ASSOCIATIONS BETWEEN HUNTINGTIN INTERACTING PROTEIN 1 (HIP1) AND RECEPTOR TYROSINE KINASES IN CANCER

By

Grace Y. Liu

An Honors Thesis submitted in partial fulfillment of the requirements for the Honors concentration in Cellular & Molecular Biology

Department of Molecular, Cellular and Development Biology
College of Literature, Science & Art
Honors Program
University of Michigan

April, 2009

Thesis Advisor:
Theodora Ross, MD/PhD
Associate Professor, Internal Medicine Hematology/Oncology

Thesis Co-Sponsor:
Matthew Chapman, PhD
Assistant Professor, MCDB

Thesis Reader:
Erik Nielsen, PhD
Assistant Professor, MCDB

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ACKNOWLEDGEMENTS

First and foremost, I thank my thesis advisor Dr. Theodora Ross, MD/PhD for her academic mentorship and personal support in the past three years. Her broad scope of knowledge in science and medicine, endless scientific enthusiasm, diligent academic attitude, and consistent and disciplined work ethic have all been great inspirations to me as a young scientist and an aspiring physician. I especially appreciate her early efforts to engage me in projects. Even as a freshman, I was entrusted with interesting and meaningful projects and received enormous support and feedback from her. The skills and confidence that I gained from these positive early experiences with science have been immensely influential to my decisions to pursue an honors degree in Cell & Molecular Biology and a career in academic medicine.

I thank Heather Ames, a current student of the MD/PhD program at the Medical School, for her mentorship and friendship in the past year. My thesis is a part of Heather's PhD thesis project, and we worked in tandem throughout most of the year. Heather's attention to details, methodical scientific approach, and keen understanding of scientific ideas have guided me in the right direction in moments of frustration and confusion.

Many others have contributed greatly to the materials presented in this thesis and to my intellectual growth. Sarah Bradley provided guidance in my initial work on the association between HIP1 and EGFR in 2007. Brendan Crawford, whose scientific knowledge and technical skills I greatly admire, helped me tremendously in subcloning IGF-1R. Chiron Graves and Steve Philips have taught me concepts and techniques that

are peripheral to this project but meaningful to my overall scientific education. Kay Oravecz-Wilson was helpful in the logistics of many experiments, and Alice Gauvin and Tina Wang performed genotyping for the HIP1 transgenic mice, the tissues from which served as the samples of some experiments.

I thank the University of Michigan Undergraduate Research Opportunities Program for awarding me the Biomedical Summer Fellowship, which partly funded this work. This thesis contains data that is being written for publication and data already published in the journal Cancer Research:

BRADLEY, S. V., HOLLAND, E. C., LIU, G. Y., THOMAS, D., HYUN, T. S. & ROSS, T. S. (2007a). Huntingtin interacting protein 1 is a novel brain tumor marker that associates with epidermal growth factor receptor. *Cancer Res* **67,** 3609-15.

Finally, I thank my parents, Rong and Xiaoming, for their unconditional faith and support throughout my educational pursuits, and my boyfriend George Ni, for being understanding and tolerant of my hectic schedules.

ABSTRACT

Huntingtin Interacting Protein 1 (HIP1) is a lipid-, actin-, and clathrin-binding endocytic protein. It is required for the growth of normal cells and, when over-expressed, transforms fibroblasts. To study its mechanism of transformation, we focused on the interactions between HIP1 and receptor tyrosine kinases for several reasons. In human brain cancer, the overexpression of HIP1 correlates with that of EGFR, a well-characterized receptor tyrosine kinase that is implicated in a variety of human epithelial tumors; elevated HIP1 expression stabilizes EGFR; and in HIP1-transformed fibroblasts, the trafficking and signaling capacities of membrane receptors are altered. We hypothesized that HIP1 interacts with EGFR to mediate cellular changes, and experimentally identified that HIP1 indeed associates with EGFR. We then proceeded to test the hypothesis that HIP1 interacts with other receptor tyrosine kinases and found that HIP1 interacts with IGF-1R and c-Kit, two receptors which demonstrate high expression levels in the skin malignancy Merkel cell carcinoma, a cancer type that also highly expresses HIP1 but lacks EGFR.

The involvement of endocytic proteins in tumorigenesis is a newly emerging feature of cancer biology. These proteins have the potential to serve as molecular targets for cancer therapy, affecting multiple receptors and signaling cascades simultaneously. The finding that HIP1 interacts with multiple receptor tyrosine kinases validates HIP1 as a candidate for such approach.

INTRODUCTION

Brief overview of cancer

Tumorigenesis is a multi-step process by which normal cells are transformed to cancer cells. It results from mutations, acquired spontaneously or through external carcinogens, which have escaped DNA repair mechanisms. Cancer cells are characterized by growth factor-independence growth and evasion of apoptosis, which lead to the hallmark of limitless and unregulated proliferative potential. Benign tumors exhibit these altered properties but remain constrained to their original locale. In malignant transformation, beyond the basic properties, cells acquire mutations that enable them to sustain angiogenesis and become invasive.

There are several currently known mechanisms of transformation. There are gain-of-function mutations in proto-oncogenes and loss-of-function mutations in tumor-suppressor genes. Among the proteins encoded by proto-oncogenes are growth-promoting protein ligands and their corresponding receptors, members of the signal transduction pathway, transcriptional and translational regulators, and auto-apoptotic proteins. The current investigation focuses on the interactions between receptor tyrosine kinases, well-known tumorigenic proteins that initiate multiple signal transduction pathways, and Huntingtin-Interacting Protein1 (HIP1), one of the first endocytic proteins implicated in cancer.

Receptor tyrosine kinases in oncogenic transformation

Receptor tyrosine kinases (RTK) are transmembrane proteins on the cell surface that respond to extracellular growth-promoting ligands and relay intracellular signals. RTKs that are commonly associated with cancers include the epidermal growth factor receptor (EGFR), insulin-like growth factor 1 receptor (IGF-1R), and c-Kit, all of which have been the subject of my thesis project. Binding to ligands activates the receptors through the dimerization and auto-phosphorylation of the cytoplasmic kinase domains. Phosphorylated tyrosine residues serve as docking sites for the recruitment of various catalytic and scaffolding proteins, which transduce signals that ultimately lead to proliferative or anti-apoptotic cellular responses.

Endocytosis of the receptor-ligand complex and the subsequent lysosomal degradation is the principal mechanism of signal termination (Figure 1A, left) (Di Fiore & De Camilli, 2001). The ubiquitin ligase c-Cbl is one of the proteins recruited by active receptors and marks the receptor-hormone complex for internalization into clathrin-coated vesicles (CCV), with the help of several coat and adaptor proteins. CCVs then fuse with other vesicles to become early endosomes. It is important to note that the receptors remain activated, thus signaling continues, until the limiting endosomal membranes are sequestered into the intraluminal space of multivesicular bodies. This sorting, facilitated again by mono-ubiquitination, is specific for receptors destined for lysosomal degradation. Unsorted receptors recycle back to the plasma membrane.

While parts of molecular mechanisms of receptor activation and down-regulation remain

unclear, it is evident that this pathway harbors the potential to cause cancer. In fact, the over-expression and/or dysregulation of dozens of RTKs have been implicated in various human cancers (Floyd & De Camilli, 1998; Rosell, 2007).

The mechanisms of RTK dysregulation involve either over-activation or lack of deactivation of the receptors (Bache, Slagsvold & Stenmark, 2004). Genetic alterations that cause ligand-independent receptor autophosphorylation lead to constitutive activation and neoplastic growth. Disruptions in receptor-ligand internalization and subsequent degradation also result in sustained proliferative signaling (Figure 1B, right). The latter case is substantiated by a recently generated interest in the alterations of endocytic proteins such as HIP1.

HIP1 in endocytosis

HIP1 is the mammalian orthologue of the yeast Sla2p, a protein component of the cortical actin cytoskeleton and involved in the regulation of endocytosis. It was initially identified through a yeast two-hybrid screen as a binding partner of huntingtin (Kalchman et al., 1997), the protein altered in the neurodegenerative Huntington's Disease. Since then, HIP1 has been shown to be a novel and important component of the endocytic machinery.

All known members of the Sla2p/End4 family of proteins are involved in actin binding and membrane trafficking (Chen & Brodsky, 2005). Several groups have independently shown that HIP1 interacts directly with the light chain of clathrin through a LMDMD

domain (Figure 1B) (Chen & Brodsky, 2005; Legendre-Guillemin et al., 2002; Metzler et al., 2001; Waelter et al., 2001). A variety of other endocytic proteins possess related sequences that mediate their interactions with clathrin (Metzler et al., 2001). The disruption of this association abrogates clathrin assembly activity (Chen & Brodsky, 2005). In addition to clathrin, HIP1 contains a classic DPF motif that mediates its association with AP2 (Metzler et al., 2003), an important element of the adaptor complex that facilitates the formation of CCVs. Furthermore, HIP1 associates with the cytoskeletal F-actin through its TALIN homology domain (Engqvist-Goldstein et al., 1999). It is also worth noting that HIP1-related (HIP1r), the only known mammalian homologue of HIP1, also associates with clathrin and actin, but not with AP2 or huntingtin (Seki et al., 1998).

Aside from direct associations with endocytic and cytoskeletal proteins, HIP1 also binds 3-phosophoinositol lipids through its AP180 N-terminal homology (ANTH) domain and has been shown to be localized to endocytic vesicles and enriched in CCVs (Metzler et al., 2001). Thus, HIP1's participation in endocytosis is well-established.

HIP1 in cell survival and tumorigenesis

As a trafficking protein, HIP1 is involved in the survival and normal function of many cell types. In mice, the inactivation of HIP1 leads to spinal and hematopoietic defects (Oravecz-Wilson et al., 2004), and wild type HIP1 is essential for the differentiation of spermatogenic progenitors (Rao et al., 2001). In Huntington's disease, the mutant huntingtin protein has weakened interactions with HIP1, suggesting that Huntington's Disease may be partially due to the dysregulation of normal HIP1 functioning in the brain

(Kalchman et al., 1997; Rao et al., 2001).

Evidence accumulated more recently has identified HIP1 as one of the first endocytic proteins directly involved in tumorigenesis (Rao et al., 2003). The over-expression of HIP1 in NIH/3T3 cells transforms them as assayed by growth in soft agar assays, and the subcutaneous injection of HIP1-transformed cells initiates tumors in nude mice (Rao et al., 2003). HIP1 was first implicated in cancer when it was identified as the chromosome 7 break point region in a t(5;7) chromosomal translocation in a human patient with chronic myelomonocytic leukemia. The chromosome 5 break point was the platelet-derived growth factor beta receptor (PDGFβR) gene (Ross et al., 1998). Translocations involving the PDGFβR, a RTK, produce cytoplasmic fusion kinases that are constitutively active. For example, in the t(5;7) translocation, the HIP1/ PDGFβR fusion is cytoplasmic protein that contains constitutive kinase activity. Interestingly, the transforming properties of this fusion protein depend on the intact structure of HIP1 as well as the kinase domain of the PDGFβR (Ross & Gilliland, 1999).

Subsequent to discovery of the HIP1/ PDGFβR fusion in cancer, HIP1 has been found to be elevated in a variety of human cancers, including prostate, colon, breast, brain, lymphoid, and skin cancers. In fact, the overexpression of HIP1 serves as a reliable marker of brain tumors, including glioma, oligodendroglioma, and glioblastoma (Bradley et al., 2007a). In prostate cancers, HIP1 expression level correlates with relapse after prostatectomy (Rao et al., 2002). More recent studies have demonstrated that anti-HIP1 serum antibody levels can distinguish between localized and metastatic Merkel cell

carcinoma (unpublished). Additionally, the level of HIP1 auto-antibodies in the blood serum is significantly increased in both mice and human patients with prostate and brain cancers (Bradley et al., 2007a; Bradley et al., 2005), findings that provide a new HIP1-based blood test for the diagnosis and prognosis of these cancers. Thus, studying HIP1 in cancer is relevant to not only understanding the molecular and cellular mechanisms in tumorigenesis, but also in identifying and improving existing clinical tools.

HIP1 and receptor tyrosine kinase

In trying to elucidate the mechanism by which HIP1 causes transformation, receptor tyrosine kinases came to our attention when it was discovered that HIP1-transformed cells had altered EGFR regulation (Rao et al., 2003); these cells showed over-expression and over-activation of EGFR under normal growth conditions. In subsequent experiments, we found that high levels of HIP1 can stabilize EGFR by inhibiting its degradation post stimulation (Hyun et al., 2004). In human brain tumors, HIP1 over-expression correlates with over-expression of EGFR and PDGFβR (Bradley et al., 2007a).

In this study, we investigated the physical interactions between HIP1 and three RTKs – EGFR, IGF-1R, and c-Kit. We also discuss the biological significance of these interactions in cancer and consider a mechanism by which the trafficking of receptors in normal and neoplastic cells is dependent upon the levels of HIP1 expression.

Epidermal Growth Factor Receptor

Members of the ErbB family of receptor tyrosine kinases, such as the epidermal growth factor receptor (EGFR), or ErbB-1, have been found to be expressed and altered in a variety of epithelial human cancers, including the head and neck, pancreas, kidney, colorectal, breast, ovary, prostatic, bladder, brain, and lung (Sibilia et al., 2007). EGFR dimerization activates multiple downstream signaling pathways, including Ras/ERK, STAT, and the PI3K/Akt pathways (Bianco et al., 2007). The mechanisms of EGFR-associated oncogenic activation are well-studied and include processes that result in the overexpression of EGFR, autocrine ligand expression, altered heterodimerization and cross-talk with other receptors, and unsuccessful receptor downregulation (Zandi et al., 2007). Due to its frequent activation in cancer, EGFR is the target of many drugs used to treat several human malignancies (Klein & Levitzki, 2009). The EGFR inhibitors include monoclonal antibodies that competitively bind to the receptor, small molecule tyrosine kinase inhibitors that disrupt tyrosine phosphorylation, and drugs that target transduction molecules downstream of EGFR (Klein & Levitzki, 2009).

In this paper, we present evidence for the direct binding of HIP1 to EGFR (Bradley et al., 2007a), an interaction that implicates HIP1 in neoplastic biology. We discuss the biological implications of this interaction in the initiation and progression of cancer.

Insulin-Like Growth Factor 1 Receptor

IGF-1R is a RTK that has a unique tetrameric structure. It has one extracellular alpha-subunit and one transmembrane β-subunit that possesses tyrosine kinase activity (Adams et al, 2000). IGF-1R is activated by the insulin-like growth factor 1 (IGF-1), a polypeptide hormone similar in structure to insulin. Insulin and IGF-1 have critical roles in regulating cellular proliferation and apoptosis in relation to diet (Pollak, Schernhammer & Hankinson, 2004). IGF-1R was once thought to be a redundant receptor whose function overlaps with IR, but IGF-1R has characteristics distinct from IR. In humans, IGF-1R is widely expressed in normal tissues. It functions in cellular proliferation, transformation, and survival, functions that render the IGF-1R particularly significant in cancer (Baserga, Peruzzi & Reiss, 2003). Its over-expression induces tumor formation (Lopez & Hanahan, 2002) and metastasis presumably by sending proliferative signals required for transformation (Jones et al., 2007).

Since we have found that HIP1 is elevated in the human skin malignancy Merkel cell carcinoma, which overexpresses IGF-1R, we posited that HIP1 and IGF-1R interact. Indeed, we found that HIP1 physically interacts with IGF-1R in mammalian cells. We discuss the functional significance of this interaction as it relates to Merkel cell carcinoma.

c-Kit Receptor

The receptor c-Kit, also known as CD117, binds stem cell factor (SCF), also known as mast cell factor. It is a surface marker of hematopoietic stem cells, and the Kit-SCF combination is required for the normal differentiation of hematopoietic cells (Edling & Hallberg, 2007). The overexpression and overactivation of Kit is associated with some cancer types, most commonly gastrointestinal stromal tumors and myeloid leukemia (Boissel et al., 2006). The Kit tyrosine kinase activates an array of biochemical signaling cascades including the phosphoinositide 3'kinase, JAK-STAT, Ras-Erk, SFK, and phospholipase C pathways (Lennartsson et al., 2005).

In the human disease Merkel cell carcinoma, HIP1 and c-Kit are expressed at high levels. Because of this, we propose the hypothesis that c-Kit and HIP1 interact with each other. We have begun testing if, upon SCF stimulation, HIP1 physically associates with c-Kit receptors and discuss the functional significance of this proposed interaction in the context of cancer induction and maintenance.

MATERIALS & METHODS

Animal Tissue Samples:

Transgenic mice with human HIP1 overexpression (Bradley et al., 2007b) were sacrificed and their liver tissues harvested for protein extraction. After confirming the high expression of human HIP1 and high expression of EGFR in the tissues, co-immunoprecipitation experiments were performed with 1 ml of extracts at 2 mg/ml protein concentration.

Expression Plasmids:

The full length IGF-1R cDNA was subcloned from a non-expression vector into the expression vector pcDNA3. The IGF-1R cDNA-containing pCR-XL-TOPO vector (NIH MGC 348) was purchased and amplified in DH5a bacterial cells, followed by DNA extraction and purification (maxiprep). The plasmid and empty pcDNA3 vector were digested with EcoRI restriction enzyme overnight at 37^{o}C. The IGF-1R sample was then subjected to agarose gel electrophoresis, and the IGF-1R band (4.5 kb) was extracted under ultraviolet light and purified (Qiagen PCR Purification Kit). The empty pcDNA3 vector was dephosphorylated with calf intestine phosphatase for 30 minutes at 37^{o}C and purified. IGF-1R cDNA was ligated into pcDNA3 vector by incubation with T4 ligase for 1 hour at 37^{o}C. Following transformation of the mixture into DH5a cells, 40 colonies were screened to give one colony containing the IGF-1R cDNA insertion in the correct orientation (Figure 3C). The pcDNA3-IGF-1R construct has been confirmed by the University of Michigan sequencing core. The pcDNA3-HIP1 construct had been

previously cloned. The pRKS-EGFR and pCMV6-c-Kit (Origene) expression constructs were commercially purchased.

Tissue Culture and Transfection:

The human embryonic kidney cell line 293T was used for all of the transfection experiments. The cells were maintained at 37^{o}C in a 10-cm dish with medium containing DMEM, 10% FBS, Penicillin/Streptomycin, and 1mM sodium pyruvate until transfection at 70% confluence. Cells are transfected with 20 ug of total DNA and 60 uL of Superfect reagent (Qiagen). Approximately 24 hours after transfection, the cells were lysed for protein exraction.

Lysate Preparations:

Both animal tissues and cultured cells are lysed using an all-purpose lysis buffer containing 50 mmol/L Tris (pH 7.4), 150 mmol/L NaCl, 1% Triton X-100, 1.5 mmol/L $MgCl_2$, 5 mmol/L EGTA, 10% glycerol, complete EDTA-free protease inhibitor tablets (Roche), 30 mmol/L sodium pyrophosphate, 50 mmol/L NaF, and 100 umol/L sodium orthovandate. To ensure maximum protein extraction, cells were manually ground approximately 50 times with plastic pestles and incubated for 1 hour in 4^{o}C with rotation. The mixture was centrifuged for 15 minutes in 4^{o}C at 13,200 rpm, and the supernatant stored in -80^{o}C.

Co-Immunoprecipitations:

One milligram of protein lysate was pre-cleared by incubating with 20 microliters of the appropriate beads for 30 minutes in 4^{o}C with rotations. After centrifuging the mixture, the protein-containing supernatant was incubated with rabbit pre-immune serum or the appropriate polyclonal antibodies overnight at 4^{o}C with rotation. One hundred microliters of a 50:50 slurry of beads and buffer were added to the protein-antibody mixture for 1 hour at room temperature with rotation. The pellet, containing bead-protein complexes, is washed three times by mixing with lysis buffer and centrifuging for 5 minutes in 4^{o}C at 13,200 rpm. The pellet is then dissolved with 20 microliters of 6X SDS sample buffer, boiled for 5 minutes, and subjected to Western Blot analysis.

Beads and antibodies in immunoprecipitation:

For EGFR co-immunoprecipitation with HIP1: Protein G sepharose beads and UM323, a rabbit polyclonal antibody against the C-terminal end of HIP1, were used to pull down HIP1.

For IGF-1R co-immunoprecipitation with HIP1: Trueblot beads (Bioscieces) and UM410, a rabbit polyclonal antibody against the C-terminal end of HIP1 (similar to UM323), were used to pull down HIP1.

For HIP1co-immunoprecipitation with IGF-1R: Protein A sepharose and rabbit anti-IGF-1Rβ (Santa Cruz Biotechnology) were used to pull down IGF-1R.

For c-Kit co-immunoprecipitation with HIP1: Protein G sepharose and UM410 were used to pull down HIP1.

Western Blot Analyses:

Immunoprecipitated pellets were electrophoresed on 6% SDS-PAGE and transferred to nitrocellulose membranes. The membranes were incubated in 20 milliliters of blocking solution (1X TBS-T with 5% non-fat dry milk) one hour at room temperature with rocking. Membranes were then incubated with solution containing the appropriate primary antibody overnight at 4^{o}C with rocking, washed three times with 1X TBS-T, incubated with solution containing HRP-conjugated secondary antibody for 1 hour at room temperature, and washed three times with 1X TBS-T. Chemiluminescent substrate (Thermo Scientific) was used to develop film to visualize the protein bands on the nitrocellulose.

Mouse monoclonal antibody 4B10 was used for HIP1 detection; sheep polyclonal antibody (Upstate Biotechnology) for EGFR; rabbit polyclonal anti-IGF-1Rα (Santa Cruz Biotechnology) for IGF-1R; and mouse monoclonal anti-c-Kit (Cell Signaling) for c-Kit.

RESULTS

HIP1 associates with EGFR

In investigating the interactions between HIP1 and RTKs in oncogenic transformation, we first focused on EGFR. In human glioblastomas, HIP1 overexpression correlated with EGFR overexpression, so we tested if there was physical association between the two. The liver lysates of a human HIP1 transgenic mouse line were used for this analysis because levels of EGFR in murine liver were higher than in other tissues, and HIP1 was expressed at high levels, conditions that were necessary for the detection of the endogenous interaction. Shown in Figure 2 (Bradley et al., 2007a), a small fraction of the total endogenous EGFR co-immunoprecipitated with HIP1 (lane 2). This result has since been reproduced in multiple independent experiments in lysates from HIP1 transgenic murine tissues and cultured HEK293T cells (data not shown). Efforts to identify the EGFR-binding domain of HIP1 are ongoing.

HIP1 associates with IGF-1R

Next we wanted to investigate if HIP1 associates with other RTKs. In a simultaneous study into the expression of HIP1 in Merkel cell carcinoma (MCC), we found that HIP1 is over-expressed in the tumor (Figure 3) and hypothesized that HIP1 and IGF-1R may interact, since both proteins are elevated in the disease.

Indeed, with brain lysates of the human HIP1 transgenic mouse line, a small fraction of endogenous IGF-1R co-immunoprecipitated with HIP1 (Figure 5A). We next wanted to confirm this interaction by the reverse of this approach and determine if HIP1 could be co-immunoprecipitated with IGF-1R.

Due to the limited availability of murine tissues that express high levels of HIP1 and IGF-1R, we wanted to confirm this interaction in transfected HEK293T cells. Prior to being able to perform the assay, it was necessary to subclone the IGF-1R cDNA into the expression vector pcDNA3. The cloning scheme (Figure 4A) and subsequent selection of the correct clone (Figure 4B, C) are described in detail in the Materials and Methods section.

With protein extracts from the HIP1 and IGF-1R co-transfected 293T cells, HIP1 was co-immunoprecipitated with the IGF-1Rβ subunit. This result has been reproduced in several independent experiments with cell lysates obtained from independent transfections. Thus, we have confirmed that HIP1 and IGF-1R physically associate with each other as assayed by two different approaches.

HIP1 associates with c-Kit receptor

We next examined if HIP1 interacts with the c-Kit receptor, another RTK that is co-expressed at high levels of MCC. Since c-Kit is normally stimulated by stem cell factor (SCF), a cytokine present only in trace amounts in standard HEK growth media, we were also interested in seeing if there was a change in binding affinity between SCF-stimulated and SCF-deficient cells. In hematopoietic stem cells, the stimulation of c-Kit by SCF is important for the survival, growth, and differentiation of the cells. Thus, we hypothesized that, if the mechanism of HIP1-mediated tumorigenesis is dependent on its interactions with growth factor receptors, then HIP1 may bind to c-Kit more strongly under the proliferative SCF-stimulated conditions.

In HIP1 and c-Kit co-transfected 293T cells, we have begun investigating the interactions between the two proteins in the presence or absence of SCF. In preliminary studies, we have found that, upon SCF stimulation, c-Kit does co-immunoprecipitate with HIP1 (Figure 6A). We have not found interaction of c-Kit with HIP1 in the absence of SCF stimulation (6B). I emphasize that this data is preliminary as replicate experiments are still pending.

DISCUSSIONS

We report that HIP1 physically interacts with EGFR, IGF-1R and c-Kit. We hypothesized that, possibly independent of its participation in trafficking, HIP1 can affect and bind multiple receptors. In fact, a recent study by another group has demonstrated that HIP1 binds the fibroblast growth factor receptor 4 (FGFR4) (Wang et al., 2008). Studies are ongoing to determine the association between HIP1 and other RTKs such as the PDGFβR.

We and others (Wang et al.) have reported that HIP1 prolongs the half-life of RTKs such as the EGFR and FGFR4 following ligand-mediated stimulation by preventing protein degradation (Bradley et al., 2007a; Wang et al., 2008). These data suggest that HIP1 may directly interact with receptors to prolong their half-lives and increase cellular levels. This function may or may not be independent of HIP1's role in receptor mediated endocytosis.

Currently there exist cancer therapy approaches targeting RTKs that are altered in different cancers. Trastuzumab (Herceptin) treats breast cancer by interfering with the HER2/neu receptor, and Imatinib (Gleevec) competitively binds the tyrosine kinase site on various RTKs and inhibits their activity in leukemia. But targeting individual RTKs may not be fruitful in all cancers, as concomitant upregulation of multiple distinct RTKs may be the consequence of an oncogenic alteration, such as HIP1 upregulation. Thus, it is necessary to identify additional molecules that can serve as markers of prognosis and

targets of therapy. We believe that HIP1 has the potential to be such a molecular marker and target that can affect parallel changes in multiple RTKs and signaling pathways. HIP1 is overexpressed in many cancers, its over-expression causes cellular transformation associated with altered EGFR signaling, and it post-translationally stabilizes EGFR. Thus, HIP1 as an endocytic protein defines a relatively novel cellular pathway to consider targeting as we design new cancer therapies.

In addition to HIP1, other components of the trafficking machinery, including CBL (Abbas et al., 2008; Caligiuri et al., 2007; Sargin et al., 2007), SNAP91 (Dreyling et al., 1996), EPS15 (So et al., 2003), RAB25 (Cheng et al., 2004), and caveolin 1 (Shatz & Liscovitch, 2008), have been found to have oncogenic properties. These proteins regulate crucial features of signal transduction, both in amplitude and spatial distribution. It is likely that their effects extend beyond initiating cancer and may alter many processes involved in the maintenance and progression of cancer. Examples of additional effects include changes in extracellular matrix composition, establishment of cell polarity, and migration (Mosesson, Mills & Yarden, 2008).

To make HIP1 a possible target for therapy, it will be important to determine the domains on HIP1 that are necessary for binding to RTKs. Our lab has begun to map the EGFR-binding domain of HIP1 by expressing a series of HIP1 deletion mutants in 293T cells (Bradley et al., 2007a). These experiments demonstrated that HIP1 binds to EGFR independent of its lipid-, clathrin-, and actin-binding domains. Based on these mutant analyses, the EGFR binding domain is between amino acids 381-814. However, this

experimental approach, and the data inferred, may be problematic. The putative EGFR associating region includes a coiled-coil domain, a common protein binding motif that can serve as the site of HIP1 dimerization. When constructs with truncated HIP1 mutants are introduced to 293T cells, the endogenous intact proteins are produced along with the exogenous mutants. Thus, as long as the coiled-coil domain remains in the mutant protein, it can dimerize with an intact HIP1 partner, which may bind to EGFR.

More recently, the requirement of the coiled-coil domain of HIP1 has been reported in the HIP1/FGFR4 association (Wang et al., 2008). This group used a slightly altered technique. A series of HIP1 mutant proteins were expressed in bacteria, purified, and incubated with 293T lysates containing exogenous FGFR4. It was concluded that the region of HIP1 spanning amino acids 601-798, which contains the coiled-coil domain, is required for FGFR4 association. Similar to the approach of our lab, the use of lysates from 293T cells, and the possible dimerization between mutant and wildtype HIP1, is problematic in mapping the interaction domain.

Working toward availing HIP1 as a target for drug therapy, efforts are ongoing to definitively map the RTK-associating domain of HIP1. HIP1 deletion constructs will be introduced into cells which do not contain endogenous HIP1. The human promyelokemic leukemia cell line HL60 is the current candidate as it is HIP1-deficient (Figure 7) and transfectable (Olesen and Gough, 2006).

In conclusion, we found that HIP1 interacts with EGFR, IGF-1R and c-Kit receptor, three RTKs that are frequently altered in cancer. These findings identify HIP1 as a potential target of drug therapy in cancer that simultaneously affects multiple signaling pathways.

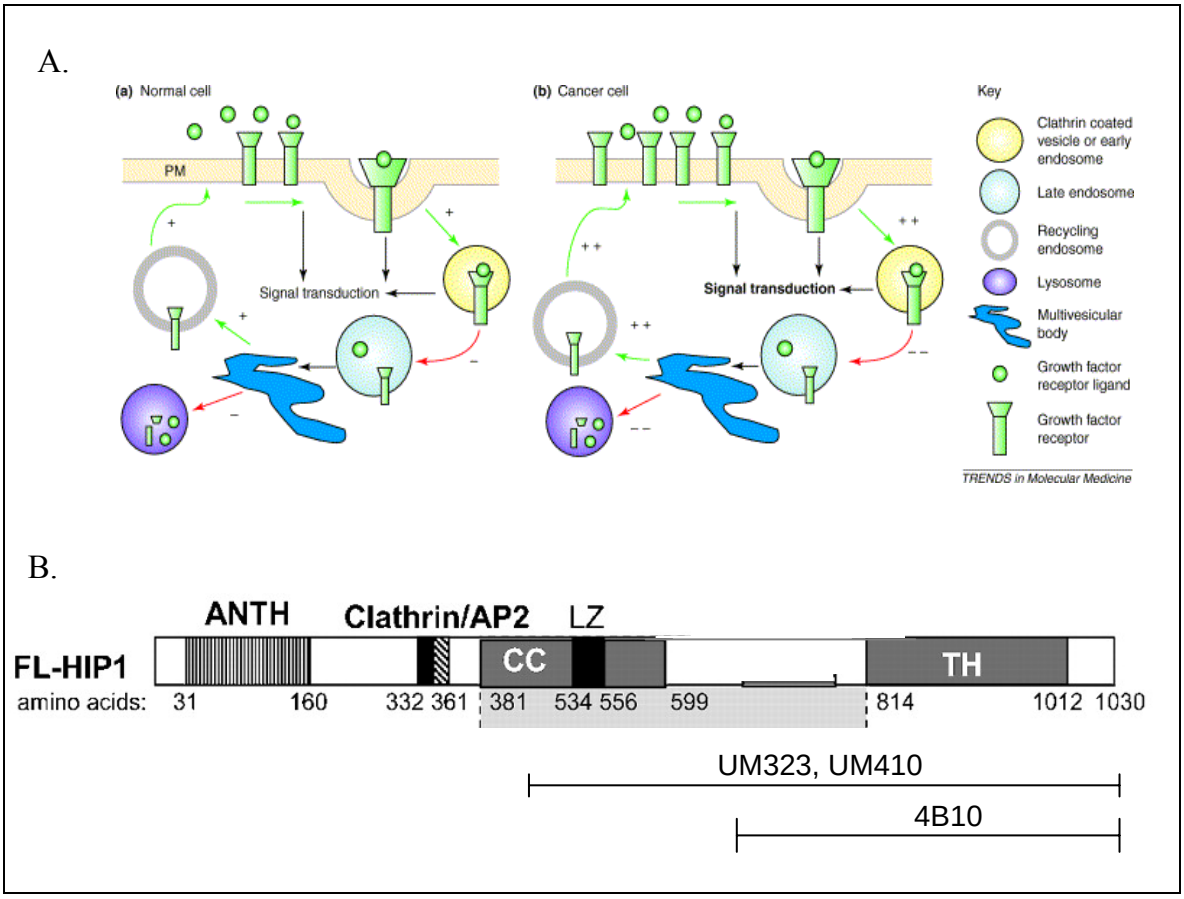


Figure 1. RTK trafficking and HIP1 domains.

A: CCV-mediated endocytosis of receptor tyrosine kinases in (a) normal cells and (b) cancer cells (Hyun & Ross, 2004). Abbreviations: PM, plasma membrane; +, possible positive regulation by HIP1; -, possible negative regulation by HIP1.

B: Structural domains of the full-length HIP1 protein and recognition sites for antibodies used in immunoprecipitation and Western blot experiments. ANTH: AP180 N-terminal homology; CC: coiled-coil; LZ: leucine zipper; TH: talin homology.

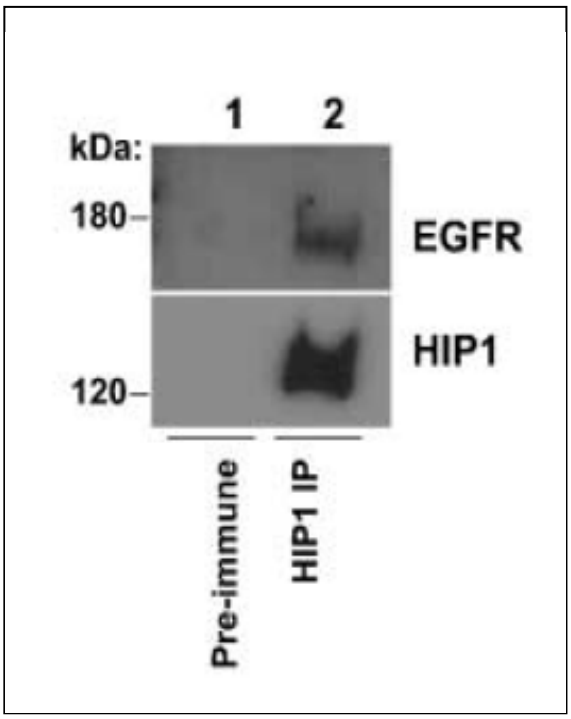
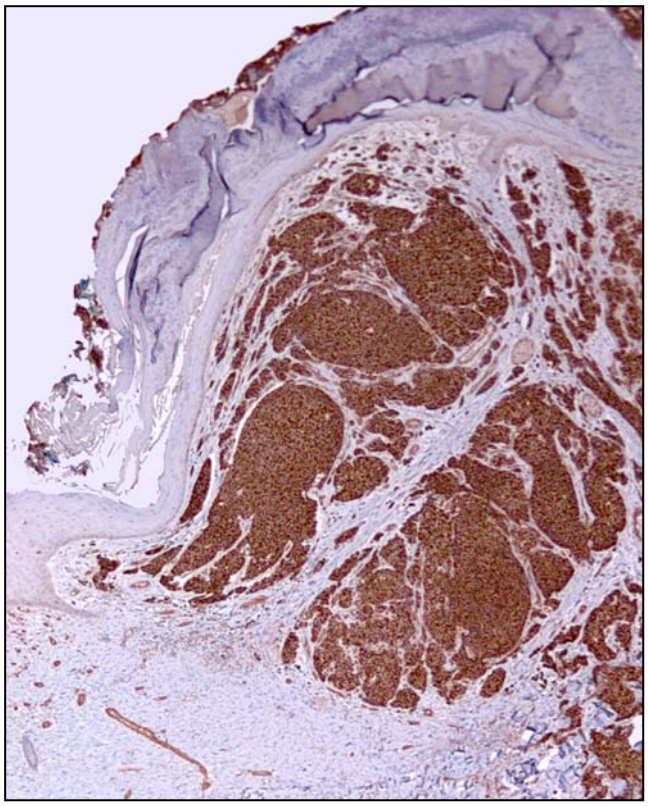


Figure 2. Association of HIP1 with EGFR.

Liver extracts from HIP1 transgenic mice were immunoprecipitated with either preimmune sera (lane 1) or UM323, a polyclonal antibody specific to the COOH-terminal of HIP1 (lane 2), electrophoresed b 6% SDS-PAGE and blotted for HIP1 and EGFR. The immunoprecipitated pellet contains both HIP1 and EGFR.



Merkel Cell Carcinoma

Figure 3. HIP1 in Merkel cell carcinoma

Compared to the surrounding cells, HIP1 stains intensely in cancerous Merkel cells.

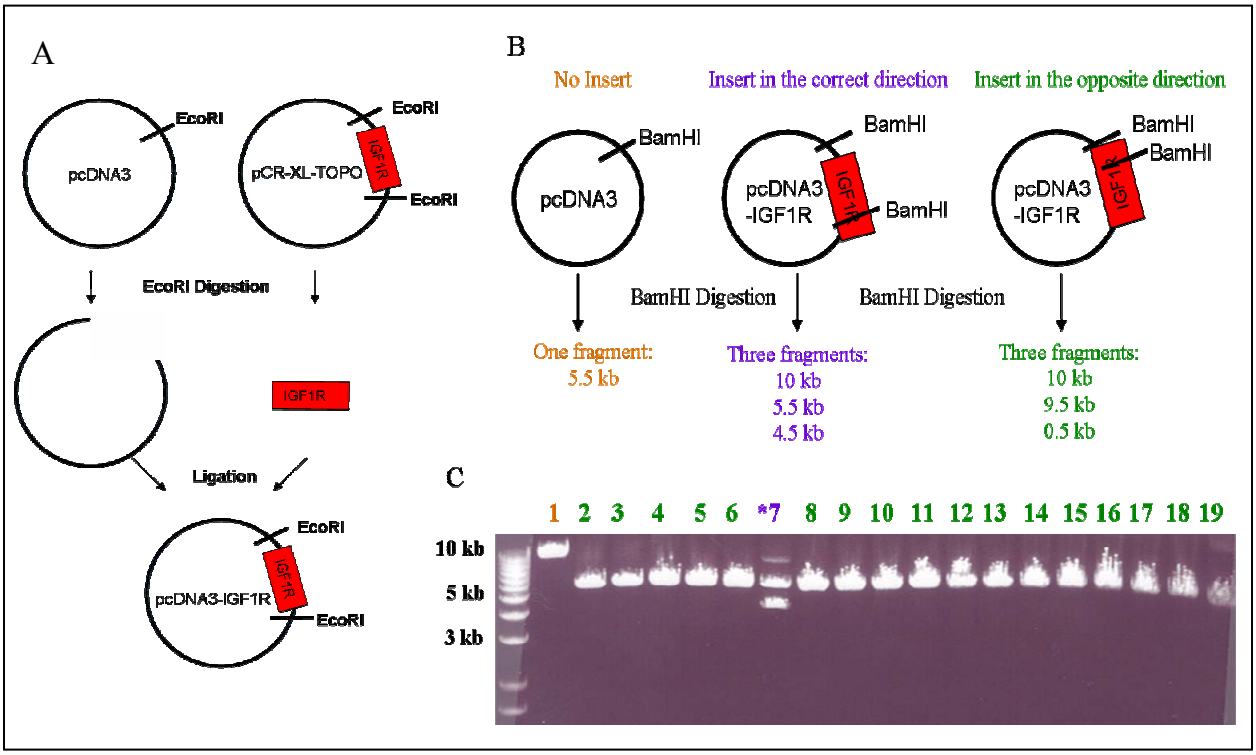


Figure 4. IGF-1R subcloning

A. Scheme for subcloning IGF-1R full length cDNA (4.5 kb) into empty pcDNA3 vector (5.5 kb) using EcoRI restriction enzyme digestion and ligation.

B. Scheme for the selection of the appropriate bacterial clone containing the IGF-1R cDNA insert in the correct orientation. Selection is based on the number and sizes of DNA fragments that remain after BamHI restriction enzyme digestion.

C. Clones were grown in culture medium and DNA was extracted, purified, digested by BamHI for 1 hour at 37^{o}C. Digested DNA was separated on a 0.8% agarose gel containing Ethidium Bromide and visualized under UV light. Of the 19 colonies shown, colony 7 contained the IGF-1R in the correct orientation, colony1 contained IGF-1R in the incorrect orientation, and the others contained no IGF-1R.

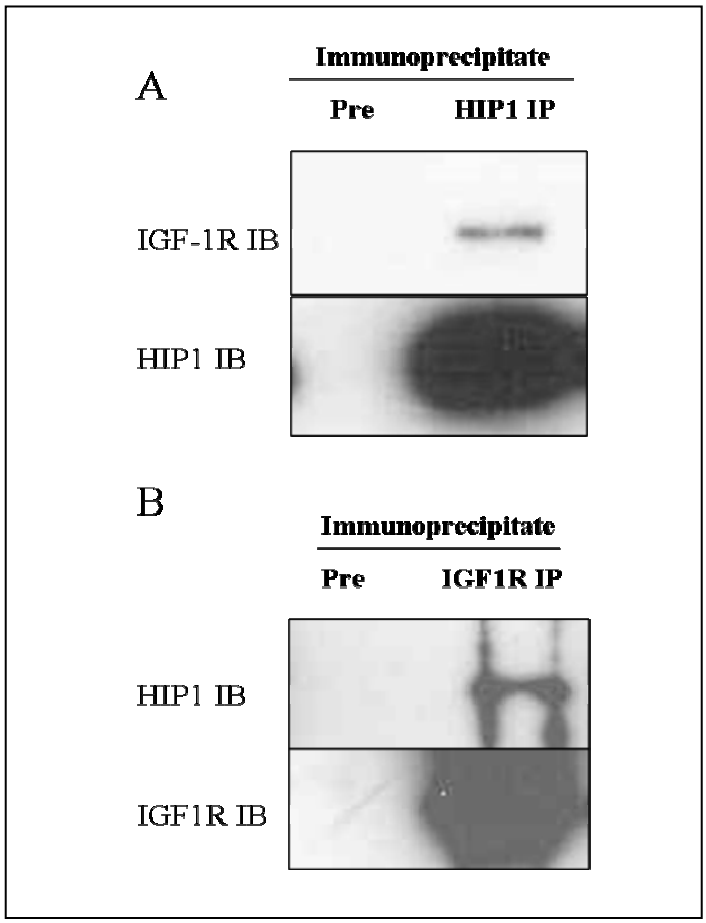


Figure 5. Association of HIP1 and IGF-1R.

A. Brain extracts from HIP1 transgenic mice were immunoprecipitated (IP) with either preimmune sera (lane 1) or UM410, a polyclonal antibody specific to the COOH-terminal of HIP1 (lane 2), electrophoresed by 6% SDS-PAGE and immunoblotted (IB) for HIP1 and IGF-1R. The immunoprecipitated pellet contains both HIP1 and IGF-1R.

B. Lysates of HIP1 and IGF-1R co-transfected HEK293T cells were immunoprecipitated with either preimmune sera (lane 1) or IGF-1Rβ (lane 2), electrophoresed by 6% SDS-PAGE and immunoblotted for IGF-1R and HIP1. The immunoprecipitated pellet contains both IGF-1R and HIP1.

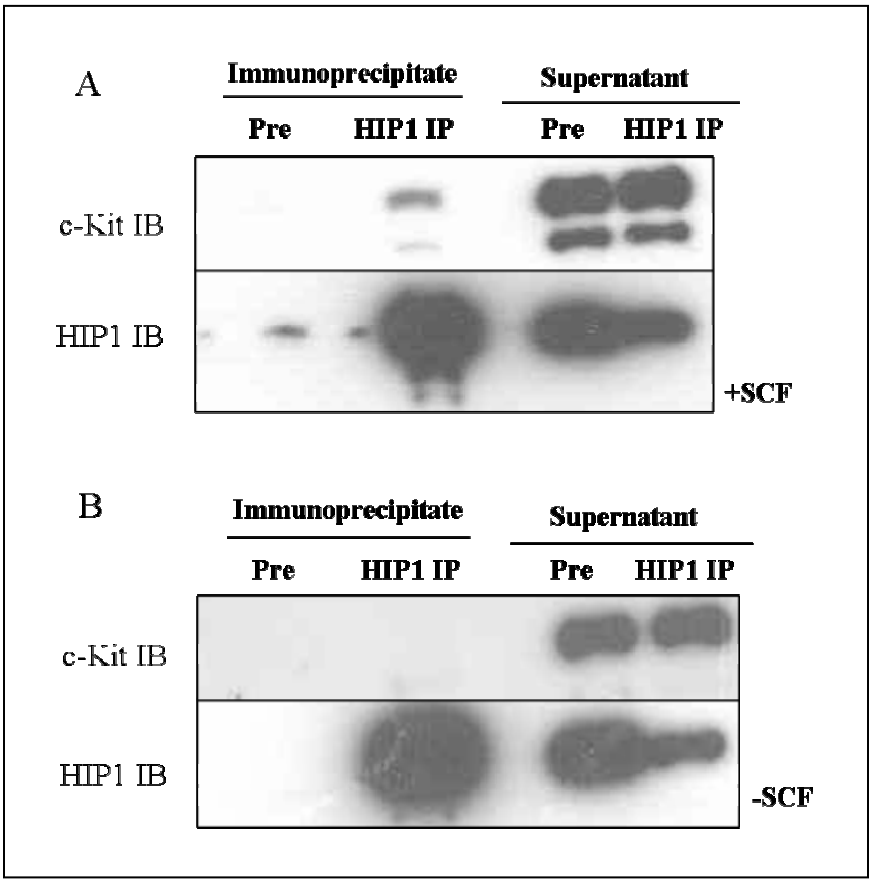


Figure 6. Association of HIP1 and c-Kit.

A. Lysates HEK293T cells, transfected with HIP1 and c-Kit in the presence of SCF, were immunoprecipitated (IP) with either preimmune sera (lane 1) or UM410 (lane 2), electrophoresed by 6% SDS-PAGE and immunoblotted (IB) for HIP1 and c-Kit. The immunoprecipitated pellet (lane 2) contains both HIP1 and c-Kit.

B. Lysates HEK293T cells, transfected with HIP1 and c-Kit in the absence of SCF, were immunoprecipitated with either preimmune sera (lane 1) or UM410 (lane 2), electrophoresed by 6% SDS-PAGE and immunoblotted for HIP1 and c-Kit. The immunoprecipitated pellet (lane 2) does not c-Kit.

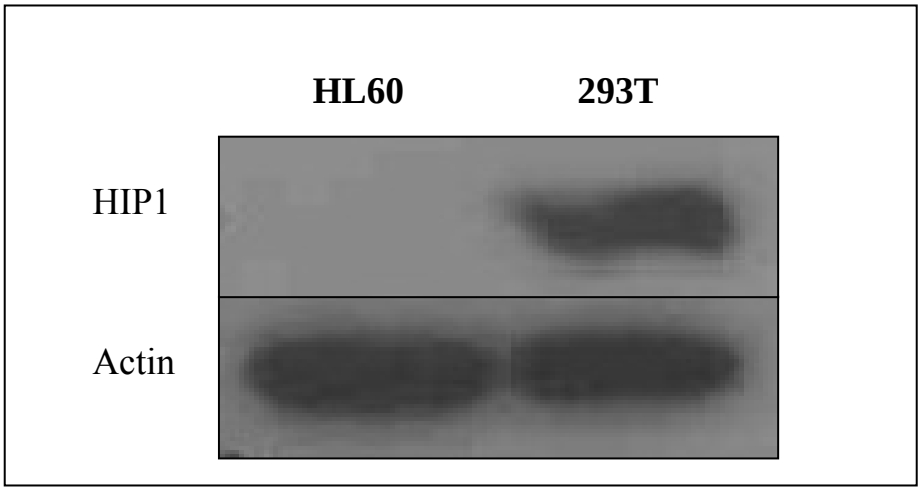


Figure 7. Expression of HIP1 in HL60 cell line.

In identifying possible cell lines in which to perform deletion mutant analyses to map the EGFR-associating region of HIP1, lysates of HL60 (lane 1) and 293T (lane 2) cells are compared by Western blot analysis. At comparable levels cytoplasmic protein, measured by actin, HIP1 expression in HL60 is significantly reduced from in 293T.

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