstatin expression reduced overall IGF-IR levels and attenuated IGF-I-induced activation of both the IGF-IR and of Akt/PKB. However, herstatin-expression increased IGF-I-mediated activation of Erk compared to parental cells. Furthermore, herstatin-expression resulted in reduced IGF-I- or Insulin-stimulated proliferation *in vitro*. These studies demonstrate that, in addition to binding to and blocking activation of the EGF receptor family, herstatin binds to the IGF-IR and IR and modulates IGF-I-stimulated proliferation and survival signaling, either through direct interaction with the IGF-IR or indirectly by modulating crosstalk with the EGF family of receptors. In summary, herstatin is a unique ligand of both the EGF and IGF-I receptor families that functions to modulate the action of these receptors.

CHAPTER 1

Thesis Introduction

Receptor Tyrosine Kinases

The translation of extracellular stimuli to activation of intracellular effector molecules is essential to proper cellular growth and metabolism. This translation is accomplished in great part by receptor tyrosine kinases (RTKs). RTKs are divided into four subclasses and contain approximately 20 different families. However, they all share a similar gross morphology. RTKs all have a large, glycosolated extracellular ligand binding domain (ECD), a single-pass transmembrane domain, and a tyrosine-kinase-containing intracellular domain (Ullrich and Schlessinger 1990). Subclass I RTKs, which include the Epidermal Growth Factor Recptor (EGFR) family, are monomeric receptors that contain two cysteine-rich segments in the extracellular domain. Subclass II RTKs, which include the Insulin-Like Growth Factor-I Receptor (IGF-IR) family, also contain similar cysteine-rich repeats, but functional receptor dimers are formed by disulfide-linked heterotetrameric ($\alpha_2\beta_2$) receptor subunits. Subclasses III and IV RTKs each contain either five or three immunoglobulin (IgG)-like repeats in their extracellular domains and also have characteristically split kinase domains (Ullrich and Schlessinger 1990). Despite differences in protein structure, these receptors function in a highly analogous manner.

Activation of RTKs occurs through ligand binding and receptor dimerization. Either a monovalent ligand (subclass I) or a bivalent ligand (subclasses III and IV) induces receptor dimerization, placing the intracellular kinase domains in close enough proximity to cross-phosphorylate the intracellular tyrosines. However, in the case of subclass II, where the receptors already exist

in a pre-formed dimer, ligand binding is thought to induce a conformational change within the dimer making it signaling-competent (Ullrich and Schlessinger 1990). Signaling emanating from the dimeric RTK complexes, results in recruitment of intracellular effectors containing Src homology-2 (SH-2) and phosphotyrosine-binding (PTB) domains, such as Shc, Grb2, Src, and phopholipase Cγ. These effector molecules eventually lead to the activation of important downstream signaling cascades such as the mitogen activated kinase (MAPK), the phosphoinositide 3-kinase (PI3K)/Akt, and JAK/STAT pathways (Ullrich and Schlessinger 1990; Rommel, Clarke et al. 1999; Blume-Jensen and Hunter 2001; Johnston, Pirola et al. 2003; O'Connor 2003; Marmor, Skaria et al. 2004). Selective activation and modulation of timing and intensity lead to differing signaling outcomes such as growth, differentiation, and cellular metabolism.

This thesis focuses specifically on the EGF and IGF-I receptor families. The EGFR family has been shown to mediate cell growth and differentiation. The IGF-IR family has also been shown to play a role in cellular growth and proliferation, as well as to mediate unique, biological processes, such as glucose metabolism (Sell, Dumenil et al. 1994; Baserga 1997; Baserga, Resnicoff et al. 1997; Burden and Yarden 1997; O'Connor, Kauffmann-Zeh et al. 1997; Morrione, Romano et al. 2000; Olayioye, Neve et al. 2000; Sweeney and Carraway 2000; Holzenberger, Dupont et al. 2003; Kuribayashi, Kataoka et al. 2004). While these receptor families are essential for normal growth and development, aberrant expression by mutational activation, autocrine activation, or by receptor over-

expression leads to a variety of human cancers (Blume-Jensen and Hunter 2001; Holbro, Civenni et al. 2003; Rochester, Riedemann et al. 2005).

The EGF Receptor Family - Evolution

The EGF receptor family is highly conserved. There are four members of this receptor family found in mammals. However, there is only one receptor type present in invertebrates that has highest homology to the EGFR; this includes Let-23 in *C. elegans* (Aroian, Koga et al. 1990) and DER in *D. melanogaster* (Livneh, Glazer et al. 1985). In *C. elegans* there has only been one ligand found for Let-23, making a simple signaling network of one receptor-ligand pair (Stein and Staros 2000). In contrast, *D. melanogaster* has three activating ligands, Spitz, Vein, and Gurken, and the only known inhibitory EGF-like ligand, Argos (Figure 1.1) (Stein and Staros 2000). Argos is a secreted protein that contains an atypical EGF motif and was thought to be a canonical ligand that directly induced receptor inactivation (Schweitzer, Howes et al. 1995). However, recent evidence shows that Argos is not a DER ligand, rather it inhibits DER activation by binding to and sequestering Spitz, an activating ligand (Klein, Nappi et al. 2004). This mode of action is a novel mechanism for downregulating the potent signal potentiated by members of the EGF receptor family that has not yet been found in mammals.

In mammals, the EGF receptor family consists of four members: EGFR/ErbB1/ Human Epidermal growth factor Receptor -1 (HER-1), ErbB2/HER-2/*neu*, ErbB3/HER-3, and ErbB4/HER-4. Correspondingly, there are eleven

mammalian ligands for these receptors (Figure 1.1) (Riese and Stern 1998). Evolutionary analysis suggests that gene duplication resulted in two ancestral receptor precursors: one for EGFR/ErbB1 and one for ErbB3/ErbB4 (Stein and Staros 2000). This is also reflected in the segregation of ligands, with the majority of ligands either binding to EGFR or coordinately to ErbB3 and ErbB4 (Stein and Staros 2000).

Significant homology exists amongst all four receptors. Sequence alignment of the human EGF receptor family members reveals an approximately 30% amino acid identity among the four receptors (Stein and Staros 2000). This identity is highest in the kinase domain, followed by the ECD (Stein and Staros 2000). Despite high homology, the individual receptor-types discriminate between ligands and participate in unique heterodimer pairs that result in different signaling patterns (Figure 1.2) (Sweeney and Carraway 2000). Moreover, ErbB2 and ErbB3 are unique in function. ErbB2 is the only one of the four unable to bind ligand, and is the preferred co-receptor in heterodimer pairs (Figure 1.2) (Alimandi, Romano et al. 1995; Wallasch, Weiss et al. 1995; Pinkas-Kramarski, Shelly et al. 1996; Tzahar and Yarden 1998). In addition, ErbB3 is kinase-impaired and therefore must heterodimerize and be tyrosine phosphorylated to signal (Figure 1.2) (Schaefer, Akita et al. 1999). ErbB3 has an abundance of PI3K docking sites at its C-terminus, making it a potent heterodimer partner and strong activator of the anti-apoptotic Akt/PKB signaling cascade (Stein and Staros 2000). In addition to having canonical PI3K docking site motifs, receptor chimera studies between the EGFR

and ErbB-3 confirmed that the C-terminus of ErbB-3 is responsible for coupling directly to PI3K and activating Akt (Fedi, Pierce et al. 1994; Prigent and Gullick 1994).

The EGF-Receptor Family Ligands

The EGF receptor family growth factors are all produced as membrane-bound precursors. While these membrane-bound ligands can engage in limited juxtacrine-signaling, the soluble form of the ligand is by far the most potent (Sweeney and Carraway 2000; Harris, Chung et al. 2003). Recent evidence has shown that suppression of matrixmetalloprotease (MMP) activity significantly decreases the signaling of the EGF receptor family, thus suggesting that MMP sheddases control the shedding of the EGF ligands. More recently, a family of sheddases, a disintegrin and metalloprotease (ADAM), has been shown to be the major convertase family for the EGF ligands (Sahin, Weskamp et al. 2004). Both *in vitro* and mouse knockout studies have revealed the importance of ADAMs in release of ligand, leading to competent receptor signaling (Holbro and Hynes 2004; Sahin, Weskamp et al. 2004).

The eleven human ligands for the EGF receptor family are all activating ligands that directly bind to specific receptors (Stein and Staros 2000). EGF, transforming growth factor-α (TGF-α), amphiregulin (AR), and epigen all bind specifically to the EGF receptor, while heparin-binding EGF-like growth factor (HB-EGF), betacellulin (BTC), and epiregulin (EPR) bind to both EGFR and HER-4 (Sweeney and Carraway 2000; Harris, Chung et al. 2003). Additionally,

neuregulin (NRG) [heregulin (HRG)] 1 and 2 bind only to HER-3 and HER-4, while NRG-3 and 4 are specific only to HER-4. Finally, NRG 1 and 2 as well as BTC have been shown to bind HER-2/HER-3 heterodimers (Figure 1.2) (Sweeney and Carraway 2000; Harris, Chung et al. 2003). This plurality of ligands exerts ligand-induced specificity by dictating which receptor dimer-pairs are formed (Sweeney and Carraway 2000). For example, Hrg-1 or -2, preferentially induces HER-2/HER-3 heterodimers and activation of the anti-apoptotic Akt/PKB signaling cascade through direct recruitment of the p85 subunit of PI3K (Sweeney and Carraway 2000). In contrast, BTC preferentially activates HER-4 homodimers and promotes cellular growth and proliferation by recruiting the adaptor proteins Shc and Grb2 (Sweeney and Carraway 2000).

Despite their receptor-binding specificity, all eleven ligands contain a canonical EGF-like motif containing six spatially conserved cysteine residues. These six cysteines form three intramolecular disulphide bonds in the following manner: C1-C3, C2-C4, and C5-C6 (Harris, Chung et al. 2003). This EGF motif is critical for binding to the EGF receptor family, though binding specificity is mediated through specific side-chain moieties interacting with the receptors.

The EGF Receptor Family Protein Structure

The EGF receptor family ECD is further characterized by four subdomains I (L1), II, (S1, CR1), III (L2), and IV (S2, CR2) (Figure 1.2). Subdomains I and III have been termed the "ligand binding domains" and are known to coordinate ligand binding, while subdomains II and IV are the cysteine rich (CR) domains

that do not directly participate in ligand binding (Bajaj, Waterfield et al. 1987). The CR domains contain a total of 50 cysteine residues that form 25 intramolecular disulphide bonds, the spacing of which is conserved throughout the receptor family. The cysteines are configured into groups of eight, each set forming a cysteine knot with a repeat pattern of 1-3, 2-4, 5-6 and 7-8 disulphide pairing (Abe, Odaka et al. 1998).

Recent crystallographic data have provided important new information on how these subdomains function in ligand binding and receptor dimerization. With the exception of HER-2, the EGF receptor family ECD is found in two conformations: the unliganded open conformation and the ligand-bound closed conformation (Cho and Leahy 2002; Garrett, McKern et al. 2002; Ogiso, Ishitani et al. 2002; Cho, Mason et al. 2003; Garrett, McKern et al. 2003) (Figure 1.3). Previous studies determined that subdomains I and III form the ligand binding pocket. In the closed receptor conformation, a ß-strand in subdomain I, lies parallel with ß-strands in the ligand, forming strong contact points (Garrett, McKern et al. 2002). These contact points between subdomain III and the ligands occur mainly though side chain interactions from loops that surround ß-sheets (Garrett, McKern et al. 2002). Interestingly, these crystal structures reveal that lack of conservation of specific residues in subdomain I of HER-2 contribute to its inability to bind ligand though both steric clash and loss of a critical hydrogen bond (Garrett, McKern et al. 2002).

In addition to substantiating previous data about the ligand binding domains, the crystal structures revealed unique information about the dimer-

orientation of the ECD. While most receptors either exist in a pre-formed dimer, or dimerize though association of a bivalent ligand or direct contacts of two monovalent ligands, the EGF receptor family was found to dimerize in a completely different fashion. Surprisingly, the dimer interface was found to be mediated through receptor contact points, primarily in subdomain II and to a lesser extent, in subdomain IV. Subdomain II contains the "dimerization loop" that is intramolecularly bound to the outer portion of subdomain IV in the absence of ligand and extends to form the dimer contact point in the ligand-bound receptor (Cho and Leahy 2002; Garrett, McKern et al. 2002; Ogiso, Ishitani et al. 2002) (Figure 1.3). The conformation that involves release of the dimerization arm, also allows the ligand binding pocket formed by subdomains I and III to close, forming ligand-binding contact points. Though crystal structures give a glimpse of active and resting receptors, it is yet to be established whether binding of ligand triggers the conformational change, or if the open and closed conformations are in equilibrium with each other, with the active conformation stabilized by ligand binding.

The crystal structures also help to explain both the inability of HER-2 to bind ligand and its preferred heterodimer status. The dimerization arm in subdomain II of HER-2 is constitutively extended and the ligand-binding pocket is closed, helping to explain both its preferred dimer partner status and its inability to bind ligand (Cho, Mason et al. 2003; Garrett, McKern et al. 2003). The inability of HER-2 to bind ligand is further explained by substitutions of key conserved residues in the binding pocket of subdomain I. In HER-2 these residues, which

normally form contact points with the ligand, are replaced by amino acids with bulky side chains that are likely to sterically inhibit binding of any ligand (Garrett, McKern et al. 2002). Both the extension of the dimerization arm, which closes the binding pocket, and the substitution of key conserved residues explain the unique status of the HER-2 receptor.

The full ECD crystal structures of HER-2, and its rat ortholog, *neu,* have given insight into the role of subdomain IV. The human and rat structures are superimposable, and dimers were not detected in the either crystal structure. Modeling based on the dimers present in EGFR ECD crystal structures suggest that subdomain IV pairs in dimers reside in close proximity (Cho, Mason et al. 2003; Bagossi, Horvath et al. 2005). This is in agreement with recent biological data that indicate that receptor transmembrane domains must be juxtaposed for competent signaling to occur (Bagossi, Horvath et al. 2005).

The EGF Receptor Family – Signal Transduction

The capacity to homo- and hetero-dimerize, the plurality of ligands, and the ability to recruit multiple intracellular effectors makes the EGF receptor family a highly regulated orchestrator of mitogenic signaling. Binding of the EGF growth factor ligands to the receptors causes dimerization, activation, and C-terminal tyrosine phosphorylation (Schlessinger, Ullrich et al. 1988; Dougall, Qian et al. 1994; Hynes and Stern 1994; Riese and Stern 1998; Tzahar and Yarden 1998; Sweeney and Carraway 2000). Phosphorylation of the C-terminal tyrosines promotes formation of SH-2 and PTB docking sites, leading to the potentiation of

intracellular signaling cascades, typified by activation of the mitogenic MAPK, anti-apoptotic Akt, and JAK/STAT signaling molecules (Rommel, Clarke et al. 1999; Busse, Doughty et al. 2000; Blume-Jensen and Hunter 2001). Recruitment of a specific adaptor protein, which couples receptor activation to downstream intracellular signaling molecules, is heavily dependent on the 5-8 amino acid residues surrounding the phosphotyrosine (Rotin, Margolis et al. 1992; van der Geer, Wiley et al. 1995; Sweeney and Carraway 2000). Therefore, extracellular ligand-induction of intracellular effectors is modulated by differential phosphorylation of the C-terminal tails of the receptors.

Though differential ligand stimulation may lead to different signaling outcomes, all receptor/ligand combinations stimulate the MAPK pathway, either directly through the Grb-2/Sos pathway or indirectly through Shc (Marmor, Skaria et al. 2004). Activation of MAPK (Erk1/2) leads to its rapid translocation into the nucleus where it activates several transcription factors such as, Sp1, E2F, Elk-1, and AP1. Activated MAPK also plays a role in the cytoplasm where it phosphorylates a variety of cytoplasmic and cytoskeletal proteins (Marmor, Skaria et al. 2004). This multiplicity of MAPK action makes it a potent mitogen in normal growth and development.

On the other hand, stimulation of the Akt, the primary effector of PI3K, is different by receptor type. EGFR and HER-2 do not directly couple to the Akt signaling cascade. Rather, they bind the p85 regulatory subunit of PI3K through the use of multiple adaptor proteins, such as Gab1 (Marmor, Skaria et al. 2004). In contrast, HER-3 has six and HER-4 has one p85 binding sites, allowing the

receptors to directly couple to the PI3K lipid kinase (Soltoff and Cantley 1996). Akt promotes cell-survival by phosphorylating the pro-apoptotic protein, Bad, consequently blocking its association with the anti-apoptotic proteins, Bcl-2 and Bcl-X (Danielsen and Maihle 2002; Marmor, Skaria et al. 2004). Otherwise, upon Bad activation, Bcl-2 and Bcl-X insert into the outer mitochondrial membrane (OMM) and block membrane permeabilization, a critical step in mitochondrial-mediated apoptosis (Adrain, Creagh et al. 2003). Additionally, Akt promotes cell-cycle progression by phosphorylating and thus downregulating the cyclin-E dependent kinase inhibitor (CKI), $p27^{KIP1}$. Therefore, the EGF receptor family is capable of regulating many proliferative and cell-survival signaling events, through the Akt pathway.

In addition to activating secondary effectors and intracellular signaling cascades, the EGF receptor family is capable of directly and indirectly targeting transcription factors. The EGFR directly phosphorylates STAT1, STAT3, and STAT5. These transcription factors then dimerize through exposed SH2 domains and translocate to the nucleus where they are involved in activating pro-proliferation genes. Additionally, it has recently been shown that proteolytic fragments of the EGF, HER-3 and HER-4 receptors translocate to the nucleus where they may function to activate transcription factors (Wells and Marti 2002).

The EGF Receptor Family in Normal Growth and Development

The EGFR is the prototypical receptor in regulating normal growth and development of epithelial tissues. The *in vivo* evidence for the function of the

EGF receptor family has come primarily from murine knockout studies. Targeted deletion of the receptors and/or their ligands frequently leads to embryonic or perinatal death, both suggesting the importance of these receptors and highlighting their unique functions (Holbro and Hynes 2004).

Murine studies targeting the EGF receptor in a similar fashion show a variety of phenotypes depending on mouse background. This variation suggests that other proteins may partially compensate for one or several of the roles in different tissues. However, until mouse-strain specific modifiers, which lead to unique backgrouds, are fully identified, it will be difficult identify which specific proteins participate in these compensatory functions. Embryonic defects were as severe as pre-implantation death and inability to support the inner cell mass, but ranged to include placental defects and mid-gestation lethality (Threadgill, Dlugosz et al. 1995). In backgrounds that supported live birth, abnormalities compared to matched litter-mates were found in epithelial derived tissues, specifically: skin, kidney, liver, brain, and GI tract, and resulted in death at no later than three months (Threadgill, Dlugosz et al. 1995). These studies suggest that the EGF receptor is involved in a variety of cellular activities essential for normal growth and development.

Additional studies targeting the HER-2 ortholog, *neu*, have highlighted its importance as a heterodimeric signaling partner for other EGF family receptors. HER-2/HER-3 knockouts were shown to be of critical importance for the proper development of the peripheral nervous system (Burden and Yarden 1997; Holbro and Hynes 2004). Furthermore, specific knockouts of HER-2, HER-4, or NRG-1

were all lethal at E10.5 due to improper cardiac development (Burden and Yarden 1997; Holbro and Hynes 2004). To ensure that these HER-2 based phenotypes were not due to a scaffolding effect, a kinase-dead HER-2 was knocked in. The resultant phenotype mimicked the HER-2 null mouse (Burden and Yarden 1997; Holbro and Hynes 2004). These data suggest specific roles for different heterodimers in different tissue-types.

Due to the propensity of EGF receptor family deletions to be embryonic lethal, it has been difficult to study the role of these receptors in the mature adult. Several studies have used conditional knockouts/knockins to investigate the tissue-specific roles of these receptors. The EGFR was found to play an important role in adult skin and hair follicles. HER-2/*neu*, on the other hand, was found to be essential in cardiac and neuromuscular synaptic function, as well as muscle spindle formation and regeneration (Miettinen, Berger et al. 1995; Holbro and Hynes 2004). Other studies have highlighted the importance of HER-2 heterodimers in mammary gland development. Co-localization and activation of EGFR and HER-2 is observed in the murine mammary gland at puberty, late pregnancy and lactation (Stern 2003; Holbro and Hynes 2004). Additionally, HER-3 and HER-4 expression is greatly increased in the developing, mature, and lactating gland compared to that of the pre-pubescent gland, and their expression is cell-type specific (Stern 2003; Holbro and Hynes 2004). This evidence points to both unique and overlapping roles of these receptors.

These data suggest that the EGF-receptor family and ligands all participate in cell-type specific signaling in normal growth and development.

Though the receptors share a high-degree of homology and common intracellular signaling pathways, their functions are non-overlapping to a certain extent. Expression of one receptor, or ligand, cannot rescue the mutant phenotype resulting from the knockout of another receptor. Thus it can be suggested, primarily through heteromeric interactions, that these receptors and ligands exist in a delicate balance with one another necessary for proper growth and development. Disruption of this balance may lead to mutant phenotypes or lethality in embryogenesis, and possibly cancer in the adult organism.

Soluble EGF Receptors

In addition to full-length membrane-bound receptors, soluble EGF family receptor isoforms have been detected. To date, several alternate transcripts encoding these soluble receptor isoforms have been found for EGFR, HER-2, and HER-3, but none have been reported for HER-4. First characterized, was a secreted form of the EGF receptor found in human epidermoid carcinoma cells (Ullrich, Coussens et al. 1984). This receptor isoform, sEGFR, which contains the ectodomain of the EGFR followed by a novel 18-amino acid C-terminal extension, is the result of alternate splicing in intron 16 due to chromosomal translocation (Ullrich, Coussens et al. 1984; Reiter, Threadgill et al. 2001). Early biochemical analysis showed that this soluble EGFR mutant is capable of binding EGF-ligand, but with reduced affinity compared to the full-length receptor (Gunther, Betzel et al. 1990). However, this mutant remains in a monomeric state independent of ligand-status (Gunther, Betzel et al. 1990). Recently, another

human EGF receptor alternate transcript was found in normal placenta and liver (Reiter, Threadgill et al. 2001). The putative coding sequence of this transcript encodes for the full-EGFR ectodomain followed by a unique 78 amino acid C-terminal sequence, generated by splicing to the alternative exon 15B (Reiter, Threadgill et al. 2001). To date, the function of this transcript has not been characterized. Finally, using expressed sequence tags (ESTs) two additional EGFR alternate transcripts were detected. Both contained the EGFR ectodomain followed by 2 and 1 unique amino acids (Reiter, Threadgill et al. 2001). While ESTs provide a good method for identifying potential novel transcripts, the validity of these two alternate transcripts needs to be confirmed either by detection by Northern Blot or through an RNA-protection assay on various tissues.

Characterization of the HER-2 receptor has also revealed splice variants. A mutant 2.3 kb mRNA was found in gastric cancer cell lines and in gastric tumors (Aigner, Juhl et al. 2001). This isoform, which encodes p100HER-2, contains the HER-2 ECD with no additional sequence. When transfected into breast carcinoma cells, p100HER-2 was found to inhibit Heregulin-mediated dimerization and activation of HER-4 and downstream MAPK signaling, as well as limiting soft-agar colony formation (Aigner, Juhl et al. 2001). Both p100HER-2 and its transcript appear only to be found in tumor samples and cell-lines, but not in normal tissues, suggesting a non-native, albeit inhibitory, role for the protein.

A HER-2 splice variant, Herstatin, which acts as a naturally-occurring EGF receptor family autoinhibitor, has also been described (Doherty, Bond et al.

1999). Herstatin is encoded by either a 4.8 kb or a 2.6 kb transcript in which intron 8 is retained (Doherty, Bond et al. 1999). The herstatin transcript was originally found in fetal kidney and liver, at lower levels in fetal lung, and not at all in fetal brain, thus suggesting a tissue-specific role in normal growth and development (Doherty, Bond et al. 1999). Herstatin contains subdomains I and II of the HER-2 ECD followed by a unique 79-amio acid C-terminal domain created by read-through into intron 8 (Doherty, Bond et al. 1999). The function of herstatin will be further described at the end of this chapter.

Furthermore, several soluble isoforms of HER-3 have been characterized in ovarian carcinoma cell lines and normal human tissues (Lee and Maihle 1998). The presence of these transcripts in normal tissues suggests a role in regulating normal cellular growth and development; however the presence of these transcripts in EGF receptor family-driven tumors, suggests that they may not function in a strong inhibitory role. Two of these alternate transcripts that contain retained introns encode for p45HER-3 and p85HER-3 and were originally found in ovarian carcinoma cell lines, but were later found to be expressed in normal human placenta (Lee and Maihle 1998). Most notable is p85HER-3, which is secreted, and consists of subdomains I, II, and part of subdomain III of the HER-3 ectodomain followed by a novel 24-amino acid C-terminal intron-encoded domain (Lee and Maihle 1998; Lee, Akita et al. 2001). P85HER-3 has been shown to bind Hrg with similar affinity as the full-length HER-3 receptor, and can compete with HER-2/HER-3 heterodimers for Hrg binding (Lee, Akita et al.

2001). Additionally, p85HER-2 inhibits Hrg-mediated activation and downstream signaling of HER-2, HER-3 and HER-4 in breast carcinoma cells (Lee, Akita et al. 2001).

The EGF Receptor Family and Its Role in Cancer

Since the EGF receptor family plays a key role in regulating the growth of epithelial cells, it is consequently heavily implicated in a variety of carcinomas. Of these receptors, the involvement of HER-2 in oncogenic growth is best described. HER-2 is overexpressed in breast, ovarian, gastric and endometrial carcinomas. Amplification of HER-2 by gene duplication occurs in 25-30% of breast cancers, and correlates with a more aggressive phenotype, shorter time to relapse and lower survival (Slamon, Godolphin et al. 1989). Additionally, HER-2 overexpressing breast and ovarian tumors have decreased responsiveness to adjuvant chemotherapy and anti-hormone treatments, such as tamoxifen (Benz, Scott et al. 1993; Felip, Encabo et al. 1995; Pegram, Finn et al. 1997). The conventional paradigm has been that an increase in receptor density further attenuates the constitutive activity of HER-2 homodimers, and is the likely reason for the transforming capabilities of the HER-2 receptor. However, recent studies have shown that the oncogenic capabilities of HER-2 in breast cancer are likely mediated through HER-2/HER-3 heterodimers (Holbro, Beerli et al. 2003). Therefore, though one receptor-type may be overexpressed, oncogenic signaling is a complex network of events that extends beyond one receptor-type.

The HER-2 rat ortholog, *neu*, has been extensively characterized and is best known for an activating mutation within its transmembrane domain, V664E, that confers oncogenic transformation (Bargmann, Hung et al. 1986; Bargmann and Weinberg 1988). The corresponding mutation is not found in humans despite over 90% amino acid identity between rat *neu* and HER-2 (Suda, Aizawa et al. 1990; Hynes and Stern 1994; Stein and Staros 2000). Rather, wt HER-2 is constitutively active and its overexpression is sufficient to confer oncogenic transformation.

Increasingly, the EGF receptor is being recognized for its role in oncogenesis. Approximately 80% of all head and neck tumors are EGFR-positive (Mendelsohn 2001; Harari 2004). In these tumors, the presence of the EGF receptor is a strong prognostic marker (70%) for reduced survival rates. EGFR overexpression is primarily due to gene amplification (Libermann, Nusbaum et al. 1985; Zwick, Bange et al. 2002). However, recently many mutations in the kinase domain of the EGFR have been identified that add to its oncogenic potency. Additionally, a naturally occurring mutant of the EGF-receptor, ΔEGFR (EGFRvIII), which contains a truncated extracellular domain and is constitutively active, is present in approximately half of *de novo* glioblastoma tumors (Nishikawa, Ji et al. 1994). Though best characterized in head and neck cancers, the role of the EGF receptor has also been documented in ovarian, cervical, bladder, esophegal, gastric, breast, endometrial, colorectal and non-small cell lung (NSCLC) carcinomas (Yarden and Sliwkowski 2001). In addition to receptor-overexpression and kinase domain mutants, there is evidence that many

carcinomas also express growth factors to the EGF receptor, suggesting autocrine activation that supports tumor growth (Yarden and Sliwkowski 2001).

Targeted Therapies for the EGF Receptor Family

Due to the important role of the EGF receptor family in malignant growth, there has been extensive effort directed toward the development and characterization of inhibitors that target these receptors. Effective tumor inhibition has been achieved clinically with inhibitors that antagonize the EGFR and HER-2. Both small molecule tyrosine-kinase inhibitors and monoclonal antibodies have been used to target both EGFR and HER-2 (Ross, Schenkein et al. 2004; Agus, Gordon et al. 2005).

Herceptin (trastuzumab), a partially humanized monoclonal antibody, whose antigenic heavy chains have been humanized, but whose variable arms bind subdomain IV of the HER-2 receptor, has been widely used as a therapeutic for HER-2 positive tumors. It has achieved positive results in metastatic disease and is now being tested for efficacy in earlier stages of breast cancer (Ross, Schenkein et al. 2004). More recently, Omnitarg (pertuzumab; 2C4), a partially-humanized monoclonal antibody, which binds the dimerization arm of the HER-2 receptor, has been developed by Genentech (Ross, Schenkein et al. 2004; Agus, Gordon et al. 2005). Additionally, a monoclonal antibody to the EGF receptor, Erbitux (cetuximab), was approved last year for combinatorial use with chemotherapy for patients with advanced and refractory metatstatic colorectal cancer (Ross, Schenkein et al. 2004).

In addition to monoclonal antibodies, several small molecule tyrosine kinase inhibitors (TKIs) have been developed to target the EGF receptor family. Iressa (gefitinib) interacts with high affinity with the kinase domain of the EGF receptor. Tarceva (erlotinib), another TKI, also has nanomolar affinity for the kinase domain of the EGF receptor. Despite its high-specificity for the EGFR kinase domain, preclinical studies also suggest that Tarceva may inhibit HER-2-dependant neoplastic growth (Ross, Schenkein et al. 2004; Agus, Gordon et al. 2005).

The IGF-IR Family

The IGF-I receptor (IGF-IR) family, consisting of the insulin receptor (IR), the IGF-I receptor, and the insulin receptor –related receptor (IRR), is activated by insulin-like growth factor (IGF)-I or IGF-II ligands or by insulin (O'Connor 2003). This family of receptors consists of a disulphide linked heterotetrameric structure of two extracellular α-subunits and two transmembrane ß-subunits, which is distinct from other RTK families. (Massague and Czech 1982; Ward, Garrett et al. 2001). Despite the different subunit organization, the IGF-IR α-subunit ectodomain has sequence and structural homology with the ectodomain of the EGFR (Garrett, McKern et al. 1998; Ward, Garrett et al. 2001; Cho and Leahy 2002; Ogiso, Ishitani et al. 2002) (Figure 1.4). Ligand binding to the both the IGF-IR and IR leads to receptor activation and autophosphorylation of tyrosine residues (Johnston, Pirola et al. 2003) (O'Connor 2003). This phosphorylation of tyrosines in the carboxyl-terminal domains of the ß-subunits

provides docking sites for PTB and SH2-domain-containing scaffolding and adapter proteins such as Shc and the insulin receptor substrates (IRS), leading to activation of two major signaling cascades: the PI3K/Akt pathway and the MAPK/ERK pathway (Johnston, Pirola et al. 2003) (O'Connor 2003).

Unlike other RTKs that directly activate a myriad of secondary effector molecules, the majority of intracellular signaling by the IR and IGF-IR occurs through activation of the IRS family of proteins. The IRS proteins bind to phosphotyrosines on the C-terminal tails of the receptors, become phosphorylated, and then recruit down-stream SH-2-containing effector molecules (Myers, Sun et al. 1994; Johnston, Pirola et al. 2003). Most notably, IRS-1 and IRS-2 function to potentiate the insulin, IGF-I, or IGF-II stimulation of the IR and IGF-IR. It has been shown that basal levels of serine and threonine phosphorylation must be present for the proper action and further activation of IRS-1 and IRS-2, thus possibly allowing for tighter regulation of IR and IGF-IR signaling (Greene and Garofalo 2002; Johnston, Pirola et al. 2003).

Structural Homology between EGFR and IGF-IR

Subdomains I, II and III of the ectodomain of the EGF receptor each share approximately 25% sequence identity with the corresponding subdomains of the a-subunit of the IGF-IR (Ogiso, Ishitani et al. 2002) (Figure 1.4). Sequence alignment shows that this homology is centered in the ligand-binding domains, subdomains I and III. The crystal structures of the ligand-bound EGF receptor and the unliganded IGF-I receptor ECDs, reveals that subdomains I and III each

fold into "single-stranded right-handed ß-helical barrels" (Garrett, McKern et al. 1998; Ogiso, Ishitani et al. 2002). Receptor-specific interactions are then primarily mediated by side-chain interactions, as well as non-conserved α-helices. Additionally, subdomains II of both receptors are cysteine rich and also share similar topology, as mediated by the EGF-like fold of the cysteines. However, there are some striking differences in the gross-topology of these subdomains. Most notably is the dimerization loop, present in the EGF-receptor but absent in the IGF-IR.

EGFR – IGF-IR Crosstalk

Both the EGF and IGF receptor families are potent orchestrators of cell growth and proliferation in normal and malignant tissues. Crosstalk between these receptor tyrosine kinases may allow coordinated control of cellular responses in both normal and tumor cells (Adams, McKern et al. 2004).

Coordination of signal transduction and crosstalk between the EGF and IGF1 receptor families has been examined primarily using *in vitro* studies (Adams, McKern et al. 2004). Sustained activation of MAPK by the EGFR requires a functional IGF-IR, and conversely, activation of MAPK by IGF-IR requires a functional EGF receptor (Swantek and Baserga 1999; Gilmore, Valentijn et al. 2002; Ahmad, Farnie et al. 2004; Kuribayashi, Kataoka et al. 2004). While EGFR-IGF-IR crosstalk is crucial for the activation of MAPK, it is not required for the activation of Akt. IRS-1 activation by the IGF-IR, which leads to the activation of Akt, is unaffected by EGFR-specific inhibitors (Roudabush,

Pierce et al. 2000; Gilmore, Valentijn et al. 2002). Furthermore, the EGFR does not tightly couple to the Akt/PKB signaling cascade (Marmor, Skaria et al. 2004). Therefore, these data suggest that crosstalk between the EGFR and IGF-IR coordinately controls activation of the mitogenic MAPK signaling pathway, but not the anti-apoptotic Akt/PKB pathway.

Redundant signaling through IGF-IR may support activation of pathways essential for survival in the presence of EGFR family inhibitors. IGF-IR signaling in MCF7/HER-2 and SKBR-3 breast carcinoma cells protects against growth inhibition by Herceptin, a HER-2 inhibitor (Lu, Zi et al. 2001). Overexpression of the IGF-IR and increased IGF-I signaling can also overcome the effects of AG1478, an EGFR tyrosine kinase inhibitor, in glioblastoma multiforme cells (Chakravarti, Loeffler et al. 2002). Additionally, it has been shown in cell culture models that acquired resistance to Iressa, an EGFR kinase inhibitor, occurs through increased activation and signaling of IGF-IR (Jones, Goddard et al. 2004; Camp, Summy et al. 2005).

Herstatin is an Autoinhibitor of the EGF Receptor Family

Herstatin is a recently described, naturally occurring, autoinhibitor of the EGF receptor family. Initial characterization of the protein product showed that both full-length herstatin and its intron 8-encoded domain bind to the HER-2 receptor with nanomolar affinity. Additionally, herstatin was shown to disrupt HER-2 dimerization and receptor activation as measured by tyrosine phosphorylation (Doherty, Bond et al. 1999).

Further characterization revealed that herstatin blocks EGF- and TGF-α-induced EGFR dimerization and receptor activation. In both NIH-3T3 and CHO cells transfected with the EGF receptor, herstatin was found to modulate downstream signaling. In the presence of herstatin, ligand-induced EGF receptor activation resulted in activation of the mitogenic MAPK cascade, but in abrogation of the anti-apoptotic Akt/PKB (Justman and Clinton 2002). Ultimately, this resulted in inhibition of proliferation.

Examination of the effects of herstatin on HRG-induced signaling revealed that herstatin also inhibits transactivation of HER-3 by HER-2 in transfected CHO cells (Azios, Romero et al. 2001). Additionally, herstatin was found to inhibit HRG-induced proliferation of MCF7 and BT474 breast carcinoma cells (Jhabvala-Romero, Evans et al. 2003). Constitutive expression of herstatin in MCF7 cells abrogated HRG signaling through both the MAPK and Akt pathways and prevented HRG-mediated cellular proliferation. Herstatin expression in these cells also resulted in a down-regulation of the HER-3 and HER-4 receptors, both of which are HRG-receptors (Jhabvala-Romero, Evans et al. 2003).

Previous studies have characterized the *in vitro* signaling and growth outcomes of herstatin interacting with cells expressing receptors from the EGFR family. This thesis focuses on the peptide-receptor interactions that underlie these processes. I investigate the unique aspects of herstatin, namely the novel 79-amino acid C-terminal intron-encoded domain, and examine the receptor-binding specificities and affinities of both full-length herstatin and its intron-encoded domain. I show that herstatin and the intron-encoded domain bind to the

EGF and IGF receptor families, and suggest that the intron-encoded domain is a receptor-binding module.

This thesis seeks to define the role of herstatin binding. In doing so, I hypothesize the following:

1) Herstatin binds to all four members of the EGF receptor family with the similar affinity.
2) The intron 8-encoded domain functions as the binding domain of herstatin.
3) Herstatin and its intron 8-encoded domain may bind to other receptor tyrosine kinase families, such as the IGF-IR family, that share ectodomain homology with the EGF receptor family.
4) If herstatin binds to the IGF-I receptor, it may affect IGF-I-induced cellular signaling and growth.

Figure 1.1. Schematic of the EGF receptor family signaling network throughout evolution. In *C. elegans*, there is a simple signaling network of one receptor, LET-23, and one ligand, Lin-3. In *D. melanogaster*, there are now three ligands, Spitz, Gurken, and Vein, though still only one receptor, the DER. In mammals, multiplicity of both ligands and receptors results in a complex signaling network. There are eleven mammalian ligands. With the exception of HER-2 which does not bind ligand, the other members of the EGF receptor family: EGFR, HER-3, and HER-4, bind a variety of ligands, each of which result in different homomeric and heteromeric receptor dimer combinations.

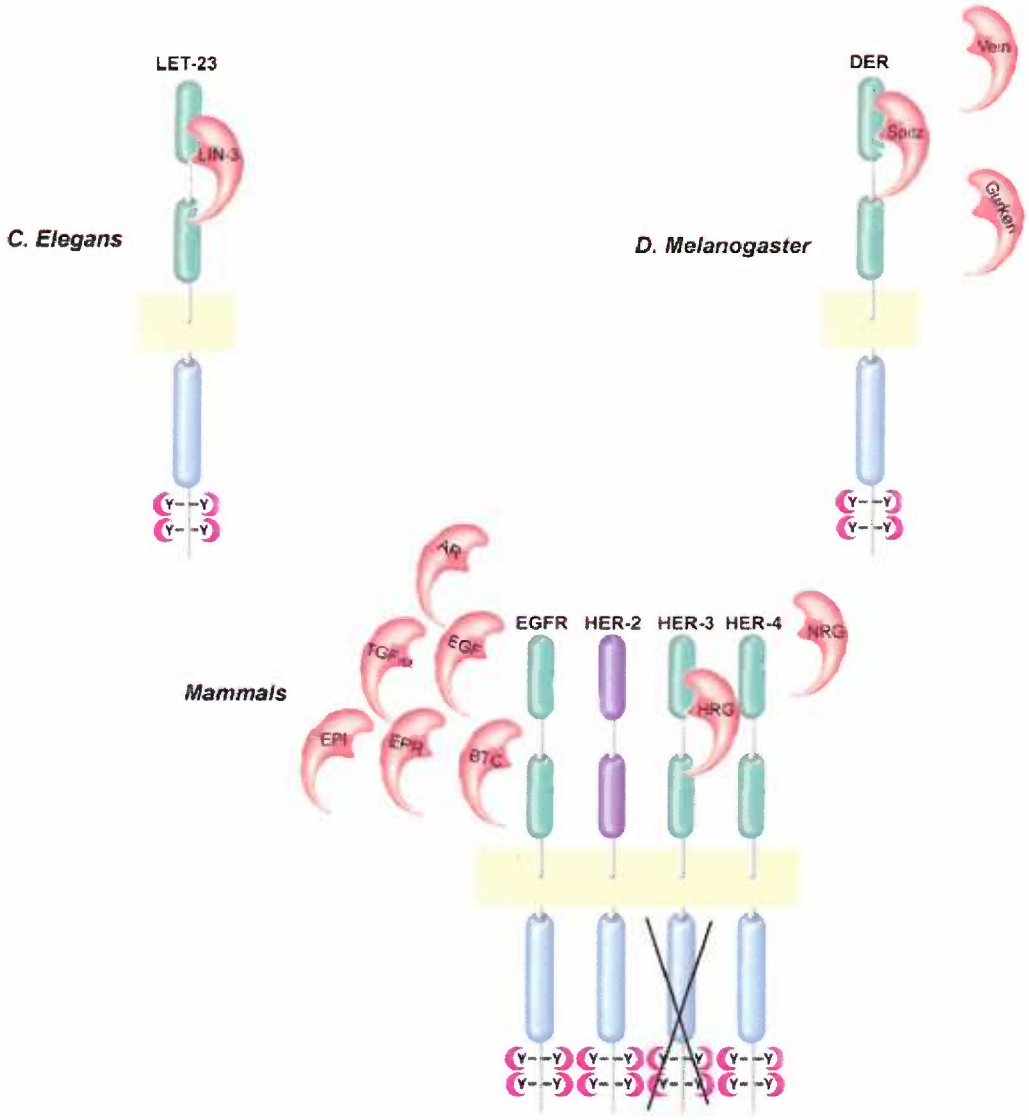

LET-23
LIN-3
C. Elegans
DER
Vein
Spitz
Gurken
D. Melanogaster
Mammals
AR
TGF-α
EGF
EPI
EPR
BTC
EGFR
HER-2
HER-3
HER-4
NRG
HRG
Y
Y
Y
Y

Figure 1.2. The EGF receptor family binds many ligands and has unique properties. A schematic of the EGF receptor family: subdomains I, II, III, and IV of the receptor extracelllular domain (ECD), followed by the transmembrane domain, and intracellular kinase domain and tyrosine residues. HER-3 is unique in that it is kinase-dead and must heterodimerize to be phosphorylated. HER-2, also is the only one of four receptors that does not bind ligand. The eleven mammalian ligands are depicted below each receptor they bind.

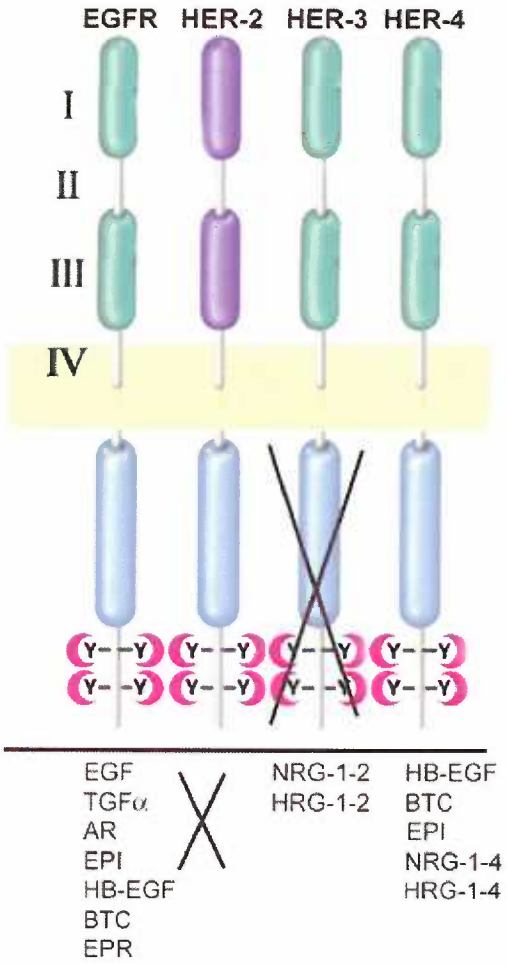

EGFR
HER-2
HER-3
HER-4
I
II
III
IV
Y
EGF
TGFα
AR
EPI
HB-EGF
BTC
EPR
NRG-1-2
HRG-1-2
HB-EGF
BTC
EPI
NRG-1-4
HRG-1-4

Figure 1.3. Comparison of the crystal structures of the ectodmains EGF-bound EGFR dimer, HER-2, and unliganded-HER-3. Ribbon diagram of EGFR, HER-2, and HER-3 soluble ectodomains: subdomain I (magenta), subdomain II (teal), subdomain III (orange), and subdomain IV (not present in EGFR) (green). The EGF molecule is in grey. Helices are indicated by curled ribbons and ß-strands by broad arrows. The EGFR dimer is mediated by interreceptor contacts through the "dimerization arm" in subdomain II. The HER-2 receptor is in a constitutively active position, poised to accept a dimer partner. The "dimerization arm" of the unliganded HER-3 is intramolecularly bound to the outermost portion of subdomain IV (Cho and Leahy 2002; Ogiso, Ishitani et al. 2002; Cho, Mason et al. 2003).

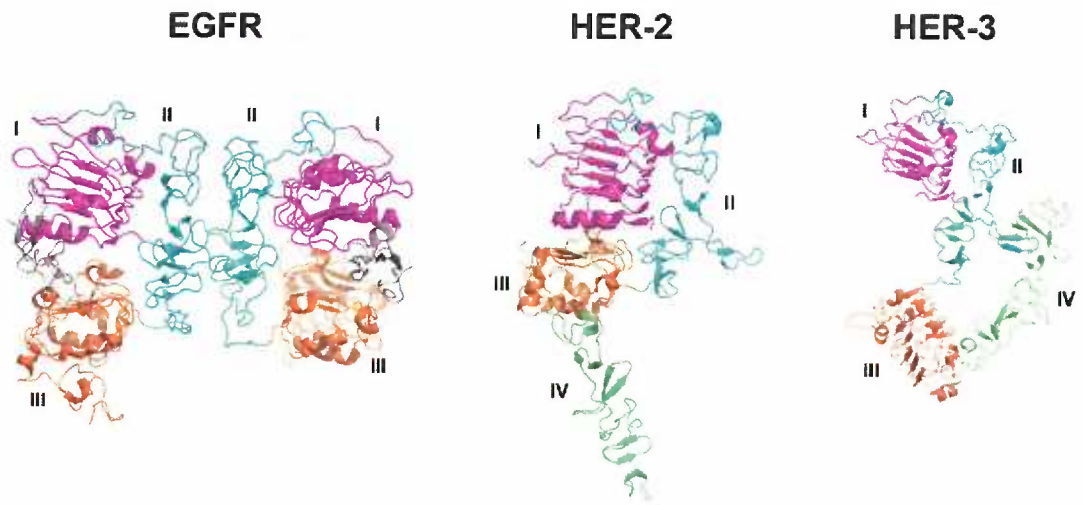
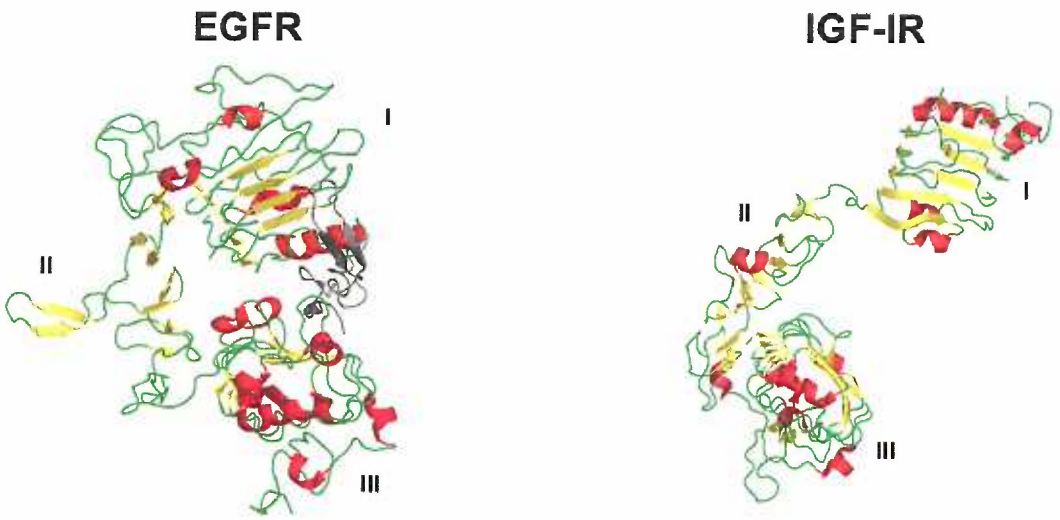

EGFR
HER-2
HER-3
I
II
II
I
III
III
I
II
III
IV
I
II
IV
III

Figure 1.4. Comparison of the crystal structures of the ectodmains of EGFR and IGF-IR. Ribbon diagram of EGFR and IGF-IR soluble ectodomains. Helices are indicated by curled ribbons (red), ß-strands by broad arrows (yellow), and loops (green). The EGF molecule is in grey. Approximately 25% sequence identity exists in subdomains I, II, and III of the EGF and IGF-I receptors (Garrett, McKern et al. 1998; Ogiso, Ishitani et al. 2002). This high identity is reflected in the structural similarity shown here.


EGFR
I
II
III
IGF-IR
I
II
III

CHAPTER 2

Receptor Binding Specificities of Herstatin and Its Intron 8-Encoded Domain

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by

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Key words: ErbB receptors; HER-1, HER-2, HER-3, HER-4; IGF-I receptor, Herstatin.

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Abstract

Retention of intron 8 in alternative HER-2 mRNA generates an inhibitory secreted ligand, Herstatin, with a novel receptor-binding domain (RBD) encoded by the intron. This study examines binding interactions with several receptors and investigates sequence variations in intron 8. The RBD, expressed as a peptide, binds at nM concentrations to HER-2, the EGFR, ΔEGFR, HER-4 and to the IGF-I receptor, but not to HER-3 nor to the FGF-3 receptor, whereas a rare mutation in the RBD (Arg to Ile) eliminates receptor binding. The full length Herstatin binds with 3-4 fold higher affinity than its RBD, but with ~10 fold lower affinity to the IGF-IR. Sequence conservation in rhesus monkey but not in rat suggests that intron 8 recently diverged as a receptor-binding module critical for the function of Herstatin.

I. Introduction

The ErbB receptor family consists of four receptor tyrosine kinases: EGFR (HER-1, erbB-1), HER-2 (erbB-2), HER-3 (erbB-3) and HER-4 (erbB-4). Aberrant expression of ErbB receptors by mutational activation, receptor overexpression, and tumor production of ligands contributes to the development and maintenance of a variety of human cancers (Blume-Jensen and Hunter 2001; Holbro, Civenni et al. 2003).

The ErbB receptors are activated by several ligands consisting of an EGF core domain (Groenen, Nice et al. 1994). The exception is the HER-2 receptor, which is recruited as a preferred dimer partner with other ligand binding erbB receptors. While the eleven mammalian EGF-like ligands are all agonists, the ligand Argos, in *Drosophila*, inhibits activation of the EGFR (Jin, Sawamoto et al. 2000; Vinos and Freeman 2000).

Although the HER-2 receptor does not directly bind EGF-like ligands, a secreted product of an HER-2 alternative transcript, Herstatin, binds with nM affinity to the ectodomain of HER-2. Herstatin consists of a segment of the HER-2 ectodomain followed by 79 novel amino acids, encoded by intron 8, which function as a receptor-binding domain (RBD) (Doherty, Bond et al. 1999). Herstatin blocks homomeric and heteromeric ErbB receptor interactions, inhibits activation of the PI3K/Akt pathway initiated by EGF, TGF-α, and Heregulin, and causes growth arrest suggesting potential as an anti-cancer agent (Doherty, Bond et al. 1999; Azios, Romero et al. 2001; Justman and Clinton 2002; Jhabvala-Romero, Evans et al. 2003). However, no study has yet addressed the

receptor specificity of Herstatin. To identify receptor binding targets and to further assess the significance of the novel intron 8-encoded RBD, we investigated binding to several receptors expressed in transfected cells, examined the consequence of a rare mutation in intron 8, and compared the sequence in human, rat and rhesus monkey.

II. Materials and Methods

2.1 Cell lines, transfections, and Western blots

The 3T3/HER-2 cells were previously described (Lin and Clinton 1991). The 3T3/IGF-IR cells were from Dr. Charles Roberts, OHSU, Portland, OR. For transient transfections, 2 µg of empty vector or 2 µg EGFR, HER-2, HER-3, HER-4, ΔEGFR, or FGFR-3-myc expression vectors were added with Lipofectamine (GIBCO-BRL) to Cos-7 cells in 6 cm plates. The HER-2 and EGFR expression plasmids were previously described (Azios, Romero et al. 2001), ΔEGFR was a gift from Dr. Webster Cavenee (Ludwig Institute, UCSD, La Jolla, CA), the FGFR-3-myc construct was from Dr. William Horton (Shriners Research Hospital, Portland, OR), and the HER-4 expression plasmid was a gift of Dr. Nancy Hynes (Friedrich Miescher-Institute for Biomedical Research, Basel, Switzerland). To analyze receptors by Western blot analysis, proteins were resolved by SDS-PAGE and electro-transferred onto nitrocellulose membranes (BioRad, Hercules, CA). Blots were blocked in 5% milk and incubated with primary antibody overnight at 4°C. The antibodies included anti-HER-2 (Christianson, Doherty et al. 1998) anti-EGFR, anti-HER-3, anti-HER-4, which were all rabbit polyclonal antibodies against the receptor C-terminal domains (Santa Cruz Biotechnology). Antibodies against the ß subunit of IGF-IR were from Dr. Charles Roberts. After washing, the blots were incubated with secondary antibody conjugated to HRP for 30 min (BioRad, Hercules, CA). The membranes were developed with SuperSignal West Dura (Pierce, Rockford, IL) and exposed to x-ray film.

2.2. Sequencing of Intron 8

Human genomic DNA was obtained from blood samples (supplied by Dr. David Henner, OHSU) from individuals 18 years or more, after giving informed consent, with approval by the Institutional Review Board of OHSU. The samples, assigned random four-digit numbers, could not be traced to patient identity. The polymerase chain reaction (PCR) was employed to amplify intron 8 using primers: 3' AACACAGCGGTGTGAGAAGTGC (exon 8) and 5' GTATCGGTAGTTCATTTCCTTTGGTTGC (intron 9). The reactions were cycled 95°C for 120"; 69°C for 30"; 72°C for 30"; for 30 cycles. PCR products were purified and sequenced. Electropherograms were individually reviewed to detect polymorphic alleles. Samples found to contain a polymorphism were sequenced at least twice to confirm the mutation. Rhesus monkey DNA, provided by Dr. Scott Wong (ORPC, Portland, OR) was amplified and sequenced using the above primers. Intron 8 in rat genomic DNA was amplified by PCR using rat specific primers: 5'-CTACCTGTCTACGGAAGTGG-3' and 5'-TTCCGGGCAGAAATGCCAGG-3'. The cycling parameters were: 94°C for 30"; 62°C for 30"; 72° C for 60", for 25 cycles.

2.3. Expression and purification of intron 8-encoded peptide (Int8) and Herstatin

The intron 8 cDNA was cloned into the pET 30 bacterial expression vector (Novagen, Madison, WI), expressed in bacteria (BL-21), and purified by nickel affinity chromatography as described (Doherty, Bond et al. 1999). For purification of insect Herstatin, S2 insect cells, stably transfected with 6xHis tagged-Herstatin

in the pMT/BiP expression plasmid (Invitrogen, Carlsbad, CA), were induced with 100 μM cupric sulfate for ~16hrs. Herstatin was purified to ~90% purity by Ni-NTA (Qiagen, Valencia, CA) affinity chromatography as previously described (Jhabvala-Romero, Evans et al. 2003).

2.4. Cell binding studies

About 2 x 10^6 cells in 6-well plates were incubated with purified Herstatin or int8 peptide for 2 hours at 4°C in serum-free media. Cells were washed with Phosphate Buffered Saline (PBS) and extracted in 50mM Tris·HCl, pH 7.0, 1.0% NP-40. Int8 peptide or Herstatin bound to cells were quantified using a sandwich Herstatin ELISA per manufacturer's instructions (Upstate Biotechnology, Lake Placid, NY). The dissociation constant (K_d) and maximal binding (B_{max}) of Herstatin or the int8 peptide were determined by nonlinear regression analysis of the plot of pmol of bound *versus* nM of Herstatin or int8 peptide added. Statistical comparisons between different binding curves were performed by extra sums-of-squares F-test nonlinear regression coefficients. All tests were performed (α = 0.05) using GraphPad Prism 4 software (GraphPad Software, 1994-2003).

2.5. Pull-downs with int8 peptide immobilized on protein S agarose

About 100 μl of a 50% suspension of S-protein agarose (Novagen) was incubated with or without 100 μg of int8 peptide with an S-protein tag, at room temperature for 1hr, and then washed twice with 500 μl PBS. The agarose samples were then incubated at room temperature for 1 hr with 200 μg of

transfected Cos-7 cell extract and washed twice with 500 µl of PBS with 1% NP40. The proteins were eluted from the resin at 92°C for 2 min in 40µl of SDS-sample buffer, and analyzed as a Western blot.

III. Results

3.1 Sequence of human, rhesus monkey, and rat intron 8

Herstatin is generated by retention of HER-2 intron 8, which encodes the unique C-terminal proline-rich domain of 79 amino acids (Fig 2.1a). Because of its critical function in receptor binding (Doherty, Bond et al. 1999), we sequenced genomic HER-2 intron 8 from 215 humans, rhesus monkey, and rat. The HER-2 intron 8 deduced amino acid sequence, originally determined from SKOV3 ovarian cancer cells (AF177761), was found to be the most common in germ line DNA. In addition, we identified a sequence variation in intron 8 (G1112T in AF177761) resulting in an Arg to Ile substitution at residue 31 in Figure 2.1. This mutant allele was found in only one of 215 (<0.5%). The deduced amino acid sequence of intron 8 from rhesus monkey was 85% identical to that of humans (Fig. 1) and the nucleotide sequence, up to the stop codon, was 93% identical. However, there was no conservation between rat and human intron 8 (Fig 2.1), in contrast to the HER-2 receptor coding sequence, which is highly conserved in rat *neu* (Stein and Staros 2000).

3.2 Receptor binding of the HER-2 intron 8-encoded peptide.

To identify other potential receptor targets of Herstatin, we examined binding of the intron 8-encoded RBD, expressed as a bacterial peptide (Int8). Protein S agarose, with or without immobilized int 8 peptide, was incubated with extracts from Cos-7 cells transiently transfected with several different receptors. Following washing steps, the protein bound to the agarose was analyzed as a

Western blot with receptor-specific antibodies. As previously observed (Doherty, Bond et al. 1999; Azios, Romero et al. 2001) EGFR and HER-2 from the transfected cell extracts bound specifically to the agarose with int8 peptide (Fig. 2.2A). In contrast, the int8 peptide with the Arg to Ile mutation at residue 31 (see Fig. 2.1) did not pull-down the HER-2 receptor (Fig. 2.2B). Figure 2.2A also demonstrates that ΔEGFR, a tumor variant of the EGFR missing its N-terminal subdomains I and II specifically associated with int8 peptide (Nishikawa, Ji et al. 1994). Another member of the erbB family, HER-4, was also pulled-down by int8. However, there was no detectable association of HER-3 with int8 peptide agarose despite abundant expression in the transfected cells (Fig. 2.2A). We also investigated the possible interaction with the IGF-I receptor (IGF-IR), which contains regions of ectodomain sequence homology with the EGFR (Garrett, McKern et al. 2002). Interestingly, we observed specific pull-down of the ß subunit of the IGF-IR from transfected cell extracts (Fig. 2.2A). The FGFR-3, a receptor tyrosine kinase with Ig-like motifs and no structural homology with the ErbB family ectodomains, did not bind to the int8 peptide.

To further examine interaction of the int8 peptide with the extracellular domain of receptors at the cell surface, an Herstatin ELISA was used to quantify bound peptide. The int8 peptide bound in a specific and dose-dependent manner to EGFR, HER-2, HER-4, and ΔEGFR, but not to HER-3, FGFR-3, or mock-transfected cells (Fig. 2.2C) in agreement with results obtained by the pull-down assay. Binding affinities were further characterized by generating saturation-binding curves. Int8 peptide bound to HER-2 transfected Cos-7 cells (K_d =50 ±

6nM) and to EGFR transfected Cos-7 cells (K_d=78 ± 10nM) with binding affinities, assessed by comparative nonlinear regression analysis, that were not significantly different (*P*=.40) (Fig. 2.3A). Further, int8 peptide bound to the IGF-IR/3T3 cells (K_d =70 ± 21nM) and to HER-2/3T3 cells (K_d=66 ± 16nM) with similar affinities (*P*=0.96) (Fig. 2.3B). In contrast the mutant int8 peptide with Arg31Ile did not significantly bind to the HER-2 receptor overexpressing cells at any of the peptide concentrations tested (Fig. 2.3C) even though the Herstatin ELISA detected the wildtype and mutant peptide equally (Fig. 2.3D). These results suggested that the int8 peptide bound to EGFR, HER-2, and IGF-IR with overlapping binding affinities and that the Arg-Ile mutation inhibited receptor binding without destroying antibody binding epitopes.

3.3 Receptor binding properties of full length Herstatin.

The full length Herstatin bound to 3T3/HER-2 cells with a K_d = 14.7 ±1.8 nM, which is significantly different from the binding affinity of int8 peptide (*P*<.0001) by 3-4 fold . A direct comparison of the binding of Herstatin to 3T3/HER-2 and 3T3/IGF-IR cells revealed that the affinity for the IGF-IR (K_d ~151 nM) was lower (*P*<.0001) by about 10 fold (Fig. 2.4A). The dissociation constant of Herstatin for EGFR was similar to that of HER-2, and was unaffected by ligand occupation indicated by a K_d = 16.4 ± 3.6 nM versus 16.3 ±3.6 nM (respectively) for Cos-7/EGFR treated or not with 10 nM EGF (Fig. 2.4B). Herstatin bound with saturation to endogenous receptors in A431 epidermoid carcinoma cells, which express very high levels of EGFR and low levels of other ErbB receptors (Fig.

2.4C). At saturation, 6.9 ±0.4 pmol of Herstatin were bound indicating ~2 x 10^6 binding sites/cell, which matches the number of EGFR per A431 cell at 2 x 10^6 (Filmus, Pollak et al. 1985). Comparison of nonlinear models indicated that a hyperbolic one-affinity site binding model was the best fit for EGFR-specific binding of Herstatin, in the presence and absence of EGF.

IV. Discussion

We present evidence that intron 8 of the HER-2 gene, retained in an alternative HER-2 transcript, encodes a receptor binding domain. We also report that a nonlethal, point mutation of unknown physiological significance, resulting in Arg to Ile in the intron 8-encoded domain, eliminates binding to the HER-2 receptor. Unaltered interaction of this mutant RBD with two monoclonal antibodies in an ELISA suggested that global structure was unaffected and that this Arg residue may be directly involved in receptor binding. While the intron 8 encoded domain is critical for receptor binding, it does not appear to affect receptor activity suggesting a requirement for the N-terminal subdomains I and II of Herstatin for receptor inhibition (Doherty, Bond et al. 1999) (Shamieh and Clinton, unpublished observations).

While the intron 8-encoded RBD is critical for the receptor binding activity of Herstatin, it is not conserved between humans and rats despite the high degree of sequence identify between the HER-2 receptor and its rat ortholog, *neu*. There are distinct regions in their ectodomains, however, with very little identity (Stein and Staros 2000). An additional distinction is that the rat *neu* receptor is activated as an oncogene by a single point mutation in the transmembrane domain, while the human ortholog, HER-2, is oncogenic without aberrations in the coding sequence (Weiner, Liu et al. 1989). Furthermore, the activating mutation is not functionally equivalent when introduced into HER-2 (Suda, Aizawa et al. 1990; Hynes and Stern 1994). These collective observations

point to differences in regulation of the human HER-2 receptor versus its rat ortholog, *neu*.

Specific binding of the RBD suggests that the HER-4 receptor will be a target of Herstatin. Since Herstatin binds to and blocks the dimerization of the EGFR and HER-2, we predict that Herstatin will have a similar effect on the structurally similar HER-4. Effects of Herstatin on HER-4 activation and signaling are currently under investigation. Lack of Herstatin binding to the other ErbB family member, the HER-3 receptor, was surprising. HER-3 is unique, however, since it is kinase deficient and requires an active receptor partner to signal. The Herstatin binding site may be disguised when HER-3 is overexpressed without a dimer partner.

The binding of Herstatin to the IGF-IR with nM affinity was unforeseen, since ligands do not typically cross-react with receptors from different families. Interestingly, the IGF-IR has regions of ectodomain sequence homology with the EGFR and crosstalk occurs, most notably, transactivation of the EGFR by IGF-I (Ahmad, Farnie et al. 2004). Our finding that the binding affinity of Herstatin, but not its RBD, is significantly weaker for IGF-IR than for HER-2 or the EGFR suggests that stabilizing interactions between the N-terminus of Herstatin and the receptor ectodomain are lacking. Since IGF-IR does not have a homologous dimerization loop (Garrett, McKern et al. 2002) contacts between the IGF-IR ectodomain and the dimerization arm in subdomain II of Herstatin may be prohibited. The physiological significance of Herstatin binding to the IGF-IR remains to be determined.

In addition to Herstatin, there are several other examples of alternative forms of ErbB receptors that are created by intron read-through (Lee, Akita et al. 2001; Reiter, Threadgill et al. 2001). Creation of truncated receptors fused to novel C-terminal domains by read-through into introns represents a novel regulatory mechanism important in the diversification of receptor signaling. So far, Herstatin is the only known alternative receptor product that functions as a ligand, and is the only mammalian secreted ligand that inhibits the EGF receptor family (Dougall, Qian et al. 1994; Hynes and Stern 1994; Tzahar and Yarden 1998).

Acknowledgments

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Erratum

Sequencing revealed that the pET30aInt8 (wt, R→I, and R→C) expression constructs used in this manuscript all contained a frame-shift mutation immediately following the 5' cloning site. This mutation resulted in a conserved vector sequence followed by nonsense coding sequence of similar size to the intron 8-encoded domain. The conserved vector sequence was also found on the Int8 peptide used to make the Herstatin ELISA (Upstate). I, therefore, speculate that the reactivity of these mutant peptides on the Herstatin ELISA was due to the conserved vector sequence. The frame-shift mutations were corrected using the Quik-Change Site-Directed Mutagenesis Kit (Stratagene) and confirmed through sequencing. Chapter 3 of this thesis addresses experiments done with the corrected vectors

Sequencing also revealed a mutation in the HER-3 vector coding sequence. HER-3G560E was used rather than wild-type HER-3. There is no evidence in the literature of this being a naturally-occurring mutation. Further more, heregulin-signaling studies (data not shown) show that this mutant receptor signals normally. However, in the experiments described in this chapter, HER-3G560E mutation results in an abrogation of binding of the mutant peptide. This suggests a null-function in relation to herstatin binding. Experiments using wild-type HER-3 are in Chapter 3 of this thesis.

Figure 2.1. The deduced amino acid sequence encoded by HER-2 (ErbB-2) intron 8. Alignments are with the most common human intron 8 sequence from 214 individuals with non-conserved residues shown.

```
Human:  1                                                  I
Human:  1 GTHSLPPRPAAVPVPLRMQPGPAHPVLSFLRPSWDLVSAF40
Rhesus: 1                          C  N       L              P  L
Rat:    1 GTQPHSKTSLVHPALAstop

Human:  41 YSLPLAPLSPTSVPISPVSVGRGPDPDAHVAVDLSRYEGstop
Rhesus: 41  C      L          M S        DL            N     C   stop
```

Figure 2.2. Binding of intron 8-encoded peptide to different receptors expressed in transfected cells. **(A)** Extracts from transfected Cos-7 cells were incubated with protein S agarose without or with immobilized wild-type or **(B)** R31I mutant int8. Associated proteins were analyzed as a Western blot. **(C)** Transfected Cos-7 cells were incubated with purified int8 for 2 h at 4°C in serum-free media, cells were washed, extracted, and analyzed by Herstatin ELISA.

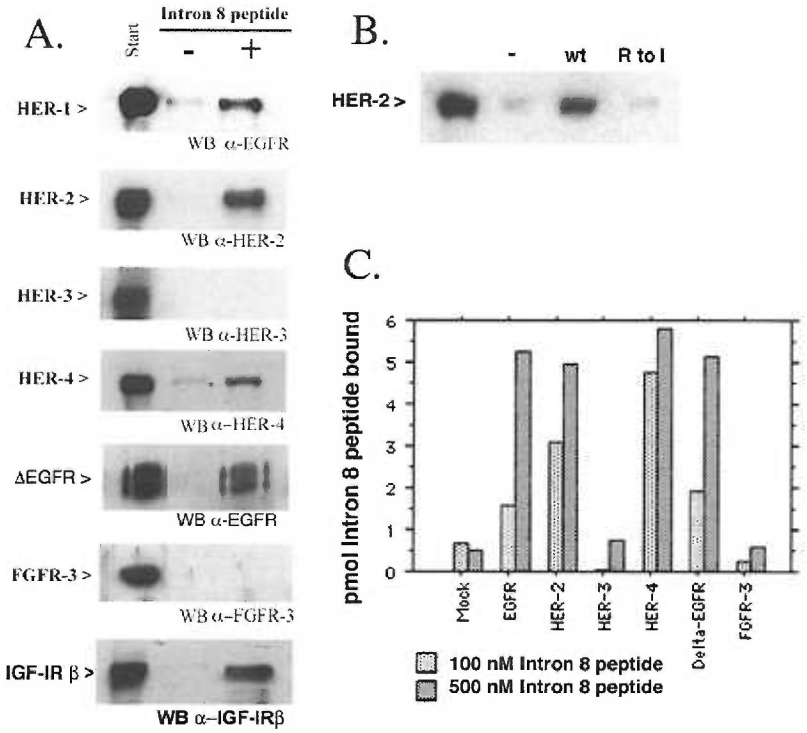

A.
Start
Intron 8 peptide
-
+
HER-1 >
WB α-EGFR
HER-2 >
WB α-HER-2
HER-3 >
WB α-HER-3
HER-4 >
WB α-HER-4
ΔEGFR >
WB α-EGFR
FGFR-3 >
WB α-FGFR-3
IGF-IR β >
WB α-IGF-IRβ
B.
-
wt
R to I
HER-2 >
C.
pmol Intron 8 peptide bound
0
1
2
3
4
5
6
Mock
EGFR
HER-2
HER-3
HER-4
Delta-EGFR
FGFR-3
100 nM Intron 8 peptide
500 nM Intron 8 peptide

Figure 2.3. Saturation binding curves of intron 8 peptide to cells transfected with HER-2, the EGFR, and the IGF-IR. Different amounts of purified int8 were added to the indicated cells and bound peptide was quantified by Herstatin ELISA. Nonlinear regression analysis of binding data was used to determine the dissociation constants (K_d) and maximal amount bound. In **(A)** parental (Cos-7) or transiently transfected Cos-7-HER-2 or Cos-7-EGFR cells, or in **(B)** 3T3 cells or stably transfected HER-2-3T3 or IGF-IR-3T3 cells were used. In **(C)** wild-type or R31I mutant Int8 peptides were incubated with HER-2-3T3 cells. In **(D)** indicated amounts of wild-type or R31I peptides were incubated in an Herstatin ELISA.

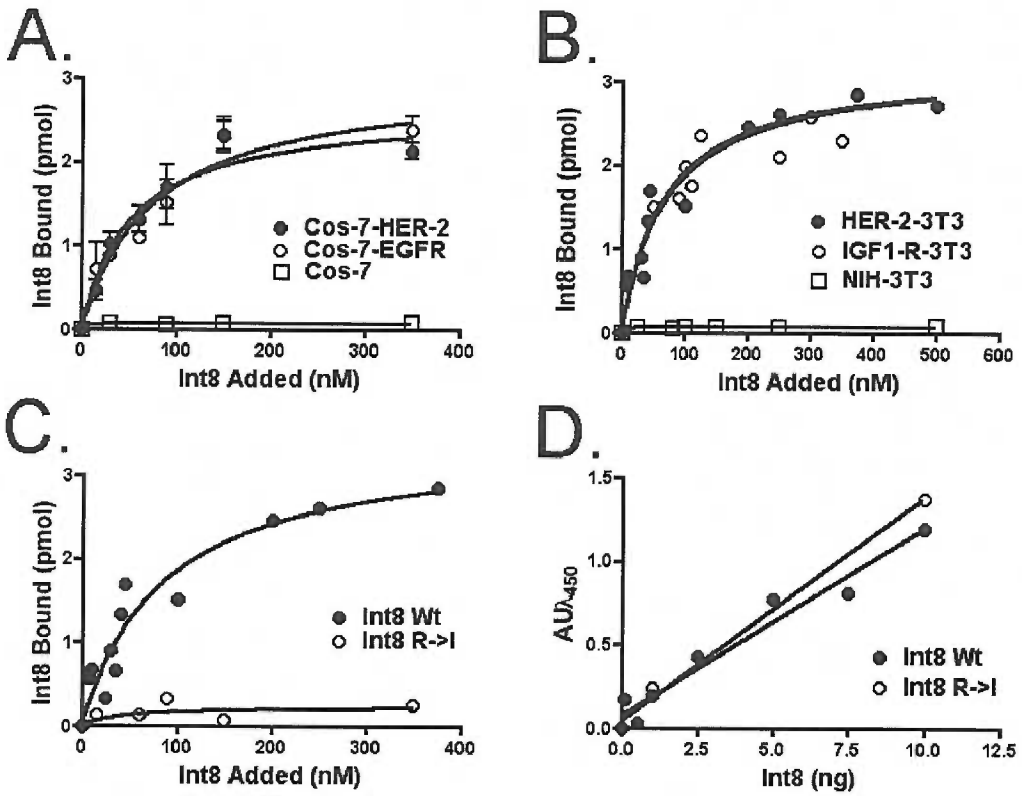
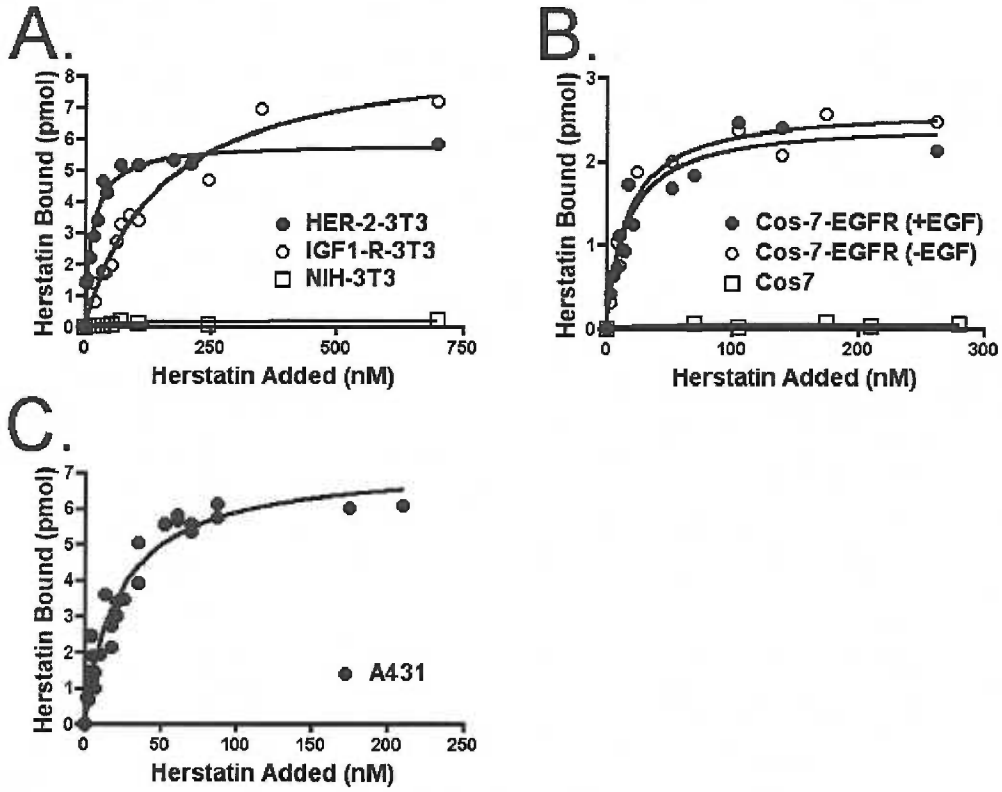

A.
Int8 Bound (pmol)
Int8 Added (nM)
Cos-7-HER-2
Cos-7-EGFR
Cos-7
B.
Int8 Bound (pmol)
Int8 Added (nM)
HER-2-3T3
IGF1-R-3T3
NIH-3T3
C.
Int8 Bound (pmol)
Int8 Added (nM)
Int8 Wt
Int8 R->I
D.
AU450
Int8 (ng)
Int8 Wt
Int8 R->I

Figure 2.4. Saturation binding curves of Herstatin to cells expressing different receptors. Herstatin purified from S2 insect cells was incubated with: **(A)** 3T3 cells, HER-2-3T3, or IGF-IR-3T3 cells or in **(B)** with parental or transiently transfected Cos-7-EGFR cells serum starved for 24 h and then treated or not for 2 h on ice with 10 nM EGF, or in **(C)** A431 epidermoid carcinoma cells.


A.
Herstatin Bound (pmol)
Herstatin Added (nM)
HER-2-3T3
IGF1-R-3T3
NIH-3T3
B.
Herstatin Bound (pmol)
Herstatin Added (nM)
Cos-7-EGFR (+EGF)
Cos-7-EGFR (-EGF)
Cos7
C.
Herstatin Bound (pmol)
Herstatin Added (nM)
A431

CHAPTER 3

Examination of the Binding of Herstatin and Its Intron-Encoded Domain to the EGFR and IGF-IR Families

by

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Abstract

Herstatin is produced from an alternately spliced HER-2 mRNA in which retention and read through of intron 8 leads to a protein product consisting of subdomains I and II of the HER-2 receptor followed by a unique 79 amino-acid C-terminal domain, encoded by intron 8 (Doherty, Bond et al. 1999). Previous studies have shown that herstatin binds to EGFR and HER-2 with a K_d of ≈ 14 nM and to a non-EGF family receptor tyrosine kinase (RTK), the Insulin-like Growth Factor Receptor (IGF-IR) with a K_d of ≈ 150 nM (Doherty, Bond et al. 1999; Shamieh, Evans et al. 2004). The purpose of this study was to examine the effects of germline polymorphisms in the intron 8-encoded domain on binding to the EGF receptor family. This study also examines the binding of herstatin to the Insulin Receptor (IR), a homologue of the IGF-IR, and investigates the impact of herstatin on insulin-mediated growth. These studies demonstrate that herstatin and its intron-encoded domain bind at nanomolar concentrations to the four members of the EGF receptor family and to a mutant EGFR missing subdomains I and II of its ECD (ΔEGFR), but not to FGFR-3. Arg31Ile and Arg17Cys, two mutations in the intron-encoded domain of herstatin, both lower the binding affinity of herstatin to EGFR and HER-2. Herstatin also binds, albeit with reduced affinity, to the insulin receptor (IR). MCF7 breast carcinoma cells stably expressing herstatin showed that herstatin expression reduced insulin-stimulated growth *in vitro*. These results suggest that both full-length herstatin and its intron-encoded domain bind to both EGF and the Insulin-like Growth Factor (IGF) receptor families, of which IR is a member.

I. Introduction

The EGF receptor family consists of four receptor tyrosine kinases (RTKs): EGFR (HER-1, erbB1), HER-2 (erbB-2), HER-3 (erbB-3), and HER-4 (erbB-4). These RTKs play a critical role in normal growth and development of epithelial tissues (Dougall, Qian et al. 1994; Hynes and Stern 1994; Olayioye, Neve et al. 2000; Blume-Jensen and Hunter 2001; Holbro and Hynes 2004). The EGF receptors are also implicated in a variety of carcinomas. Gene duplication, aberrant expression by mutational activation, and autocrine production of ligands contribute to tumor formation and progression in a variety of human cancers (Blume-Jensen and Hunter 2001; Holbro, Civenni et al. 2003; Marmor, Skaria et al. 2004). Most notably, 25-30% of all breast tumors overexpress HER-2 (Slamon, Clark et al. 1987; Slamon and Clark 1988; Slamon, Godolphin et al. 1989; Zhang, Silva et al. 1989; Press, Jones et al. 1990; Ross and Fletcher 1998).

The EGF receptors are monomeric receptors that homo- or hetero-dimerize upon ligand binding (Schlessinger, Ullrich et al. 1988; Heldin and Ostman 1996; Garrett, McKern et al. 2002; Ogiso, Ishitani et al. 2002; Cho, Mason et al. 2003; Garrett, McKern et al. 2003). The notable exception is HER-2, which does not bind ligand, but is the preferred heterodimer partner of the EGF receptor family (Hynes and Stern 1994; Tzahar and Yarden 1998). The EGF receptor family extracellular domain (ECD) consists of four subdomains: subdomains I and III which are the ligand binding domains and the cysteine-rich subdomains II and IV (Bajaj, Waterfield et al. 1987). The eleven mammalian

ligands to the EGF receptor family are all agonists and contain a core EGF-like domain (Sweeney and Carraway 2000; Harris, Chung et al. 2003). However, in Drosophila, the molecule, Argos, which also contains a modified-EGF-like domain, acts as an EGF receptor antagonist by binding to and sequestering Spitz, an activating ligand (Klein, Nappi et al. 2004).

Herstatin binds to EGFR and HER-2 with a K_d of ≈ 14 nM, and to the Insulin-Like Growth Factor Receptor (IGF-IR) with a K_d of ≈ 150 nM, and is unique as a naturally occurring EGF-receptor family autoinhibitor that also has been shown to interact with non-EGF-receptor family RTKs (Doherty, Bond et al. 1999; Shamieh, Evans et al. 2004). Herstatin, which is produced from an alternative HER-2 transcript in which intron 8 is retained, consists of subdomains I and II of the HER-2 receptor, followed by a unique 79-amino acid intron 8-encoded C-terminal domain (Doherty, Bond et al. 1999). This domain is proline-rich and is thought to be the binding module for herstatin (Doherty, Bond et al. 1999; Shamieh, Evans et al. 2004).

This study further examines the receptor-binding specificity of herstatin and its intron-encoded domain. We also investigate how germline mutations in intron 8 affect the binding of full-length herstatin to EGFR and HER-2. Finally, we examine the binding of herstatin to a second member of the IGF-IR family, the Insulin Receptor (IR) and begin to address the impact of herstatin expression on insulin-mediated growth of breast carcinoma cells.

II. Materials and Methods

Cell lines, transfections, and Western blots

All cells were maintained at 37°C and 5% CO2 in Dulbecco's modified Eagle's Medium (DMEM) containing 10% fetal bovine serum (FBS) and gentamicin (0.25 µL/mL). Media and supplements were purchased from Gibco. MCF7 breast carcinoma cells and Cos-7 cells were obtained from the American Type Culture Collection. The herstatin-expressing MCF7 clones, previously characterized in (Jhabvala-Romero, Evans et al. 2003), were maintained under the same conditions as parental MCF7 cells and supplemented with 0.5 mg/mL G418 sulfate. The IRa-3T3 cells were from Dr. Charles Roberts, Oregon Health and Sciences University, Portland, OR. For transient transfections, 2 µg of empty vector or 2 µg EGFR, HER-2, HER-3, HER-4, ΔEGFR, or FGFR-3-myc expression vectors were added with Lipofectamine (GIBCO-BRL) to Cos-7 cells in 6 cm plates. The HER-2 and EGFR expression plasmids were previously described (Azios, Romero et al. 2001; Shamieh, Evans et al. 2004). The DEGFR in pcDNA3.1 was a gift from Dr. Webster Cavenee (Ludwig Institute for Cancer Research, UCSD, La Jolla, California) and the FGFR-3-myc construct was from Dr. William Horton (Shriners Research Hospital, Portland, OR). The HER-4 expression plasmid was a gift of Dr. Nancy Hynes (Friedrich Miescher-Institute for Biomedical Research, Basel, Switzerland). To analyze receptors by Western blot analysis, proteins were resolved by SDS-PAGE and electro-transferred onto nitrocellulose membranes (BioRad, Hercules, CA). Blots were blocked in 5% milk and incubated with primary antibody overnight at 4°C. The antibodies included

anti-HER-2 (Christianson, Doherty et al. 1998) anti-EGFR, anti-HER-3, anti-HER-4, which were all rabbit polyclonal antibodies against the receptor C-terminal domains (Santa Cruz Biotechnology). After washing, the blots were incubated with secondary antibody conjugated to HRP for 30 min (BioRad, Hercules, CA). The membranes were developed with SuperSignal West Dura (Pierce, Rockford, IL) and exposed to x-ray film.

Sequencing of Intron 8

Human genomic DNA was obtained from blood samples (supplied by Dr. David Henner, Oregon Health and Sciences University) from individuals 18 years or greater, after giving informed consent, with approval by the Institutional Review Board of OHSU. The samples were assigned random four-digit numbers and could not be traced to patient identity. The polymerase chain reaction (PCR) was employed to amplify intron 8 using primers: 3' AACACAGCGGTGTGAGAAGTGC (exon 8) and 5' GTATCGGTAGTTCATTTCCTTTGGTTGC (intron 9). The reactions were cycled at 95°C for 2 minutes, 95°C for 30 seconds, 69°C for 30 seconds, 72°C for 30 seconds for 30 cycles in a PTC-200 thermal cycler (MJ Research). PCR products were purified and subjected to cycle-sequencing. Electropherograms for each sample were individually reviewed to detect polymorphic alleles. Subject DNA samples found to contain a polymorphism were amplified and sequenced at least once more to confirm the variant.

Expression and purification of intron 8-encoded peptide (Int8) and Herstatin

The intron 8 cDNA was cloned into the pET 30 bacterial expression vector (Novagen, Madison, WI), expressed in bacteria (BL-21), and purified by nickel affinity chromatography as described (Doherty, Bond et al. 1999; Shamieh, Evans et al. 2004). Monomers were enriched for using Amicon Ultra 30,000 molecular weight cut-off (MWCO) Centrifugal Filter Devices (Millipore). Sequencing revealed that previously used pET30aInt8 expression constructs contained a frame-shift mutation (Shamieh, Evans et al. 2004). This mutation was corrected using the Quik-Change Site-Directed Mutagenesis Kit (Stratagene) and the correction was confirmed through sequencing. For purification of Herstatin, S2 insect cells were transfected with 6xHis tagged-Herstatin in the pMT/BiP expression plasmid (Invitrogen, Carlsbad, CA), and induced in fresh media with cupric sulfate (100 μM) for ~16hrs. Herstatin was purified to ~90% purity by Ni-NTA (Qiagen, Valencia, CA) affinity chromatography as previously described (Jhabvala-Romero, Evans et al. 2003).

Secondary Structure Prediction:

Secondary structure prediction of the pET30a Intron8 wild-type, R→I, and R→C peptides was carried out using the GORIV secondary structure prediction program on the ExPASy Proteomics Server (http://au.expasy.org/).

Cell binding studies

Monolayer cultures of ~$2 \cdot 10^6$ cells were plated in 6-well tissue culture plates and incubated with different amounts of exogenous purified herstatin or int8 peptide for 2 hours at 4°C in serum-free DMEM. Cells were washed with Phosphate Buffered Saline (PBS) and extracted in MTG (50mM Tris·HCl (pH 7.0), 1.0% NP-40, 30mM Na_3VO_4, 1mM PMSF). Int8 peptide or herstatin bound to cells were quantified using a sandwich herstatin ELISA per manufacturer's instructions (Upstate Biotechnology, Lake Placid, NY).

The dissociation constant (K_d) and maximal binding (B_{max}) of herstatin or the int8 peptide were determined by nonlinear regression analysis of the plot of pmol of bound versus nM of herstatin or int8 peptide added. Statistical comparisons between herstatin and int8 binding to each receptor and comparisons between one or two-site binding models were performed by extra sums-of-squares F-test nonlinear regression coefficients. All tests were performed ($\alpha = 0.05$) using GraphPad Prism 4 software (GraphPad Software, 1994-2003).

Growth Assays

Cells were plated ($4 \cdot 10^4$) in quadruplicate in 24-well-plates, serum-deprived by incubation in serum-free DMEM for 24 hours, and treated with either 10nM Insulin (Sigma) or PBS as vehicle. Following serum-deprivation, for four subsequent days at 24 hour intervals, cell monolayers were washed with PBS and incubated for 30 minutes at 37°C with 30 µL of MTS reagent [3-(4,5-

dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl-2H-tetrazolium), inner salt] Aqueous One Solution (Promega) dissolved in 270 ml PBS per well. Absorbance readings were obtained at 490 nm in a Bio-Tek plate reader.

III. Results

The herstatin intron 8-encoded domain is an EGF receptor family RTK binding module

To determine whether herstatin may function as a pan-EGF receptor family inhibitor, we examined the binding specificity to all four family members. Cos-7 cells were transiently transfected with receptor and purified herstatin was added to the media. Bound herstatin was quantified using an ELISA specific to the intron 8-encoded C-terminal domain of herstatin. Two concentrations were chosen for the binding assay: 35 nM and 150 nM. The lower concentration is approximately two-fold higher than the dissociation constant ($K_d \approx 14$ nM) for herstatin binding to EGFR and HER-2, and is less than the amount required to achieve saturation (Shamieh, Evans et al. 2004). The 150 nM concentration of herstatin is approximately 10-fold higher than the K_d, and therefore is a saturating concentration. Differences in binding between the two concentrations should therefore be readily detectable, and changes in binding affinity to the different receptors may also be detected at the subsaturating concentration of 35 nM.

Herstatin binding to all four members of the EGF receptor family was above background, as determined by binding to mock-transfected cells. However, herstatin binding to cells expressing FGFR-3, a non-EGFR family RTK that has five immunoglobulin (IgG)-like domains in its ECD, was similar to that of mock-transfected cells. (Fig. 3.1). When subsaturating amounts of herstatin (35nM) were added to cells transfected with the EGF-receptor family, amounts bound were not significantly different, suggesting a similar affinity. Herstatin also

bound to cells transfected with ΔEGFR (EGFRvIII), a naturally occurring mutant of the EGF receptor that is missing subdomains I and II of the ECD (Fig. 3.1) (Nishikawa, Ji et al. 1994). This result suggests that subdomains I and II of the EGF receptor family are not required for herstatin binding.

Since the intron 8-encoded domain is thought to function as the receptor binding domain (RBD) of herstatin, we next examined its receptor binding specificity in the absence of subdomains I and II. Recombinant, monomeric intron 8-encoded RBD peptide (int8) was incubated with transiently transfected cells. Binding was quantified by the intron 8-specific ELISA. Concentrations of int8 were chosen based on previous data that determined the K_d of int8 binding to HER-2 to be approximately 70 nM (Doherty, Bond et al. 1999; Shamieh, Evans et al. 2004). The 150 nM int8 concentration is approximately 2 times the dissociation constant, while 450 nM is five-to-six-fold higher, which is in the range of saturation (Doherty, Bond et al. 1999; Shamieh, Evans et al. 2004). In agreement with what was seen with herstatin, int8 binding to EGFR, HER-2, HER-3, HER-4, and ΔEGFR, was significantly above background compared to mock-transfected cells (Fig. 3.2). Moreover, int8 bound similarly to FGFR-3 and mock-transfected cells (Fig. 3.2). At subsaturating levels (150 nM), int8 bound all four EGF family receptors, as well as to ΔEGFR to a similar extent. Taken together, this data suggests that int8 has the same receptor specificity, and can therefore function as a binding module for herstatin. Furthermore, these data indicate that the int8 domain, like full-length herstatin, binds within in subdomains III and IV of the receptor ECD.

Since int8 functions as an RBD for herstatin, the binding affinity of int8 to EGFR, HER-2, and ΔEGFR was assessed. Increasing amounts of recombinant, monomeric int8 peptide was incubated with transiently transfected Cos-7 cells. In agreement with the above study, int8 bound to EGFR, HER-2 and ΔEGFR in a specific and dose-dependent manner. Int8 bound to EGFR transfected cells (K_d = 181 ± 50 nM), to HER-2 transfected cells (K_d = 247 ± 31 nM) and to ΔEGFR transfected cells (K_d = 169 ± 35 nM) with binding affinities assessed by comparative nonlinear regression analysis. These dissociation constants do not significantly differ from each other (P = 0.3646). These data also suggest that int8 binds to EGFR and HER-2 with lower affinity than previously reported (Doherty, Bond et al. 1999). This difference is most likely due to more accurate quantitation of protein using an improved Bradford assay. Additionally, enrichment for monomers using differential filtration, rather than unfractionated int8 protein preparations is likely to result in more accurate assignment of binding affinities.

Mutations in intron 8 affect herstatin binding

Due to the importance of the intron 8-encoded domain as an RBD for herstatin, 1435 human genomic DNA samples were sequenced to look for genomic variations. The published intron 8 sequence, originally cloned from SKOV3 ovarian carcinoma cells (AF177761), was confirmed to be the most common sequence. However, two polymorphic changes in intron 8 were

identified. G1112T resulted in an Arg to Ile substitution at intron 8 residue 31, and C1069T resulted in an Arg to Cys substitution at intron 8 residue 17 (Fig. 3.4).

To examine the importance of these mutants in the context of full-length herstatin, binding studies to Cos7 cells transiently transfected with either EGFR or HER-2 were carried out and the dissociation constants were determined. $Hst_{Arg\ to\ Ile}$ (Hst R→I) bound to EGFR (K_d = 34.2 ± 9.4 nM) and to HER-2 (K_d = 36.2 ± 9.5 nM) with similar affinities (P = 0.8943) (Fig. 3.5 A and B). $Hst_{Arg\ to\ Cys}$ (Hst R→C) also bound to EGFR (K_d = 40.6 ± 10.1 nM) and to HER-2 (K_d = 60.2 ± 15.2 nM) (Fig 6 A and B). Dissociation constants for Hst R→I and Hst R→C mutants binding to EGFR and HER-2 differ from the values of wild-type herstatin binding to the same receptors (K_d = 16.3 ± 3.6 nM and K_d = 14.7 ± 1.8 nM, respectively) (P = 0.0003). This data suggests that the dissociation constants for the mutants binding to EGFR and HER-2 are 2 to 3 fold higher than the dissociation constants of wild-type herstatin binding (Shamieh, Evans et al. 2004).

Herstatin binding was saturable and maximal binding was assessed to extrapolate the binding stoichiometry of mutant herstatin to EGFR and HER-2. At saturation, 3.4 ± 0.4 pmol and 2.8 ± 0.3 pmol of Hst R→I was bound to EGFR and HER-2 respectively (Fig. 3.5 A and B). Similarly, maximal binding at saturation of Hst R→C to EGFR and HER-2 was 2.7 ± 0.2 pmol and 2.6 ± 0.2 pmol (Fig. 3.6 A and B). Since this is a transiently transfected cell system, the number of receptors expressed upon the cell surface is unknown. However, saturation was achieved and maximal binding of the herstatin mutants is similar

to the published values for wild-type herstatin binding to the same transfected cells (EGFR B_{max} = 2.6 ± 0.1 pmol) (Shamieh, Evans et al. 2004). Extrapolation from binding studies of wild-type herstatin to epidermoid carcinoma cells that overexpress EGFR, and in which the number of receptors is known, suggests a binding stoichiometry of one herstatin molecule to one receptor. Taken together, this data suggests that mutations in the intron-encoded domain of herstatin may lower the binding affinity, but do not alter binding stoichiometry.

The binding of Hst R→I to HER-2 is in disagreement with previous studies that reported no significant binding of int8 R→I to HER-2 over-expressing cells (Shamieh, Evans et al. 2004). However, sequencing revealed that the pET30aInt8R→I expression vector contained a frame-shift mutation. This mutation resulted in a protein, unrelated to the int8 peptide, which therefore did not bind to HER-2.

Herstatin binds to the insulin receptor

Previous studies showed that both herstatin and its intron-encoded domain bind to the IGF-IR (Shamieh, Evans et al. 2004). This study now examines the interaction of herstatin with another member of the IGF-receptor family, the insulin receptor (IR). Herstatin bound to IR over-expressing 3T3 (IRa-3T3) cells with a K_d = 130 ± 16 nM, which is similar to the value of herstatin binding to the IGF-I receptor (P = 0.6719) (Fig. 3.7) (Shamieh, Evans et al. 2004). Furthermore, at saturation, 6.9 ± 0.2 pmol were bound, indicating ≈ $1 \cdot 10^6$ binding sites/cell. This number matches the number of IR receptors on the cell

surface of IRa-3T3 cells: $1 \cdot 10^6$ (Faria, Blakesley et al. 1994). However, since the IR is a disulphide-bonded dimer, this suggests a binding stoichiometry of one herstatin molecule to two insulin receptor monomers, which differs from the stoichiometry of one herstatin molecule binding one EGF receptor monomer (Shamieh, Evans et al. 2004).

Herstatin expression reduces growth of insulin-stimulated MCF7 cells

Herstatin expression has been shown previously to retard the growth of cells expressing EGFR and HER-2 (Doherty, Bond et al. 1999; Azios, Romero et al. 2001; Justman and Clinton 2002; Jhabvala-Romero, Evans et al. 2003). Since herstatin binds to the insulin receptor, we examined whether herstatin expression would affect insulin-mediated growth of MCF7 breast carcinoma cells. Previous studies have shown that stable expression of herstatin in MCF7 breast carcinoma cells resulted in diminished heregulin-stimulated proliferation of cells (Jhabvala-Romero, Evans et al. 2003). Similarly, we examined the growth of parental MCF7 cells and two clones stably transfected with herstatin, MCF7/Hst#1 and MCF7/Hst#2. The MCF7Hst#1 clone expresses high levels of herstatin, while clone #2 expresses lower amounts (Jhabvala-Romero, Evans et al. 2003). Cells were serum-starved for 24 hours and treated with saturating concentrations (10 nM) of insulin or vehicle for three or four days. Growth was measured using the MTS assay at 24-hour intervals. Parental MCF7 cells grew in response to the insulin stimulus, but failed to survive in the absence of hormone over a period of three or four days (Fig. 3.8). Both MCF7/Hst clones exhibited a

similar reduction in insulin-stimulated growth, suggesting that maximal inhibition was achieved (Fig. 3.8). However, the growth reduction occurred faster in clone #1, which expresses more herstatin, implying that herstatin may affect insulin-mediated growth in a concentration dependent manner.

IV. Discussion

Previous studies have proposed that the intron 8-encoded domain of herstatin is a binding module for the inhibitor and is required for herstatin binding (Doherty, Bond et al. 1999; Shamieh, Evans et al. 2004). Here we present further evidence for the receptor binding specificity for herstatin and int8. We show that both herstatin and int8 bind to all four members of the EGF receptor family. However, neither herstatin nor int8 bind to FGFR-3, a non-EGFR RTK that contains five IgG-like repeats in its ECD.

Herstatin has previously been shown to bind to EGFR and HER-2 and is also known to inhibit these receptors by disrupting receptor dimers (Doherty, Bond et al. 1999; Azios, Romero et al. 2001; Shamieh, Evans et al. 2004). Since the four EGFR family members share a similar ectodomain topology, we now hypothesize that herstatin may also function as an inhibitor of HER-3 and HER-4 by binding to the receptors and inhibiting receptor hetero- and homo-dimers. Previous studies have shown that herstatin inhibits heregulin-stimulated HER-2 transphosphorylation of HER-3 (Azios, Romero et al. 2001). This inhibition was thought to be mediated by herstatin binding to HER-2. However, our results suggest that the inhibition may also be mediated by herstatin binding to HER-3; this co-inhibition is dependent on herstatin binding with similar affinity HER-3. While our results (Fig. 3.1) suggest that herstatin may bind to HER-2 and HER-3 with a similar affinity, a determination of the dissociation constant of herstatin binding to HER-3 is needed to confirm this.

Examination of the binding constants of int8 to Cos-7 cells transiently transfected with EGFR or HER-2 revealed that the binding affinity of int8 for these receptors is approximately 10-fold less than that of herstatin. This data, as well as previous studies, suggest that intron-encoded domain is the binding module and subdomains I and II strengthen this interaction with the EGF receptor family. Additionally, the dissociation constant of int8 binding to ΔEGFR, is similar to that of EGFR and HER-2. This data suggests that int8 alone is a receptor binding module that binds in either subdomain III or IV of the receptor ECD.

We also report that two non-lethal point mutations in the intron-encoded domain, Hst R→I and Hst R→C lower the binding affinity of herstatin to EGFR and HER-2 by approximately two to three fold. Though these changes in the dissociation constant are statistically significant, such a small increase in dissociation constant is not physiologically relevant. The R→I mutation results in an aliphatic amino acid side chain of neutral charge substituted for a bulky, positively charged amino acid side chain. This change occurs in a proposed hydrophobic region in the middle of the intron-encoded domain, where there may possibly be a salt bridge between Arg31 and Asp35. GORIV secondary-structure prediction analysis predicts a short helix immediately following Asp35 in the mutant int8 that is not found in wild-type, suggesting an effect of the mutation on the secondary structure of int8 (Shamieh and Clinton, unpublished observations). However, since herstatin R→I reacts with similar affinity as wild-type on a sandwich ELISA containing two monoclonal antibodies (data not shown), we

hypothesize that this alteration of structure, if it occurs, is localized, rather than global.

In contrast, the R→C mutation results in the substitution of an amino acid that contains a lone pair of electrons for a positively charged arginine. While the intron-encoded domain does not contain any other cysteines, the N-terminal subdomains of herstatin contain 26 cysteines. Introduction of a lone, unpaired cysteine may cause misfolding of the protein through non-native cysteine-pairing, or protein aggregation though intermolecular disulphide bonds. However, we discount both these possibilities as herstatin R→C, reacts equally well on a sandwich ELISA, as wild-type or R→I, suggesting that interactions with two monoclonal antibodies in the ELISA is unaltered (Shamieh and Clinton, data not shown). Additionally, the monomer ratio to whole protein of wild-type, R→I, and R→C herstatin are similar, suggesting that intermolecular disulphide pairing is not occurring (Shamieh and Clinton, unpublished observations). Taken together, this data suggests that the R→I and R→C mutations do not have global effects on the folding of the intron-encoded domain, but may have localized effects that alter the binding of herstatin.

Herstatin has previously been shown to bind to the IGF-IR ($K_d \approx 150$ nM), which shares approximately 25% sequence identity in subdomains I, II, and III of its ECD with the corresponding subdomains of the EGF receptor (K_d herstatin = 14 nM) (Ogiso, Ishitani et al. 2002; Shamieh, Evans et al. 2004). Here, we show that herstatin also binds with similar affinity to the IR ($K_d = 130 \pm 16$ nM). The IR, which is a member of the IGF-receptor family, shares 58% sequence identity in

subdomains I, II, and III of its ECD with the corresponding domains of the IGF-IR (Garrett, McKern et al. 1998). This high homology amongst the receptor ECDs, as well as herstatin binding to receptors from both families, suggests that there may be a motif, common to both the EGF- and IGF1- receptor families, that is the consensus-binding motif for the intron-encoded RBD of herstatin.

The stoichiometry of herstatin binding to the IR is 1 to 2, which is in contrast to that of EGFR (1 to 1) (Shamieh, Evans et al. 2004). Herstatin binds to and disrupts EGFR and HER-2 dimers, so that one herstatin monomer binds to one receptor monomer (Doherty, Bond et al. 1999; Azios, Romero et al. 2001). However, the IR is a disulphide-bonded dimer, which cannot be split by protein-protein interactions. Taken together, this data suggests that only one herstatin monomer may fit in the binding space allotted by a receptor dimer.

Growth studies in MCF7 breast carcinoma cells reveal that expression of herstatin reduces insulin-induced growth of these cells. Recent evidence points to crosstalk between the EGF and IGF receptor families, mediated primarily through the EGF and IGF-I receptors (Swantek and Baserga 1999; Roudabush, Pierce et al. 2000; Jones, Goddard et al. 2004; Camp, Summy et al. 2005). Although herstatin binds directly to the IR, these results may also be explained by the effect of herstatin on EGF and IGF-I receptor family crosstalk. MCF7 cells have been shown previously to express HER-2, HER-3, and HER-4 (Jhabvala-Romero, Evans et al. 2003). Herstatin expression in these cells abrogates HER-3 and HER-4 receptor expression (Jhabvala-Romero, Evans et al. 2003). Additionally, herstatin binds with higher affinity to the EGF receptor family than to

the IR (Shamieh, Evans et al. 2004). This growth study does not distinguish between herstatin directly inhibiting the insulin receptor and herstatin affecting the insulin receptor through crosstalk with the EGF receptor family. More studies are needed to further elucidate the mechanism of this inhibition.

We therefore suggest a binding model for herstatin, in which the intron-encoded RBD of herstatin directs binding to either subdomain III or IV of the full-length RTKs. This binding may then juxtapose subdomains I and II of herstatin with subdomains I and II of its cognate receptor, and therefore stabilize RBD through interactions similar to receptor dimerization. Subdomain II of these receptors contains a dimerization arm that is also present in herstatin (Garrett, McKern et al. 2002; Ogiso, Ishitani et al. 2002; Cho, Mason et al. 2003; Garrett, McKern et al. 2003). This dimerization arm, which is not present in the IGF1 receptor family, may explain why the affinity of herstatin binding to IGF-IR and IR is similar to that of int8, but lower than that of herstatin, binding to the EGF receptor family (Garrett, McKern et al. 1998; Shamieh, Evans et al. 2004). Despite this lower binding affinity, herstatin is still the only known mammalian ligand that binds to two receptor families.

Figure 3.1. Binding of herstatin to different receptors expressed in transfected cells. Transfected Cos-7 cells were incubated with purified herstatin for 30 minutes at 37°C in serum-free media. Cells were washed, extracted, and analyzed by herstatin ELISA.

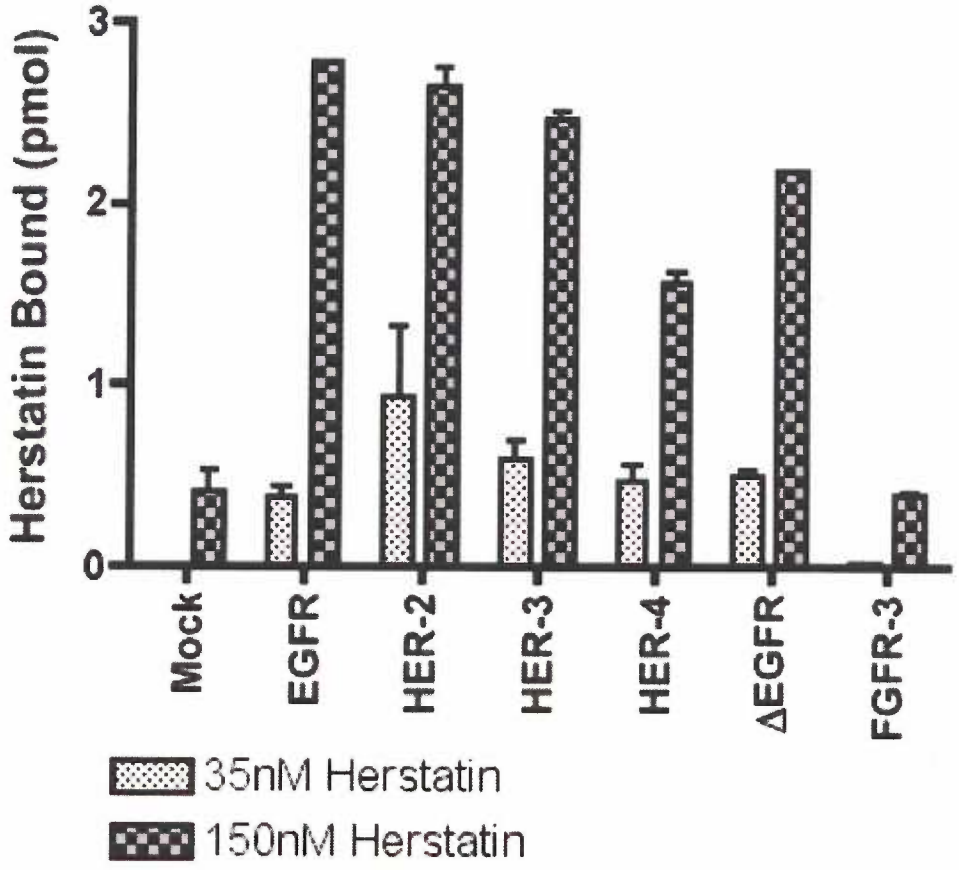
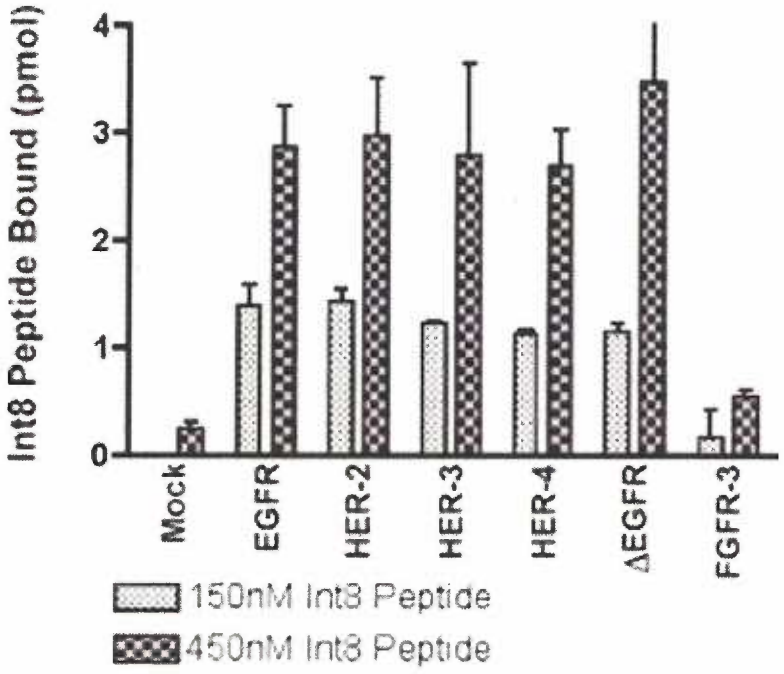

Herstatin Bound (pmol)
3
2
1
0
Mock
EGFR
HER-2
HER-3
HER-4
ΔEGFR
FGFR-3
35nM Herstatin
150nM Herstatin

Figure 3.2. Binding of intron 8 peptide to different receptors expressed in transfected cells. (A) Transfected Cos-7 cells were incubated with purified int8 peptide for 30 minutes at 37°C in serum-free media. Cells were washed, extracted, and analyzed by herstatin ELISA. **(B)** Western blot analysis of receptor expression in mock- and receptor-transfected Cos-7 cells.

A.


Int8 Peptide Bound (pmol)
0
1
2
3
4
Mock
EGFR
HER-2
HER-3
HER-4
ΔEGFR
FGFR-3
150nM Int8 Peptide
450nM Int8 Peptide

B.

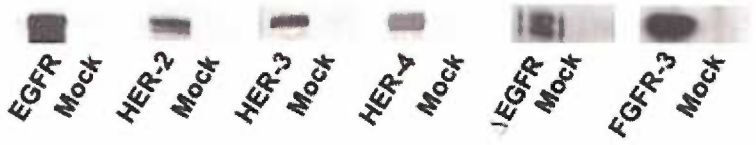

EGFR
Mock
HER-2
Mock
HER-3
Mock
HER-4
Mock
EGFR
Mock
FGFR-3
Mock

Figure 3.3. Saturation binding curves of intron 8 peptide to cells transfected with EGFR, HER-2, and ΔEGFR. Different amounts of purified int8 peptide were added to the indicated cells. Bound peptide was quantified by herstatin ELISA. Nonlinear regression analysis was used to determine the dissociation constants (K_d) and maximal bound (B_{max}). Parental Cos-7 and transiently transfected **(A)** EGFR, **(B)** HER-2, or **(C)** ΔEGFR Cos7 cells were used.

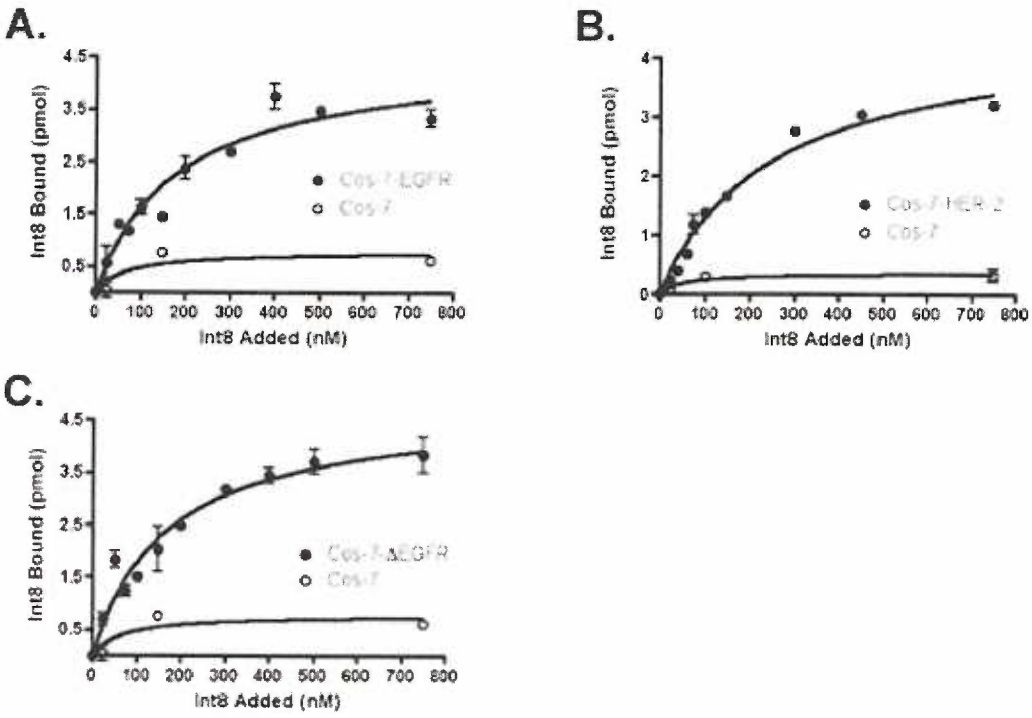

A.
Int8 Bound (pmol)
Int8 Added (nM)
B.
Int8 Bound (pmol)
Int8 Added (nM)
C.
Int8 Bound (pmol)
Int8 Added (nM)

Figure 3.4. The DNA and deduced amino acid sequence of HER-2 intron 8. The sequence is the most common human intron 8 sequence. Base substitutions and non-conserved residues, encoded by polymorphisms, are shown.

```
     1 G  T  H  S  L  P  P  R  P  A  A  V  P  V  P  L  R  M  Q 19
Int8: 1ggt acc cac tca ctg ccc ccg agg cca gct gca gtt cct gtc cct ctg cgc atg cag 57
                                                                  t
                                                                  C

20 P  G  P  A  H  P  V  L  S  F  L  R  P  S  W  D  L  V  S  A  39
58 cct ggc cca gcc cac cct gtc cta tcc ttc ctc aga ccc tct tgg gac cta gtc tct gcc 117
                                           t
                                           I

40 F  Y  S  L  P  L  A  P  L  S  P  T  S  V  P  I  S  P  V  S  59
118 ttc tac tct cta ccc ctg gcc ccc ctc agc cct aca agt gtc cct ata tcc cct gtc agt 177

60 V  G  R  G  P  D  P  D  A  H  V  A  V  D  L  S  R  Y  E  G STOP
178 gtg ggg agg ggc ccg gac cct gat gct cat gtg gct gtt gac ctg tcc cgg tat gaa ggc tga
```

Figure 3.5. Saturation binding curves of mutant herstatin (R→I) binding to cells transfected with EGFR and HER-2. Herstatin (R→I) purified from S2 insect cells was incubated with serum-starved parental Cos-7 cells and with Cos-7 cells expressing **(A)** EGFR, or **(B)** HER-2.

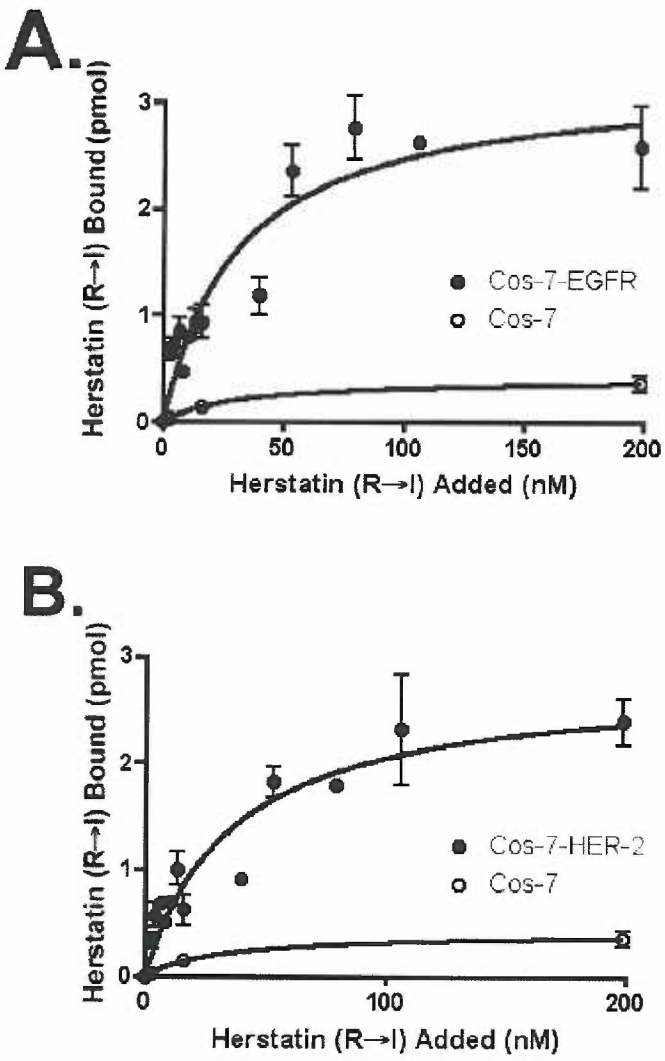

A.
Herstatin (R→I) Bound (pmol)
3
2
1
0
0
50
100
150
200
Herstatin (R→I) Added (nM)
Cos-7-EGFR
Cos-7
B.
Herstatin (R→I) Bound (pmol)
3
2
1
0
0
100
200
Herstatin (R→I) Added (nM)
Cos-7-HER-2
Cos-7

Figure 3.6. Saturation binding curves of mutant herstatin (R→C) binding to cells transfected with EGFR and HER-2. Herstatin (R→C) purified from S2 insect cells was incubated with serum-starved parental Cos-7 cells and with Cos-7 cells expressing **(A)** EGFR, or **(B)** HER-2.

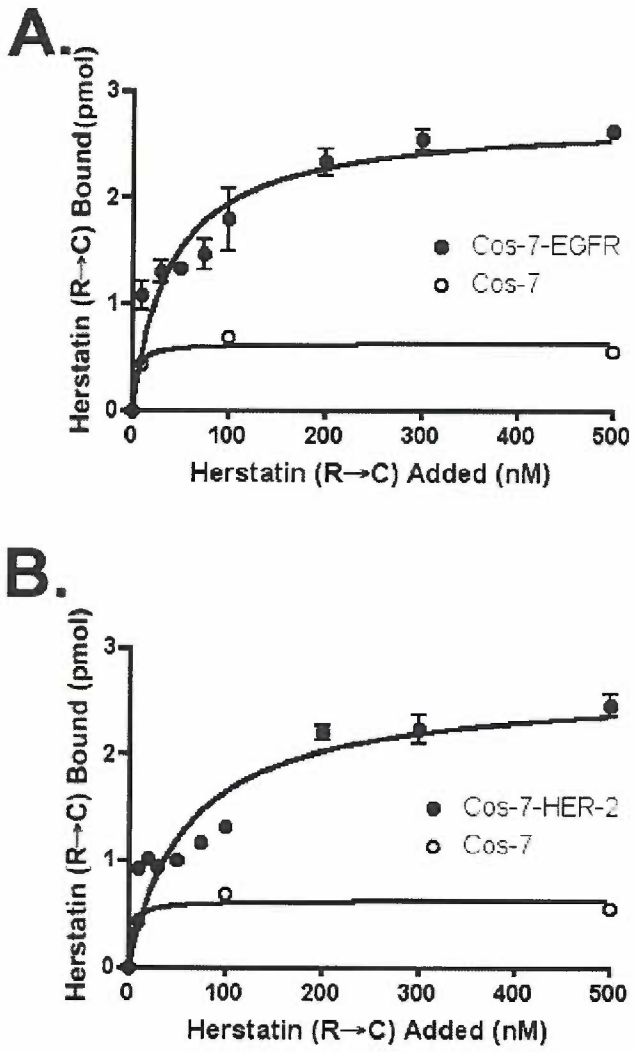

A.
Herstatin (R→C) Bound (pmol)
3
2
1
0
0
100
200
300
400
500
Herstatin (R→C) Added (nM)
Cos-7-EGFR
Cos-7
B.
Herstatin (R→C) Bound (pmol)
3
2
1
0
0
100
200
300
400
500
Herstatin (R→C) Added (nM)
Cos-7-HER-2
Cos-7

Figure 3.7. Saturation binding curve of herstatin binding to cells expressing the insulin receptor. Wild-type herstatin purified from S2 insect cells was incubated with serum-starved parental NIH-3T3 cells and 3T3 cells stably transfected with the insulin receptor (IRA-3T3).

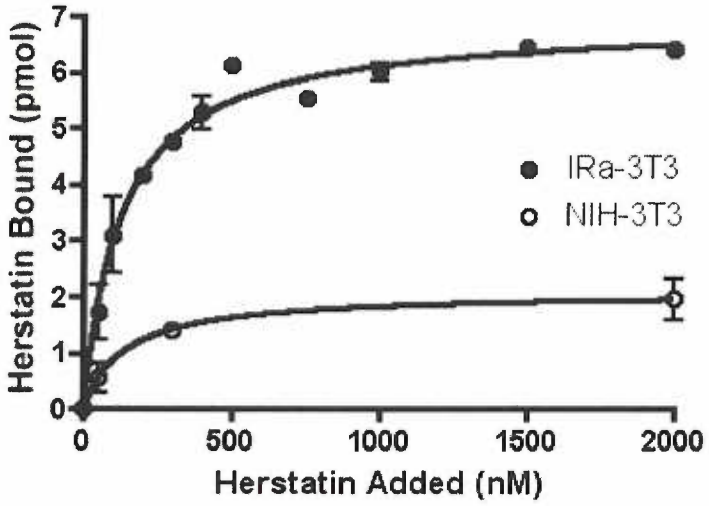
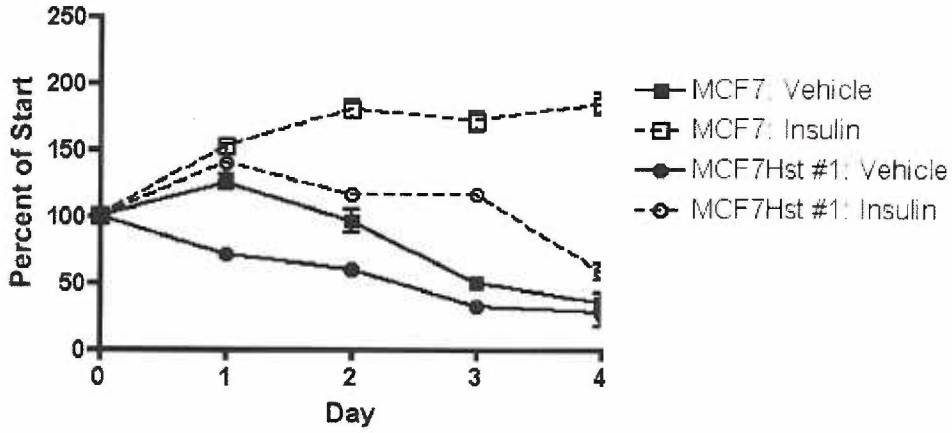
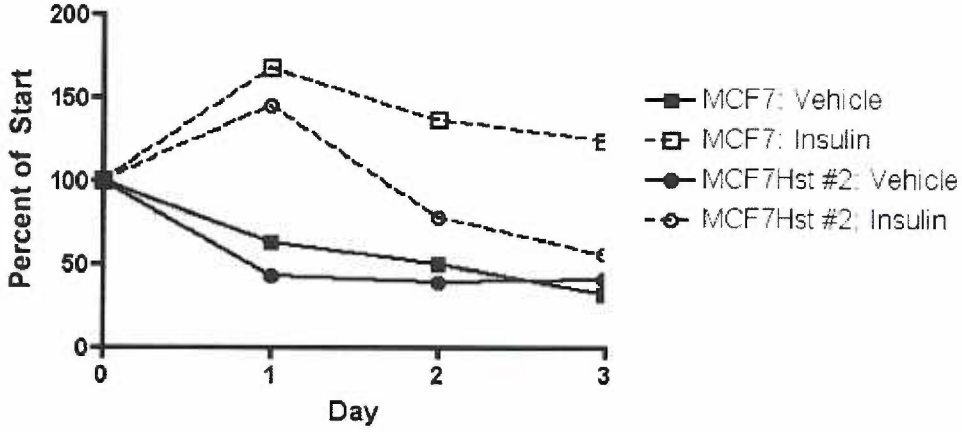

Herstatin Bound (pmol)
0
1
2
3
4
5
6
7
0
500
1000
1500
2000
Herstatin Added (nM)
IRa-3T3
NIH-3T3

Figure 3.8. Insulin-induced proliferation of breast carcinoma cells. Growth of parental MCF7 breast carcinoma cells and MCF7 cells stably transfected with herstatin, **(A)** low hst-expressing clone, and **(B)** high hst-expressing clone, was determined by the MTS assay as described in Experimental Procedures. Cells were serum-starved for 24 hours and then treated with 10nM insulin or vehicle, and growth was assessed at the indicated days.

A.


250
200
150
100
50
0
Percent of Start
0
1
2
3
4
Day
MCF7: Vehicle
MCF7: Insulin
MCF7Hst #1: Vehicle
MCF7Hst #1: Insulin

B.


200
150
100
50
0
Percent of Start
0
1
2
3
Day
MCF7: Vehicle
MCF7: Insulin
MCF7Hst #2: Vehicle
MCF7Hst #2: Insulin

CHAPTER 4

Modulation of Insulin-Like Growth Factor Signaling By Herstatin, an Alternative HER-2 (ErbB-2) Product

A manuscript in preparation

by

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ABSTRACT

Herstatin, a product of alternative splicing of the HER-2 gene, consists of subdomains I and II of the ectodomain of the HER-2 receptor tyrosine kinase, followed by a 79-amino acid C-terminal domain encoded by intron 8. Previous studies have shown that herstatin binds to the ectodomain of multiple members of the EGF receptor (EGFR) family, and that binding to EGFR and HER-2 ($K_d \approx 15$ nM) blocks receptor dimerization and ligand activation. Herstatin was recently found to also bind to the IGF-I receptor (IGF-IR) ($K_d \approx 150$ nM), which exhibits signaling crosstalk and contains regions of high homology with the ectodomain of the EGFR family (Adams, McKern et al. 2004). We, therefore, investigated the impact of herstatin expression on IGF-I signaling and proliferation in parental and herstatin-transfected MCF7 breast cancer cells. IGF-IR levels, as well as IGF-I-mediated IGF-IR tyrosine phosphorylation, were reduced several-fold in two different clonal isolates of herstatin-expressing cells. Down-regulation did not appear to be caused by herstatin-mediated inhibition of the EGFR, since treatment of parental MCF7 cells with an EGFR-specific inhibitor, AG1478, for up to 24 hours did not reduce IGF-IR levels. Examination of the impact of herstatin on IGF-I-specific signaling revealed strong inhibition of tyrosine phosphorylation of IRS-1, while IRS-2 activation was enhanced. Although IGF-IR tyrosine phosphorylation was strongly reduced, herstatin expression did not inhibit, but stimulated, IGF-I-mediated ERK activation, while IGF-I activation of the PI3K-Akt/PKB pathway was inhibited. Altered IGF-IR signaling culminated in loss of IGF-I-mediated cell growth and survival in herstatin-expressing clonal cell lines. These studies demonstrate that herstatin profoundly modulates IGF-I-stimulated signaling and proliferation in MCF7 breast cancer cells, either through direct interaction with the IGF-IR or indirectly by modulating crosstalk with the EGFR family.

I. INTRODUCTION

Receptor tyrosine kinases (RTKs), including the epidermal growth factor receptor (EGFR) and the insulin-like growth factor-I receptor (IGF-IR) families, play key signaling roles in fundamental cellular processes. The EGFR family, which includes the EGFR (HER-1/ErbB1), human epidermal growth factor receptor-2 (HER-2/*neu*/ErbB2), HER-3/ErbB3, and HER-4/ErbB4, has been shown to mediate key cellular processes such as growth and differentiation (Burden and Yarden 1997; Olayioye, Neve et al. 2000; Sweeney and Carraway 2000). The IGF-IR family, which includes the IGF-IR, the insulin receptor, and the insulin receptor-related receptor, has also been shown to participate in an overlapping array of biological processes (Sell, Dumenil et al. 1994; Baserga 1997; Baserga, Resnicoff et al. 1997; O'Connor, Kauffmann-Zeh et al. 1997; Morrione, Romano et al. 2000; Holzenberger, Dupont et al. 2003; Kuribayashi, Kataoka et al. 2004). While the expression and biological effects of these receptor families are essential for normal growth and development, aberrant expression leads to a variety of human cancers (Blume-Jensen and Hunter 2001; Holbro, Civenni et al. 2003; Pollak, Schernhammer et al. 2004; Rochester, Riedemann et al. 2005).

The four members of the EGFR family each contain an extracellular ligand binding domain, a single transmembrane domain, and a cytoplasmic tyrosine kinase domain (Hanks, Quinn et al. 1988; Schlessinger, Ullrich et al. 1988; Yarden and Ullrich 1988). Eleven growth factor ligands, each containing an EGF core domain, bind with high affinity to these receptors, except HER-2, causing the formation of receptor homo- or heterodimers. This dimerization results in receptor activation and autophosphorylation *in trans* of specific C-terminal tyrosine residues (Schlessinger, Ullrich et al. 1988; Dougall, Qian et al. 1994; Riese and Stern 1998; Tzahar and Yarden 1998; Hynes 2000; Sweeney and Carraway

2000). Phosphorylation of these residues enables the recruitment and tyrosine phosphorylation of SH2-domain-containing signaling molecules, leading to the initiation of two major intracellular signaling pathways: the (generally) anti-apoptotic PI3K-Akt/PKB and mitogenic ERK cascades (Rommel, Clarke et al. 1999; Busse, Doughty et al. 2000; Blume-Jensen and Hunter 2001).

The IGF-IR, in contrast to other RTKs, consists of a pre-formed ($\alpha_2ß_2$), disulphide-linked, heterotetramer (Massague and Czech 1982; Ward, Garrett et al. 2001). Ligand binding is thought to lead to a conformational change in the ß subunits and to activation by autophosphorylation of tyrosine residues in the catalytic domain. The subsequent phosphorylation of additional tyrosines, particularly in the juxta-membrane domain of the ß subunit, provides docking sites for PTB and SH2-domain-containing scaffolding/adapter proteins, including the insulin receptor substrates IRS-1 and IRS-2. These adaptor proteins then activate signaling pathways such as the PI3K and ERK cascades that are also activated by the EGFR family (O'Connor 2003).

By virtue of their activation of the PI3K and ERK cascades and potentially other signal transduction pathways, both the EGFR and IGF-IR families are major regulators of cell growth and survival, and dysregulation of either receptor family can lead to uncontrolled growth and tumorigenesis. Recent evidence suggests that there is crosstalk between these RTKs, which may allow coordinated control of cellular responses in normal and tumor cells (reviewed in (Adams, McKern et al. 2004)). Bidirectional crosstalk and coordination of signal transduction between the IGF-IR and EGFR families has been documented (reviewed in (Adams, McKern et al. 2004)). Sustained activation of a mitogenic ERK signal by the EGFR is heavily dependent on a functional IGF-IR (Swantek and Baserga 1999). Recently, the converse has also shown to be true, in which activation of ERK by IGF-IR

requires a functional EGFR (Gilmore, Valentijn et al. 2002; Ahmad, Farnie et al. 2004; Kuribayashi, Kataoka et al. 2004). Additionally, it has been shown in several cell types that IGF-I stimulation of the IGF-IR leads to activation of the EGFR and, coordinately, the ERK pathway, through proteolytic activation and autocrine release of HB-EGF (Roudabush, Pierce et al. 2000; Gilmore, Valentijn et al. 2002; El-Shewy, Kelly et al. 2004). IGF-I-induced coordinate activation of ERK through EGFR and IGF-IR is in contrast to IGF-I-induced activation of Akt, which is unaffected by EGFR-specific inhibitors (Roudabush, Pierce et al. 2000; Gilmore, Valentijn et al. 2002). These data suggest that crosstalk between the EGFR and IGF-IR coordinately controls activation of the ERK signaling pathway, but not the PI3K-Akt/PKB pathway. In addition to coordination of signal transduction, Ahmed et al. have recently reported that the EGFR co-immunoprecipitates with the IGF-IR in mammary epithelial cells, and that phosphorylation of the complexed EGFR is enhanced by treatment with IGF-I (Ahmad, Farnie et al. 2004). More studies, however, are needed to fully elucidate the complex interplay of these receptors.

Because of the important role of the EGFR family in malignant growth, there has been extensive effort directed toward the development and characterization of inhibitors that target these receptors. Effective tumor inhibition has been achieved clinically with inhibitors that antagonize the EGFR and HER-2 (Ross, Schenkein et al. 2004; Agus, Gordon et al. 2005). Several findings support the concept that redundant signaling through IGF-IR maintains the activation of critical pathways for survival in the presence of EGFR family inhibitors. *In vitro*, IGF-IR signaling in MCF7/HER-2 and SKBR-3 breast carcinoma cells protects against inhibition by Herceptin, a therapeutic monoclonal antibody to HER-2 (Lu, Zi et al. 2001). The inhibitory effects of AG1478, an EGFR tyrosine kinase inhibitor, can also be overcome in glioblastoma multiforme cells by overexpression and increased signaling through the IGF-IR (Chakravarti, Loeffler et al. 2002). Most recently, it has been shown in

breast and prostate cancer cell lines that acquired resistance to Iressa, an EGFR small molecule inhibitor, occurs through increased activation and signaling of the IGF-IR (Jones, Goddard et al. 2004; Camp, Summy et al. 2005).

While the EGFR family has long been an anti-cancer therapeutic target, recent attempts have also been made at targeting the IGF-IR family. Successful inhibition of tumor growth with two IGF-IR small-molecule inhibitors has been documented with solid tumor xenografts and leukemic malignancies (Garcia-Echeverria, Pearson et al. 2004; Mitsiades, Mitsiades et al. 2004). A number of specific anti-IGF-IR antibodies have been recently developed that have shown efficacy in inhibition of IGF-stimulated proliferation and tumorigenesis (Burtrum, Zhu et al. 2003; Maloney, McLaughlin et al. 2003; Sachdev, Li et al. 2003). Additionally, *in vitro* combinatorial therapy, using Herceptin to block HER-2, and a dominant-negative form of the IGF-IR in breast carcinoma cells, revealed synergy between the two treatments and led to increased growth inhibition (Camirand, Lu et al. 2002). Similarly, simultaneous use of anti-EGFR and IGF-IR antibodies was shown to exhibit increased growth inhibition compared to either antibody alone (Camirand, Lu et al. 2002). Recently, a bivalent monoclonal antibody to the EGFR and IGF-IR has been described (Lu, Zhang et al. 2004; Lu, Zhang et al. 2005). These findings all point to the utility of multi-functional inhibitors that simultaneously target both the EGFR and IGF-IR families.

This current study investigates the impact of a cellular pan-EGFR family inhibitor, herstatin, on IGF-I signaling. Herstatin, the product of alternative splicing of the HER-2 gene transcript, consists of the N-terminal portion of the HER-2 receptor ectodomain, followed by a novel 79-amino acid C-terminal domain (Doherty, Bond et al. 1999). Herstatin is unique in that it binds with nM affinity to all members of the EGFR family (Shamieh, Evans et al. 2004). Herstatin binding to the ectodomain of the EGFR and HER-2 receptors

has been shown to block receptor activation (Doherty, Bond et al. 1999; Azios, Romero et al. 2001; Justman and Clinton 2002; Jhabvala-Romero, Evans et al. 2003). We have recently demonstrated that herstatin also binds, but with reduced affinity, to the IGF-IR compared to the EGFR (K_d≈150 nM vs 15 nM) (Shamieh, Evans et al. 2004), presumably to a site in the ectodomain that has homology with the EGFR (Garrett, McKern et al. 2002).

In this study, we determine the effects of herstatin, which blocks multiple combinations of the EGFR family, on IGF-I signaling in MCF7 mammary carcinoma cell lines. We also investigate the expression and activation of IGF-IR-specific signaling proteins and IGF-I-mediated proliferation. The results of these studies demonstrate that herstatin, an alternative HER-2 gene product, provides a novel mechanism of cross-regulation between the EGFR and IGF-IR families.

II. MATERIALS AND METHODS

Cell culture

MCF7 breast carcinoma cells were obtained from the American Type Culture Collection and maintained at 37°C/5% CO_2 in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS) and gentamicin (0.25 µg/ml). Media and supplements were purchased from Gibco BRL-Life Technologies (Grand Island, NY). Herstatin-expressing MCF7 clones, previously characterized (Jhabvala-Romero, Evans et al. 2003), were maintained under the same conditions as parental MCF7 cells in media supplemented with 0.5 mg/ml G418 sulfate.

Antibodies

All primary antibodies were used at a 1:1000 dilution and incubated with Western blots overnight at 4°C, unless otherwise indicated. Polyclonal antibodies [IGF-IRb and IRS-1 (N-terminus)] and monoclonal antibody PY20 were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Monoclonal ERK 1/2 and polyclonal pERK 1/2, Akt/PKB, IRS-1 antibodies were purchased from Cell Signaling Technologies (Boston, MA). Monoclonal herstatin and polyclonal IRS-2 antibodies were obtained from Upstate Biotechnology (Lake Placid, NY). Polyclonal pAkt/PKB was purchased from Biosource International (Hopkinton, MA).

Western immunoblotting and immunoprecipitation

Cells were grown to ~80% confluency, serum-starved overnight in DMEM, and treated with 14 nM EGF or 5 nM IGF-I for the times indicated. For Western blots, cells were

washed twice with ice-cold PBS and lysed in SDS sample buffer (Sambrook, Maniatis et al. 1989) without reducing agent and boiled for 5 min. After clarification by centrifugation at 13,000 rpm for 5 min., supernatant was collected and protein concentration was determined using a detergent-compatible protein assay kit (Bio-Rad; Hercules, CA). Dithiothreitol (100 mM) and bromophenol blue (0.1% (w/v)) were then added and samples were boiled again for 5 min. Twenty-mg aliquots of protein were analyzed by 10% SDS-PAGE and electrotransferred onto nitrocellulose (Amersham Pharmacia Biotech; Piscataway, NJ). Blots were probed with a phospho-specific antibody, stripped in 5x stripping buffer (Sambrook, Maniatis et al. 1989) and reprobed with the respective pan antibody. For immunoprecipitation, cells were washed twice with ice-cold PBS, lysed in NP-40 buffer [1% NP-40, 150 mM NaCl, 10% glycerol, 20 mM Tris-HCl (pH 8.0), 1 mM EDTA (pH 8.0), 0.2% SDS], containing protease inhibitors (Roche Diagnostics; Indianapolis, IN), 1 mM $NaVO_4$, and 1 mg/ml pepstatin. Lysates were cleared and protein concentration was determined as above. For IGF-IR, 1 mg of whole-cell lysate protein was immunoprecipitated with 10 µg of anti-IGF-IR antibody and incubated overnight at 4°C while rocking. For IRS-1 and IRS-2, 500 µg of whole-cell lysate protein was incubated overnight with 5 or 10 µg antibody, respectively. 100 µl of protein A-agarose bead slurry (Amersham Pharmacia Biotech) was added for 2 hours rocking at 4°C. Three washes were performed, and the pellet was boiled in 2x SDS sample buffer (Sambrook, Maniatis et al. 1989). The beads were spun down and the supernatant loaded onto a 10% (IGF-IR) or 7% (IRS-1/2) SDS-PAGE and immunoblotted as above. Blots were probed with PY20, stripped, and reprobed with their respective antibodies. Binding of primary antibodies was detected by enhanced chemiluminescence (Amersham), and film exposures were quantified using a scanning densitometer (Bio-Rad).

Growth assays

Cells ($4x10^4$) were plated in quadruplicate in 24-well plates, incubated in serum-free DMEM for 24 hours, and treated with either 5 nM IGF-I (GroPep; Adelaide, Australia) or an equivalent volume of vehicle (10 mM HCl). At the indicated time-points, cell monolayers were washed with PBS and incubated for 30 minutes at 37°C with 30 µl of MTS reagent [3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl-2H-tetrazolium) inner salt Aqueous One Solution (Promega; Madison, WI) dissolved in 270 ml PBS] per well. Absorbance readings were obtained at 490 nm in a Bio-Tek plate reader.

EGFR inhibitor studies

Control MCF7 cells were serum-starved overnight and treated with the EGFR kinase inhibitor AG1478 (Sigma) or vehicle (DMSO) for 5 min. prior to the addition of 14 nM EGF or 5 nM IGF-I. After 5 min. of growth factor treatment, cell lysates were prepared and analyzed for ERK and Akt/PKB activation as described above.

III. RESULTS

Effect of herstatin on IGF-IR expression and activation

To evaluate the effects of herstatin expression on activation of the IGF-IR by IGF-I, we examined tyrosine phosphorylation of IGF-IR immunoprecipitated from IGF-I-treated parental and herstatin-expressing cells. In parental MCF7 cells, IGF-I robustly stimulated IGF-IR tyrosine phosphorylation, which represents the initial autophosphorylation stage of IGF-IR activation. In herstatin-expressing cells, however, there was only a small increase in IGF-IR phosphorylation, which corresponds to an approximately 8-fold reduction in activation (Fig. 4.1). This decreased activation reflects, in part, a decrease in IGF-IR expression consistently seen in herstatin-expressing cells (see Fig. 4.5), as well as diminished tyrosine phosphorylation (Fig. 4.1). Reduced IGF-IR expression and activation by IGF-I (and IGF-II) were also observed in a second clonal cell line of herstatin-expressing MCF7 cells (data not shown).

IGF-I activation of IRS-1 and IRS-2

To further investigate the effects of herstatin expression on IGF-I-mediated signaling, we examined the activation of IRS-1 and IRS-2, signaling molecules immediately downstream of the IGF-IR. IGF-I-induced phosphorylation of IRS-1 was severely reduced in MCF7/herstatin cells compared to parental controls (Fig. 4.2A & B). This decreased tyrosine phosphorylation of IRS-1 was a result of both decreased expression of IRS-1 (~5-fold; see Figure 4.5), as well as an apparent 6-fold decrease in the efficiency of IRS-1 immunoprecipitation in herstatin-expressing cells. This reduction in the amount of IRS-1 immunprecipitated from herstatin-expressing cells was also seen with a second, N-

terminally directed IRS-1 antibody (data not shown). Together, the combined effects of decreased IRS-1 expression and immunoprecipitation efficiency resulted in an ~30-fold difference in the amount of IRS-1 in immunoprecipitates from control and herstatin-expressing cells. This was similar to the difference in tyrosine-phosphorylated IRS-1; therefore, the decrease in IRS-1 protein immunoprecipitated from herstatin-expressing cells was equivalent to the decrease in IRS-1-associated phosphotyrosine. Thus, the relative activation of IRS-1 was similar in control and herstatin-expressing cells.

In contrast, the un-normalized levels of activated (tyrosine-phosphorylated) IRS-2 were actually enhanced by 50% in herstatin-expressing cells, despite the approximately 10-fold reduction in total (and immunoprecipitated) IRS-2 protein seen in herstatin-expressing cells (Fig. 4.2 C & D & Fig. 4.5). Thus, herstatin expression resulted in a 20-fold increase in IGF-I-stimulated IRS-2 tyrosine phosphorylation when the data are normalized for the decreased IRS-2 expression in herstatin-expressing cells.

IGF-I activation of ERK and PKB

Herstatin has been shown to differentially inhibit EGF-stimulated activation of the Akt/PKB versus the ERK signaling pathway in some cell types (Justman and Clinton 2002; Staverosky, Muldoon et al. 2005). Similarly, herstatin expression did not inhibit ERK signaling pathway in IGF-I-treated cells. ERK phosphorylation was rapid and transient, with a maximal response at 5 minutes in parental cells. In herstatin-expressing cells, the timing of the maximal response was the same, but the amplitude of total ERK activation, indicated by enhanced phospho-ERK, was apparently enhanced (Fig. 4.3). Interestingly, we also observed a preferential stimulation of ERK2, while there was no apparent change in the activation of ERK1. Furthermore, we consistently observed an increase in the apparent size of ERK1. This may correspond to the appearance of an ERK1 splice variant, or a post-

translational modification (Yung, Yao et al. 2000; Yung, Yao et al. 2001; Aebersold, Shaul et al. 2004). In contrast, IGF-I activation of the PI3K pathway, as assessed by the overall level of Akt/PKB phosphorylation, was reduced by 2-fold in MCF7/herstatin cells (Fig. 4.4). Thus, herstatin expression in MCF7 breast carcinoma cells does not reduce, and may slightly enhance, mitogenic ERK signaling, but attenuates the anti-apoptotic Akt/PKB signaling cascade. Similar effects, i.e., enhanced ERK2 activation and decreased Akt/PKB activation, were also seen in a second, independent herstatin-expressing MCF7 clone (data not shown).

Effect of herstatin on the expression of IGF signaling molecules

The studies described above demonstrate the effects of herstatin expression on IGF-I- signaling. Here, we examine the effect of herstatin expression on basal levels of these signaling molecules. The expression of herstatin in MCF7 cells resulted in the down-regulation of several components of the IGF signaling system (Fig. 4.5). Both IGF-IR and IRS-1 protein levels were decreased 5-fold, while IRS-2 protein levels were down-regulated by 10-fold. There was no apparent difference in the levels of total ERK; however as described above, there was a shift from a preponderance of ERK1 to ERK2, as well as an increase in the apparent size of ERK1, as illustrated in Figure 4.3. Akt/PKB levels were modestly affected, with an average 2-fold decrease seen in herstatin-expressing cells.

Herstatin reduces IGF-I-stimulated growth and survival in MCF7 cells

Previous studies have shown that stable expression of herstatin in MCF7 breast carcinoma cells blocked heregulin-stimulated proliferation (Jhabvala-Romero, Evans et al. 2003). The inhibition of IGF-IR signaling observed in herstatin-expressing cells suggested that herstatin may also interfere with IGF-I-mediated growth and survival. To further

investigate the effect of herstatin on IGF-I action, we examined the IGF-I-induced growth of parental MCF7 cells and two clones stably transfected with herstatin, MCF7/Hst#1 and MCF7/Hst#2. Parental MCF7 cells grew in response to IGF-I, whereas cell viability decreased in the absence of growth factor. Both of the MCF7/Hst clones, however, failed to exhibit IGF-I-stimulated growth (Fig. 4.6).

Herstatin blocks EGF signaling

Previous studies have demonstrated that the EGFR is involved in IGF-I signaling (Roudabush, Pierce et al. 2000; Gilmore, Valentijn et al. 2002; Adams, McKern et al. 2004; Ahmad, Farnie et al. 2004; El-Shewy, Kelly et al. 2004; Kuribayashi, Kataoka et al. 2004). Therefore, the observed effects on IGF-I signaling may have been an indirect effect of herstatin-mediated inhibition of the EGFR. To determine whether EGF-stimulated signaling was attenuated by herstatin, we compared the ability of EGF to activate the ERK and PI3K-Akt/PKB cascades in control and herstatin-expressing MCF7 cells. As shown in Fig. 4.7, EGF treatment of control cells elicited robust ERK and Akt/PKB phosphorylation, which was severely reduced in cells expressing herstatin. These data demonstrate that herstatin blocks both heregulin and EGF-stimulated signaling in MCF7 cells.

Effect of EGFR inhibition on IGF-IR expression

Herstatin expression had a striking effect on the levels of the IGF-IR. To determine if the observed effects of herstatin on IGF-IR levels were an indirect result of decreased EGFR action, we investigated whether specific inhibition of EGFR mimicked the effects of herstatin. Treatment with the EGFR inhibitor, AG1478, prevented EGF-stimulated

activation of ERK (data not shown). However, neither short-term nor long-term treatment with AG1478 resulted in the down-regulation of IGF-IR levels that was seen in herstatin-expressing cells (Fig. 4.8).

IV. DISCUSSION

An understanding of the effects of herstatin, an autoinhibitor of the EGFR family, on IGF-I signaling is critical to defining the overall mode of action of herstatin and to further clarify the mechanisms that link the actions of these two important receptor tyrosine kinase families. Our previous studies have shown that herstatin blocks heregulin signaling and proliferation in MCF7 breast carcinoma cells (Jhabvala-Romero, Evans et al. 2003). In the current study, we show that EGF signaling is also blocked in these cells. To further assess the interplay between herstatin and the IGF-IR, initially suggested by binding of herstatin at nM concentrations to the ectodomain of the IGF-IR, we examined IGF-I signaling and proliferation in MCF7 breast carcinoma cells in which signaling through the EGFR family is disabled.

We found a striking effect on IGF-I signaling. Foremost, herstatin expression resulted in down-regulation of the IGF-IR and a 8-fold decrease in IGF-I-induced phosphorylation, demonstrating a profound impact on the activation of the IGF-IR (Fig 4.1). Herstatin expression also resulted in a striking decrease in IRS-1 activation, which is immediately downstream of the IGF-IR in the IGF-I signaling pathway (Fig 4.2). Most importantly, the altered signaling culminated in a loss of IGF-I-mediated survival of MCF7 breast carcinoma cells that express herstatin (Fig. 4.6).

In contrast to the blockade of EGF and heregulin-induced ERK activation, IGF-I stimulation of ERK was not inhibited, even though IGF-IR levels were reduced several fold (Fig. 4.3). Therefore, the extent of IGF-IR activation did not parallel the effects on the downstream ERK signaling cascade. Thus, the low levels of activated IGF-IR appeared to be sufficient to fully activate ERK signaling. Though ERK1 activation was unaffected, we observed a shift in the size of ERK1 in herstatin-expressing cells. We speculate that this

size shift may be due to alternative splicing of the ERK1 gene, and may represent the ERK1b splice variant, which is 2.6 kDa larger than ERK1 (Yung, Yao et al. 2000; Yung, Yao et al. 2001; Aebersold, Shaul et al. 2004). ERK1b has an altered ability to interact with MEK1 and may, therefore, result in a differential signaling profile (Yung, Yao et al. 2001). Further studies are needed to elucidate the cause of the shift in ERK1. Interestingly, in herstatin-expressing cells, we also observed a preferential activation of ERK2 relative to ERK1, which was unaffected (Fig 4.3). Recent studies have implicated activation of ERK2, but not ERK1, in apoptosis (Frese, Pirnia et al. 2003; Castro-Obregon, Rao et al. 2004; Cheung and Slack 2004; Chen, Plotkin et al. 2005). Therefore, this preferential activation of ERK2 in herstatin-expressing cells may play a role in the loss of IGF-I-mediated survival of MCF7/Hst cells.

The effects of herstatin expression on the signaling factors immediately downstream of the IGF-IR, IRS-1 and IRS-2, were complex and distinct. Herstatin reduced both IRS-1 expression and immunoprecipitation efficiency, with a concomitant decrease in IGF-I-stimulated tyrosine phosphorylation (Fig 4.2 A & B & Figure 4.5). The mechanisms responsible for the two former effects are unclear. With respect to the differential immunoprecipitation of IRS-1 in control vs herstatin-expressing cells, it is possible that herstatin results in the altered subcellular localization, expression, or association pattern of IRS-1, such that the availability of IRS-1 to interact with multiple antibodies in attenuated. One possibility is that nuclear translocation of IRS-1, which has been observed in multiple cell types, including MCF7 cells, is affected by herstatin expression (Morelli, Garofalo et al. 2004). While herstatin expression also resulted in the down-regulation of IRS-2, there was no effect on IRS-2 immmunoprecipitation per se, and IGF-I-stimulated IRS-2 tyrosine phosphorylation was actually enhanced in herstatin-expressing cells, an effect which is very robust when accounting for the decreased IRS-2 levels (Figure 4.2 A & B & Figure 4.5).

The differential enhancement of IGF-I-stimulated IRS-1 and IRS-2 activation by herstatin may reflect the fact that feedback mechanisms, such as patterns of inhibitory serine phosphorylation, differ between IRS-1 and IRS-2 (Kim, Yeh et al. 2005). Interestingly, previous studies have shown that IRS-1, but not IRS-2, is important in IGF-I inhibition of apoptosis, an effect that may underlie the inhibitory effects of herstatin on cell viability seen in the current study (Tseng, Ueki et al. 2002). Combinatorial effects of herstatin expression that include decreased expression and activation of the IGF-IR and its immediate downstream signaling molecule, IRS-1, reduction in activation of Akt and an increase in activation of ERK2, may all contribute to the retarded growth of herstatin-expressing MCF7 cells (Fig. 4.5).

There are several potential mechanisms through which herstatin may modulate IGF-IR signal transduction and, thereby, IGF-I action. First, herstatin may directly bind to the IGF-IR, either intracellularly or the secreted herstatin may interact at the cell surface, since we have previously determined that it binds to the ectodomain of the IGF-IR with nanomolar affinity (Shamieh, Evans et al. 2004). However, since herstatin binds to all EGFR family members, and with higher affinity than to IGF-IR, the impact of herstatin on IGF-I signaling may be indirect and needs to be further investigated in cells that do not express the EGFR family.

A second possibility is that the modulation of IGF-I signaling is a secondary effect due to blockade of EGFR family signaling. Ample evidence exists for an IGF-I-stimulated autocrine loop that results in the release of heparin-binding EGF (HB-EGF) and consequently in the activation of the EGFR (Roudabush, Pierce et al. 2000). To examine whether the effect of herstatin on down regulation of the IGF-IR occurs via the EGFR, we blocked EGFR activation (using the EGFR-specific kinase inhibitor, AG1478) in parental MCF7 cells. While the inhibitor fully blocked EGF-induced ERK activation (data not shown),

it failed to mimic the results of herstatin-mediated down-regulation of the IGF-IR (Fig. 4.8). However, we cannot rule out the possibility that longer-term effects of herstatin expression are involved, that modulation of the other members of the EGFR family indirectly affects IGF-I signaling, or that the inhibitor affects IGF-IR function, but not levels.

A third possibility is that herstatin may modulate the formation of hetero-oligomers between the IGF-I and EGF receptors. Recent evidence suggests that the EGFR is present in IGF-IR immunoprecipitates, suggesting the interesting possibility that herstatin may disrupt EGFR/IGF-IR hetero-oligomers (Ahmad, Farnie et al. 2004). However, further studies are needed to validate the existence of functional hetero-oligomers between these RTK families. Regardless of whether this mechanism entails a direct or indirect effect of herstatin on the IGF-IR, the results presented here demonstrate a profound modulation of IGF-I signaling by an alternative product of the HER-2 gene.

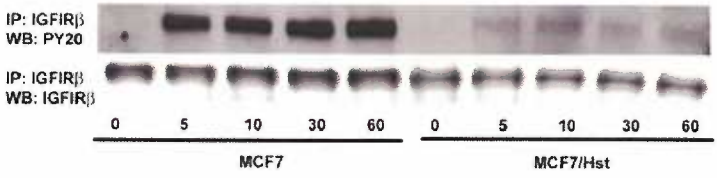
The roles of both the EGFR and IGF-IR families in neoplastic growth and malignancies have been well documented. Over-expression and autocrine stimulation of both receptor families and their ligands has been implicated in a variety of carcinomas (Slamon and Clark 1988; Khandwala, McCutcheon et al. 2000; Mendelsohn 2001; Yarden and Sliwkowski 2001; Harari 2004). Recent evidence in breast and prostate cancer cells has shown that acquired resistance to Iressa, an EGFR tyrosine kinase inhibitor, is mediated by activation and signaling of the IGF-IR (Jones, Goddard et al. 2004; Camp, Summy et al. 2005). Furthermore, IGF-IR signaling has been shown to protect HER-2 over-expressing breast carcinoma cells from the inhibitory effects of Herceptin, an anti-HER-2 monoclonal antibody (Lu, Zi et al. 2001). It is, therefore, apparent that controlling both of these receptor tyrosine kinase families would be advantageous to contain neoplastic growth. Our data suggest that herstatin is an inhibitor that may block proliferative signals from two distinct families of RTKs.

The data obtained in this study were obtained with MCF7 cells and were based on two independent herstatin-expressing clones in comparison to control cells. Limitations of this study include clonal selection of herstatin-transfected cells without the appropriate control of clonally-selected mock-transfected cells. Although MCF7 cells are a valuable established model for the study of cellular regulatory mechanisms relevant to breast cancer, it will be desirable to extend these results to other cell types, using the proper, mock-transfected controls. Constitutive expression of herstatin is, however, toxic to most other cells that we have analyzed; thus, further studies will be facilitated by exploiting conditional, regulated expression models that we are currently developing.

Currently available receptor-directed therapeutics target a single receptor or receptor family, which may explain, in part, their limited clinical efficacy. Recently, a hetero-bi-functional monoclonal antibody that targets both the EGFR and IGF-IR was found to block both EGF and IGF-I-induced activation of Akt/PKB and ERK, resulting in strong inhibition of xenograft growth (Lu, Zhang et al. 2004; Lu, Zhang et al. 2005). We suggest that herstatin may have promise as an anti-cancer agent, since it acts as a multi-functional inhibitor that suppresses signaling from both the EGFR and IGF-IR families of receptor tyrosine kinases.

Figure 4.1. Herstatin modulation of IGF-I activation of IGF-IR. MCF7 and MCF7/Hst cells were serum-starved overnight, treated with 5 nM IGF-I over a 60-minute timecourse, and harvested in NP-40 lysis buffer. 1mg of cell lysate was immunoprecipiated with IGF-IRß antibody and protein A agarose beads. Immunoprecipitates were separated on a 10% SDS-PAGE gel and analyzed for IGF-IR expression and tyrosine phosphorylation. Western blots were scanned and quantified by densitometry. **(A)** Representative Western blot of IGFI-R immunoprecipitation of IGF-I-treated MCF-7 and MCF-7/Hst cell lysates. **(B)** The graphical representation of two independent experiments of IGF-I-induced activation of the IGF-I receptor.

A.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WB: PY20
IP: IGFIRβ
WB: IGFIRβ
0 5 10 30 60 0 5 10 30 60
MCF7
MCF7/Hst

B.

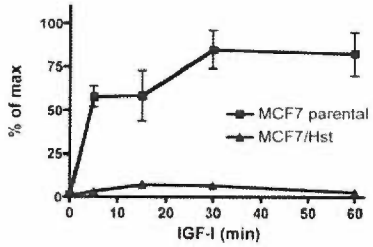

% of max
100
75
50
25
0
0 10 20 30 40 50 60
IGF-I (min)
MCF7 parental
MCF7/Hst

Figure 4.2. The effect of herstatin on IGF-I activation of IRS-1 and IRS-2. MCF7 and MCF7/Hst cells were serum-starved overnight, treated with 5 nM IGF-I over a 60-minute timecourse, and harvested in NP-40 lysis buffer. 1mg of cell lysate was immunoprecipiated with IRS-1 (A & B) or IRS-2 (C & D) antibody and protein A agarose beads. Immunoprecipitates were separated on a 10% SDS-PAGE gel and analyzed for IRS expression and tyrosine phosphorylation. Western blots were scanned and quantified by densitometry. **(A)** Representative IRS-1 immunoprecipitation and analysis with antiphosphotyrosine PY20 antibody. Both light and dark exposures of the IRS-1 immunoprecipitation are shown. **(B)** Graphical representation of 3 separate experiments. **(C)** Representative IRS-2 immunoprecipitation and analysis with anti-phosphotyrosine PY20 antibody. **(D)** Graphical representation of 3 separate experiments.

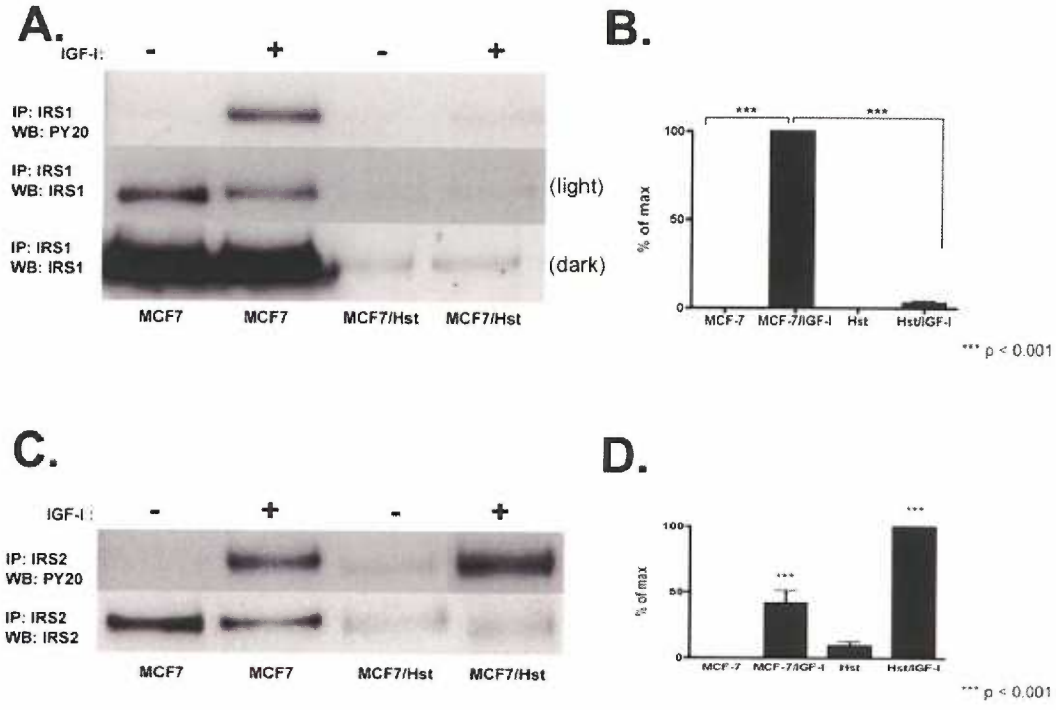

A.
IGF-I: - + - +
IP: IRS1
WB: PY20
IP: IRS1
WB: IRS1
(light)
IP: IRS1
WB: IRS1
(dark)
MCF7
MCF7
MCF7/Hst
MCF7/Hst
B.
% of max
100
50
0
***
***
MCF-7
MCF-7/IGF-I
Hst
Hst/IGF-I
*** p < 0.001
C.
IGF-I: - + - +
IP: IRS2
WB: PY20
IP: IRS2
WB: IRS2
MCF7
MCF7
MCF7/Hst
MCF7/Hst
D.
% of max
100
50
0
***
***
MCF-7
MCF-7/IGF-I
Hst
Hst/IGF-I
*** p < 0.001

Figure 4.3. The effect of herstatin on IGF-I activation of ERK. MCF7 and herstatin-expressing MCF7/Hst breast carcinoma cells were serum-starved and treated with 5nM IGF-I at 37°C over a 60 minute time period. Cell lysates were separated on a 10% SDS-PAGE gel and then analyzed by Western blot. One representative Western blot showing IGF-I-induced ERK activation in MCF7 and MCF7/Hst cells.

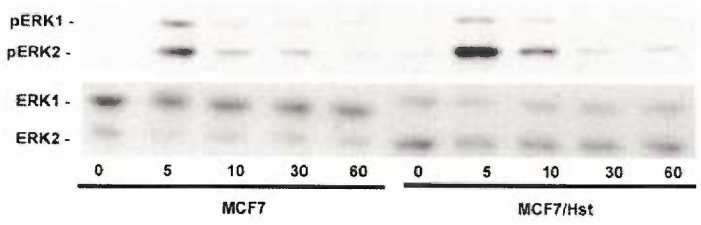
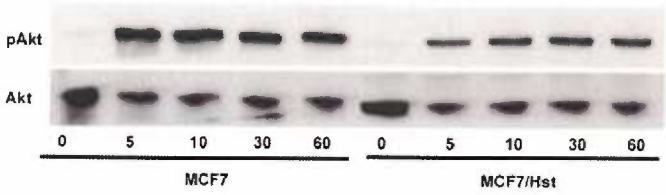

pERK1 -
pERK2 -
ERK1 -
ERK2 -
0
5
10
30
60
0
5
10
30
60
MCF7
MCF7/Hst

Figure 4.4. The effect of herstatin on IGF-I activation of Akt/PKB. MCF7 and herstatin-expressing MCF7/Hst breast carcinoma cells were serum-starved and treated with 5nM IGF-I at 37°C over a 60 minute time period. Cell lysates were separated on a 10% SDS-PAGE gel and then analyzed by Western blot. Western blots were scanned and quantified by densitometry **(A)** A representative Western blot showing IGF-I-induced Akt/PKB activation in MCF7 and MCF7/Hst cells. **(B)** The graphical representation of 3 separate experiments, as described in (A).

A.


pAkt
Akt
0
5
10
30
60
0
5
10
30
60
MCF7
MCF7/Hst

B.

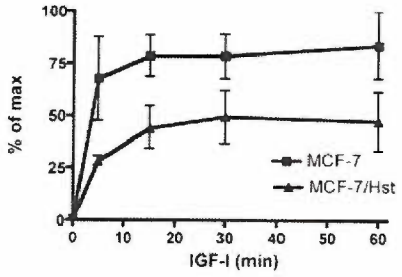

100
75
50
25
0
% of max
0
10
20
30
40
50
60
IGF-I (min)
MCF-7
MCF-7/Hst

Figure 4.5. The effect of herstatin-expression on the expression levels of various signaling proteins. Sub-confluent MCF7 and MCF7/Hst cells were extracted and signaling protein level was assessed by Western blot. Herstatin expression in MCF7 breast carcinoma cells down-regulates IGF-IR, IRS-1, IRS-2, and pKB/Akt expression, but MAPK expression is unaffected.

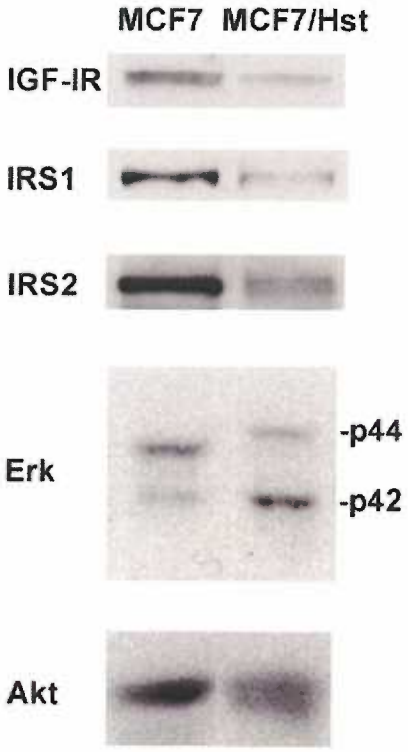
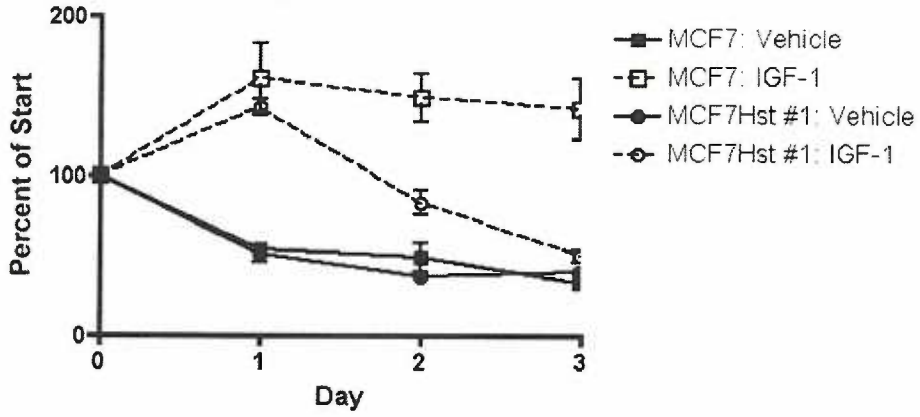

MCF7
MCF7/Hst
IGF-IR
IRS1
IRS2
Erk
-p44
-p42
Akt

Figure 4.6. The effect of herstatin on IGF-I-stimulated cell proliferation. Parental MCF7 breast carcinoma cells and MCF7 cells stably transfected with herstatin, **(A)** low hst-expressing clone, and **(B)** high hst-expressing clone, were serum-starved for 24 hours and then treated with 5nM IGF-I or vehicle. Growth was determined by the MTS assay as described in Materials and Methods and was assessed at the indicated days.

A.


200
100
0
Percent of Start
0
1
2
3
Day
MCF7: Vehicle
MCF7: IGF-1
MCF7Hst #1: Vehicle
MCF7Hst #1: IGF-1

B.

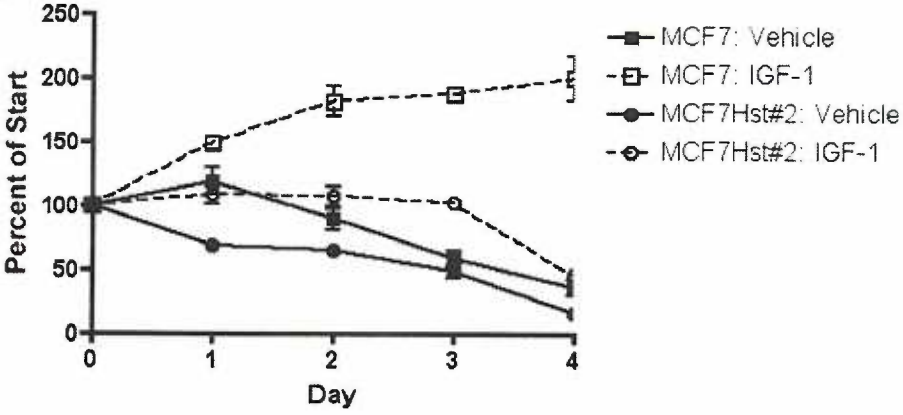

250
200
150
100
50
0
Percent of Start
0
1
2
3
4
Day
MCF7: Vehicle
MCF7: IGF-1
MCF7Hst#2: Vehicle
MCF7Hst#2: IGF-1

Figure 4.7. The effect of herstatin on EGF-stimulated signaling in parental and herstatin-expressing MCF7 cells. Parental (MCF7) and herstatin-expressing (MCF7/Hst) breast carcinoma cells were serum-starved and treated with 5nM EGF at 37°C for the durations indicated (in minutes). Cells were lysed, and lysates were run on a 10% SDS-PAGE gel and analyzed by Western blot. Western blots were scanned and quantified by densitometry **(A)** The effects of herstatin-expression on EGF-induced ERK activation. **(B)** The effects of herstatin-expression on EGF-induced Akt/PKB activation.

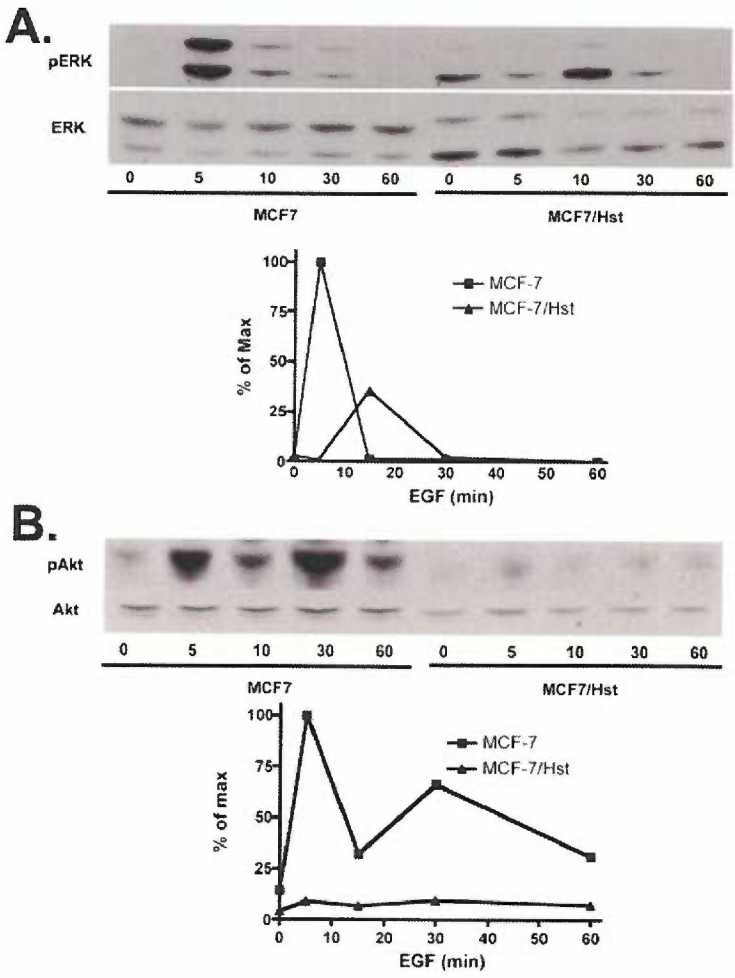
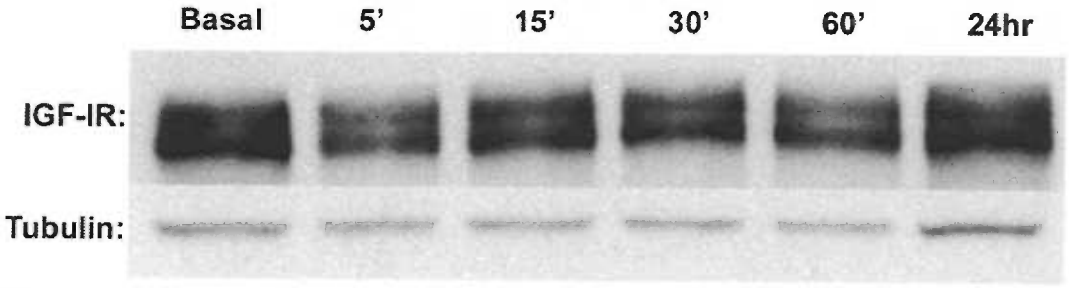

A.
pERK
ERK
0 5 10 30 60 0 5 10 30 60
MCF7
MCF7/Hst
100
75
50
25
0
% of Max
MCF-7
MCF-7/Hst
0 10 20 30 40 50 60
EGF (min)
B.
pAkt
Akt
0 5 10 30 60 0 5 10 30 60
MCF7
MCF7/Hst
100
75
50
25
0
% of max
MCF-7
MCF-7/Hst
0 10 20 30 40 50 60
EGF (min)

Figure 4.8. The effect of AG1478, an EGFR inhibitor, on IGF-IR expression. MCF7 breast carcinoma cells were treated with the EGFR inhibitor, AG1478, at 37°C for the durations indicated (in minutes). Cells were lysed, and lysates were run on a 10% SDS-PAGE gel and analyzed by Western blot. AG1478 has no effect on IGF-IR expression levels over a 24-hour period.


Basal
5'
15'
30'
60'
24hr
IGF-IR:
Tubulin:

CHAPTER 5

Discussion

Herstatin binds to both EGF and IGF-I receptor families and may function as a multi-functional inhibitor

It has been shown that herstatin binds to EGFR and HER-2 and inhibits dimerization and receptor activation (Doherty, Bond et al. 1999; Azios, Romero et al. 2001). Furthermore, studies have also shown that herstatin expression inhibits Hrg and EGFmediated signaling (Azios, Romero et al. 2001; Justman and Clinton 2002; Jhabvala-Romero, Evans et al. 2003). These studies suggest that the binding of herstatin, which is a secreted, soluble piece of the HER-2 ectodomain, is integral to its inhibitory function. However, previous studies have shown that a membrane-anchor is required for dimerization between ectodomains or pieces of ectodomains of erbB receptors (Kumagai, Davis et al. 2001). This dependence on a membrane-anchor suggests that the energetics of soluble receptor ectodomains alone are not strong enough to mediate dimerization (Kumagai, Davis et al. 2001; Garrett, McKern et al. 2003). An increase in effective local concentration is not enough to overcome this requirement of a membrane-anchor, as there is a marked absence of HER-2 ectodomain dimers in the crystal structure (Garrett, McKern et al. 2003). Since herstatin is not membrane-bound but still binds to EGFR and HER-2 with nanomolar affinity, I propose that the intron 8-encoded domain may facilitate binding of herstatin to the receptor ectodomain, overcoming the requirement for membrane immobilization.

Because of the importance of binding function of herstatin for its inhibitory activity and because of the novel sequence of the intron encoded binding module, I considered it important to define the diversity of receptors to which herstatin binds and to determine the strength of the binding interactions. I investigated whether herstatin binds to other members of the EGF receptor family. In addition to binding to EGFR and HER-2, I found that herstatin also binds to HER-3, HER-4, that is to all members of the EGF receptor family in contrast to any of the other 11 erbB ligands. This suggests that these receptors are all targets of herstatins' inhibitory effects and that there may be a common herstatin binding site in the ectdomain of all erbB receptors. Furthermore, I found that herstatin binds to ΔEGFR, a mutant missing subdomains I and II of its extracellular domain (ECD). Interestingly, even though herstatin binds to ΔEGFR, it does not block receptor tyrosine phosphorylation or down-stream signaling (Staverosky, Muldoon et al. 2005). This absence of a signaling blockade suggests that the binding function of herstatin is separate from its inhibitory function. This data also suggests that the binding site for herstatin, and its intron 8-encoded domain, may be located within subdomains III and IV of the receptor ectodomains (to be discussed below). I propose that heteromeric interactions between subdomains I and II of the receptor ECDs and herstatin function to stabilize the binding of herstatin, thus resulting in a lower K_d than binding of int8 alone. Since ΔEGFR is missing subdomains I and II of its ECD, I

predict that the K_d of herstatin binding to ΔEGFR will be similar to that of int8 binding. Future experiments are needed to determine the K_d of int8 binding to ΔEGFR.

In addition to binding to the EGF receptor family, I found that herstatin binds, albeit with reduced affinity, (10 fold) to the IGF-IR and IR. The stoichiometry of herstatin binding to the IGF-I receptor family was one herstatin molecule to one receptor dimer, as opposed to the stoichiometry of binding to the EGF receptor family (one herstatin molecule to one receptor monomer). This difference in stoiciometry may be due to steric clash between subdomains I and II of herstatin and the disulphide-bonded IGF-I receptor family ECD, and may reflect differences in herstatin's ability to regulate the two receptor families. This thesis provides the first evidence that herstatin binds to two, independent receptor tyrosine kinase families: the EGFR family and the IGF-IR family.

Herstatin not only binds to the IGF-IR family, but also inhibits IGF-I-induced signaling and growth in breast carcinoma cells. Previous studies have shown that herstatin also inhibits EGF and Hrg-induced signaling and growth in a variety of cells (Azios, Romero et al. 2001; Justman and Clinton 2002; Jhabvala-Romero, Evans et al. 2003). Due to its involvement in carcinogenesis, the EGF receptor family has been a target of anti-cancer directed therapies. Recent evidence has shown that the inhibitory effects of Iressa, an EGFR small molecule inhibitor, and Herceptin, an anti-HER-2 monoclonal antibody, can be overcome by IGF-I signaling through the EGF receptor (Lu, Zi et al. 2001; Jones, Goddard

et al. 2004; Camp, Summy et al. 2005). I suggest that herstatin, which binds to both the EGF and IGF-I receptor families, may have potential as a multi-functional therapeutic.

The physiological role of herstatin in human tissues

Since the discovery of herstatin in 1999, most studies have focused on the *in vitro* inhibitory role of the molecule and its potential as a therapeutic, rather than its normal physiological role in human tissues. In order to better understand how herstatin functions though, it is important to examine its role in normal physiology. Studies in the Clinton Lab have looked at the tissue distribution of herstatin mRNA by probing a human tissue array with an intron 8-specific probe (Evans and Clinton, unpublished results). In this study, herstatin was notably absent in five specific tissues: spleen, thymus, lung, bladder and mammary gland. In contrast, high levels of herstatin mRNA were found in adult heart and pancreas, as well as fetal heart, kidney, liver and lung. These results were confirmed by Northern Blot (Evans and Clinton, unpublished results).

Interestingly, high levels of HER-2 mRNA (placenta and kidney) did not correspond with high levels of herstatin mRNA (heart and liver). However, the tissues with high herstatin mRNA did correspond to tissues expressing moderate levels of HER-2 mRNA (Evans and Clinton, unpublished results). This data leads me to hypothesize that herstatin expression may be repressed in tissues where HER-2 is highly active. However, in normal cellular homeostasis, there is a balance between HER-2 and herstatin expression, which regulates receptor

function. Herstatin is produced from an alternate transcript of HER-2 and is likely expressed in the same cells. Since both proteins have the same signal sequence, they will traffic in through the same endosomal compartments. Therefore, it is possible that intracellular concentrations of herstatin could be quite high and may result in a physiological response.

In order to examine the developmental regulation of herstatin, Northern Blot analyis was performed on human adult and fetal tissues. With the exception of heart, fetal tissues express higher levels of herstatin mRNA than do the corresponding adult tissues (Evans and Clinton, unpublished results). This data suggests that herstatin is developmentally regulated. However, this data examines mRNA expression, which may or may not correspond to protein expression. In order to examine herstatin and HER-2 protein distribution, immunohistochemistry using HER-2 and herstatin specific antibodies must be performed on both adult and fetal tissues.

Examining mRNA and protein expression of HER-2 and herstatin is merely a first step in understanding the physiological function of herstatin. Many further experiments are needed to investigate the role of this naturally occurring inhibitor.

Retention of an intron-encoded sequence confers unique properties to herstatin

Herstatin and other products of alternative erbB receptor transcripts may function through novel mechanisms to ellicit erbB receptor control.

The results in this thesis suggest that the intron 8-encoded domain plays an important and unique role in mediating herstatin action. While the intron 8-encoded domain alone does not function to inhibit receptor signaling (data not shown), it does function as a receptor binding domain (RBD). Several erbB alternate transcripts have been identified, which encode a truncated receptor product followed by an intron encoded domain of 1 to 79 amino acids (Gunther, Betzel et al. 1990; Katoh, Yazaki et al. 1993; Lee and Maihle 1998; Aigner, Juhl et al. 2001; Lee, Akita et al. 2001; Reiter, Threadgill et al. 2001). It is therefore plausible that some of these retained introns may encode domains that have receptor binding properties akin to that of HER-2 intron 8.

Two of the erbB alternate transcripts encode proteins with unique C-terminal domains of sufficient size to possibly function as a receptor binding module: p100EGFR and p85HER-3 (Lee, Akita et al. 2001; Reiter, Threadgill et al. 2001). Sequence comparison of the unique C-terminal domains of herstatin, p85HER-3, and p100EGFR revealed no sequence similarity. However, GORIV secondary structure prediction analysis suggests that the three domains are intrinsically unstructured (over 70% random coil). This leads to the question of the utility of an intrinsically unstructured C-terminal domain in a soluble erbB

protein product. P100EGFR, found in normal placenta and liver, consists of the full-EGFR ectodomain followed by a unique 78 amino acid C-terminal sequence (Reiter, Threadgill et al. 2001). However, the function of p100EGFR has not yet been elucidated. On the other hand, p85HER-3, which consists of subdomains I, II, and III of the HER-3 receptor ECD followed by novel 24 amino acid intron-encoded C-terminal domain, has been extensively studied. P85HER-3 inhibits HER-2/HER-3 heterodimers by binding to and sequestering the Hrg ligand, which results in inhibition of Hrg-stimulated cellular growth (Lee, Akita et al. 2001). In this way, p85HER-3 appears to function in an analogous fashion to Argos, the antagonist of the DER in Drosophila, which binds to and sequesters the activating ligand, Spitz (Klein, Nappi et al. 2004). Unlike herstatin, however, p85HER-3 has not been shown to bind directly to the erbB receptors (Doherty, Bond et al. 1999; Lee, Akita et al. 2001; Shamieh, Evans et al. 2004).

The intron 8-encoded domain of herstatin is conserved in primates

The erbB receptors are potent orchestrators of mitogenic signaling. Comparison of receptor orthologs reveals that the erbB signaling network evolved from a simple one receptor: one ligand system in *C.elegans*, to a one receptor: four ligand system in *D. melanogaster*, and finally a four receptor: multiple ligand system in mammals (Stein and Staros 2000). This increased complexity of signaling may have coordinately evolved a unique mechanism for receptor attenuation reflected in alternate transcripts encoding for soluble receptors with unique C-terminal domains.

I therefore compared the intron-encoded domain of herstatin with intron8-encoded domains of orthologous erbB-2 genes and found sequence conservation with primates, but not with other animals. In this study, I sequenced the erbB-2 intron 8- of rat and rhesus monkey. Additionally, I searched the NCBI genome databases that are now available for *Bos taurus* (cow), *Canis familiaris* (dog), *Felis catus* (cat), *Mus musculus* (mouse), *Ovies aries* (sheep), *Pan troglodytes* (chimpanzee), *Rattus norvegicus* (rat), and *Sus scrofa* (pig) erbB-2 intron 8. Comparison of ErbB-2 intron 8 in these genomes with that of human, only revealed sequence identity with chimpanzee, but not with the other mammals. Interestingly, my sequencing results revealed that rat *neu* intron 8 had no conservation with that of human, even though the human and rat HER-2/*neu* receptors share 85% nucleotide identity and 83% amino acid identity. This comparison suggests that there is no herstatin-like erbB autoinhibitor in rats. However, herstatin does appear to be present in primates. Rhesus monkey erbB-2 intron 8 has 93% nucleotide identity and 85% identity in the deduced amino acid sequence to human (Table 5.1) (Shamieh, Evans et al. 2004). The chimpanzee erbB-2 intron 8 is also highly conserved compared to human: 99% nucleotide and 97% amino acid identity (Table 5.1). These comparisons suggest that the erbB-2 intron 8, and consequently herstatin, is a recent evolutionary event that is conserved in primates, but not other mammals.

Evolutionary analysis, which shows humans are more closely related to chimpanzees than to rhesus monkeys, is in agreement with my data showing that intron 8 is more highly conserved in chimpanzees than in rhesus (Goodman,

Porter et al. 1998; Goodman 1999). I next investigated the evolutionary rate of the erbB-2 intron 8, compared to the erbB-2 exonic sequence. Humans and chimpanzees diverged 6 Ma (Mega annum, million years before present), while the last common ancestor of the family Cercopithecidae (which includes the rhesus monkey) and the family Hominadae (which includes humans and chimpanzees) occurred 25 Ma (Goodman, Porter et al. 1998; Goodman 1999). This timeline suggests that over the course of 19 million years from rhesus monkeys to chimpanzees, the erbB-2 intron 8-encoded amino acid sequence evolved at an AA rate of $9.9 \cdot 10^{-9}$ (AA subsitutions/site/year) (Ye, Li et al. 2005). The AA rate over the 6 Ma time span between chimpanzees and rhesus monkeys is $4.2 \cdot 10^{-9}$ (substitutions/site/year). These evolutionary rates are in stark comparison to the rate of the evolution of the erbB-2 receptor between chimpanzees and humans [AA rate = $0.29 \cdot 10^{-9}$ (substitutions/site/year)] (Table 5.2). These numbers suggest that the putative intron–encoded domain of erbB-2 has been experiencing a period of rapid evolution for at least 25Ma. Since evolutionary rates tend to be constant within a given gene, this data suggests that the putative intron 8-encoded domain is evolving a different rate and therefore may play an important role in erbB-2 function (Ye, Li et al. 2005).

Since introns and exons may have different selective pressures, there is the possibility that they evolve at different rates (Dufour, Casane et al. 2000). To examine this possibility, I looked at the nucleotide evolutionary rate between chimpanzee and humans of ErbB-2 exonic sequence, and of intron 8 and intron 7 (Table 5.2). I find that the erbB-2 exonic sequence and the intron 7 sequence

evolved at approximately the same rates, while the sequence of intron 8 evolved approximately 4 times faster. The enhanced evolutionary rate of erbB-2 intron 8 suggests that its novel function as a receptor binding domain may be exerting selective pressure.

To definitively test whether selective pressure is occurring on the erbB-2 intron 8, a full-scale analysis of positive selection must be performed. Future studies will focus on determining whether positive selection of erbB-2 intron 8 is occurring. In order to do so, I will first obtain genomic DNA samples of multiple species of Old World and New World Monkeys. I will then PCR amplify and sequence erbB-2 intron 8 using human-based primers that flank the 3' end of exon 8 and the 5' end of exon 9. This will be performed in the same way that the Rhesus monkey DNA was sequenced (Chapter 2). Once multiple sequences are obtained, algorithms that score for non-synonymous versus synonymous substitutions will be employed to determine whether positive selection is occurring.

Model of herstatin binding to EGF and IGF-I receptor families

I propose that the intron 8-encoded domain functions as a binding module for herstatin. Both herstatin and int8 bind to ΔEGFR, an EGFR mutant missing subdomains I and II of its ECD. This suggests that the int8 binding module binds to either subdomain III or IV of the EGF receptor family. Int8 and herstatin also bind to the IGF-I and Insulin receptors. Sequence alignment the EGF, IGF-I, and Insulin receptor ECDs, reveals high homology in subdomains I, II, and III.

However, subdomain IV does not exist in the IGF-IR and IR. Taken together, these data suggest that the intron 8-encoded herstatin binding module is binding within subdomain III of the receptor ECDs.

Mapping of the 33 conserved residues onto the EGF receptor crystal structures reveals that these residues cluster in two three-dimensional sections of subdomain III (Fig. 5.1). Portions of beta sheets and alpha helices are conserved, as are several non-structured loops. Since the intron-encoded domain itself is intrinsically unstructured, I hypothesize that these conserved loops may be playing a critical role in mediating the binding of int8. One of the sections of sequence identity amongst EGFR, IGF-IR and IR clusters in the ligand-binding domain of the EGFR. Previous data showed that EGF binding is not affected by herstatin (Justman and Clinton 2002). This suggests that the conserved ligand-binding interface is not the binding site of int8, thus leaving the exposed regions on the underside of subdomain III as possible binding sites for int8.

Binding of int8 to the underside of subdomain III allows for the possibility that subdomains I and II of herstatin may be juxtaposed with subdomains I and II of the EGF receptor family. This model is in agreement with my hypothesis that subdomains I and II stabilize the binding of int8. Since, unlike the EGF receptor family, the IGF-I receptor family lacks a "dimerization" arm in subdomain II, I hypothesize that herstatin binding to the IGF-IR and the IR would not be stabilized through interactions of the N-terminal domain of herstatin and the ectodomain of IGF-IR and IR. This is consistent with data showing that full-

length herstatin binds with ten-fold lower affinity to the IGF-IR and IR than to EGFR and HER-2.

Taken together, the data in this thesis suggest that herstatin, and its intron 8-encoded domain, bind to both the EGF and IGF-I receptor families. This unique property of binding to two receptor kinase families points to the possibility that herstatin may be a multi-functional inhibitor.

Conclusions

Data in this thesis helps to define the role of herstatin binding. The following hypotheses were examined.

1) **Herstatin binds to all four members of the EGF receptor family with the similar affinity.** Previous studies have shown that herstatin binds to EGFR and HER-2 with nanomolar affinity (Doherty, Bond et al. 1999; Shamieh, Evans et al. 2004). Since all four receptors of the EGF receptor family share a similar ectodomain topology, I hypothesized that herstatin would bind to them all with similar affinity. Though the dissociation constants of herstatin binding was only determined for EGFR and HER-2, I showed that at two concentrations (35nM and 150nM) herstatin binds in a similar fashion to all four receptors. Additionally, preliminary evidence, (not presented in this thesis) suggests that herstatin binds with similar affinity to HER-3 and HER-4 (Shamieh and Clinton, unpublished results). Therefore, herstatin may bind to and inhibit homo- and hetero-dimerization of all four EGF receptor family members. Since these receptors

often function as a signaling-unit, herstatin appears to have the potential to modulate the activity of the entire system, rather than only a specific receptor.

2) The intron 8-encoded domain functions as the binding domain of herstatin. Data presented in this thesis provide evidence that the intron 8-encoded domain of herstatin functions as a binding module. The intron 8-encoded domain binds with 10-fold reduced affinity than does herstatin, but binds to the same receptors as herstatin. Furthermore, mutations in the intron-encoded domain of herstatin slightly alter the dissociation constant of herstatin binding. Even though this change in binding affinity is not physiologically relevant, it does point to the importance of the intron 8-encoded domain in herstatin binding. The data presented in this thesis are consistent with a model where the intron 8-encoded domain acts as an "anchor" for subdomains I and II of herstatin, allowing high affinity binding to the receptors, which results in inhibition of receptor activation and modulation of downstream signaling.

3) Herstatin and its intron 8-encoded domain may bind to other receptor tyrosine kinase families, such as the IGF-IR family, that share ectodomain homology with the EGF receptor family. Herstatin binds to the IGF-I receptor family (IGF-IR and IR) with approximately 10-fold reduced affinity than it does to the EGF receptor family. Furthermore, herstatin binds with a stoichiometry of one herstatin molecule to one IGF-IR family dimer (as opposed to 1:1 with the EGF receptor family). However, despite the fact that herstatin

binds with nanomolar affinity to the IGF-IR family, a detailed study of receptor and inhibitor protein levels, as well as tissue-distribution needs to be undertaken to better understand the interplay of the herstatin inhibitor with these receptors. Furthermore, *in vivo* studies are needed to determine whether herstatin participates in a physiologically relevant role in receptor inhibition.

4) **If herstatin binds to the IGF-I receptor, it may affect IGF-I-induced cellular signaling and growth.** Data presented in this thesis shows that in herstatin expression inhibits IGF-I induced activation of IGF-IR and alters down-stream signaling in MCF7 breast carcinoma cells. This signaling blockade culminates in growth inhibition as measured by MTS. Even though these experiments were done in two independent clonally derived MCF7/Hst cell lines, these cells are all derivates of clonal isolates and may have unique, compensatory mutations. Experiments are currently underway to examine the impact of the addition of exogenous herstatin to MCF7 breast carcinoma cells.

Nonetheless, the herstatin-mediated IGF-I signaling blockade may be due to either direct inhibition of the IGF-IR or through inhibition of cross-talk with the EGFR. Examination of these possibilities need to be undertaken in future experiments. However, whether through direct inhibition or through inhibition of cross-talk, this IGF-I signaling blockade points to a potential use of herstatin as a bi-functional therapeutic.

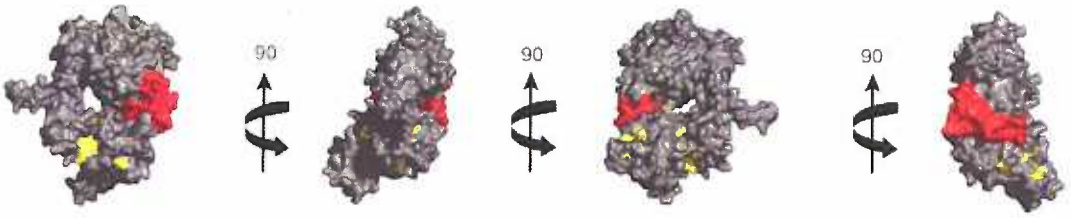
Table 5.1. Comparison of the nucleotide and amino acid identity of human, rat, rhesus monkey, and chimpanzee intron 8 sequences. The most common human intron 8 sequence (Doherty, Bond et al. 1999) was compared with chimpanzee (from the NCBI database) and PCR-determined rat and rhesus monkey sequences.

	Nucleotide Identity w/Human Intron 8	Amino Acid Identity w/Human Int8 peptide
Rat	No significant similarity	
Rhesus Monkey	93%	85%
Chimpanzee	99%	97%

Table 5.2. Comparison of the nucleotide and amino acid evolutionary rates of chimpanzee and human sequences. Human and chimpanzee nucleotide and amino acid sequences were compared for erbB-2 coding, intron 7 and intron 8 sequences, as well as the coding sequences of two housekeeping genes, GAPDH and α–actin.

	Nucleotide Rate (substitutions/site/year)	Amino Acid Rate (substitutions/site/year)
ErbB2 intron8	1.20×10^{-9}	4.20×10^{-9}
ErbB2 coding	0.26×10^{-9}	0.29×10^{-9}
ErbB2 intron7	0.34×10^{-9}	-NA-
GAPDH coding	0.60×10^{-9}	0
α-actin coding	0.58×10^{-9}	0

Figure 5.1. Surface model of subdomains I, II, and III of the EGF receptor ECD reveals clustering of conserved residues in subdomain III. The crystal structure of subdomains I, II, and III of EGFR (grey) bound with EGF bound (red) (Ogiso, Ishitani et al. 2002). Thirty-three amino acid residues (yellow) are conserved in subdomain III of the EGF, IGF-I, and Insulin receptors. These residues may represent the binding surface of int8 to these receptors.


90
90
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