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CHARACTERIZATION OF THE PUTATIVE TUMOR SUPPRESSOR GENE ST7, A NOVEL MEMBER OF THE LOW-DENSITY LIPOPROTEIN RECEPTOR SUPERFAMILY

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By

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ABSTRACT

CHARACTERIZATION OF THE PUTATIVE TUMOR SUPPRESSOR GENE ST7, A NOVEL MEMBER OF THE LOW-DENSITY LIPOPROTEIN RECEPTOR SUPERFAMILY

By

Michele Ann Battle

It is widely accepted that cancer develops through a multi-step process involving a series of genetic and/or epigenetic changes that convert a normal cell into a cancer cell. Tumors originate from a single cell that has acquired all characteristics necessary to be able to produce a malignant tumor, and the cells of the tumor are the progeny of this first malignant cell. In an effort to identify the number and nature of these changes, McCormick and Maher (Risk Anal. 14: 257, 1994) established a model system in which normal human fibroblasts in culture can be transformed into malignant fibroblasts by acquiring a series of genetic changes, each conferring a growth advantage that allows sequential clonal expansion. Using this model system and differential mRNA display to compare an infinite life span, non-tumorigenic human fibroblast cell strain to one of its carcinogen-transformed, malignant derivatives led to the identification of a novel gene, designated *ST7*, that was not expressed in the malignant derivative and was absent or expressed at a low level in a series of malignant cell lines derived from patients' tumors. Subsequent discovery of a closely related protein suggested that ST7 is a novel member of the low-density lipoprotein receptor (LDLR) superfamily. Although proteins of this superfamily are best known as endocytic receptors, recent studies demonstrate that the cytoplasmic domains of

several members interact with adaptor and scaffold proteins implicated in signal transduction. To evaluate ST7's relationship to the proteins of this superfamily, I used proteomic tools to analyze the functional motifs present in ST7. The data indicate that ST7 is, indeed, a member of an LDLR subfamily and reveal that ST7's cytoplasmic domain contains motifs implicated in endocytosis and signal transduction. Using the yeast two-hybrid system, I determined that ST7's cytoplasmic domain, like that of some other LDLR superfamily proteins, interacts with several proteins related to signal transduction and/or endocytosis. To determine if ST7 can suppress tumor formation, a malignant cell line, SHAC, derived from a patient's fibrosarcoma was transfected with a plasmid encoding ST7 or a truncated form of ST7 protein, i.e., its extracellular and transmembrane domains, but only the first ten amino acids of its 343-residue cytoplasmic domain. The tumorigenicity of cell strains expressing these proteins was determined by injection into athymic mice. SHAC cells overexpressing full-length ST7 formed tumors at a significantly lower frequency than that of control cells. Moreover, the tumors that did develop exhibited a much greater latency. In contrast, overexpression of truncated ST7 failed to inhibit the tumor-forming ability of SHAC cells. These data support the hypothesis that ST7 is a tumor suppressor. Furthermore, the fact that expression of a truncated form of ST7 failed to inhibit the tumor-forming ability of SHAC cells strongly suggests that ST7's cytoplasmic domain plays an important role in the protein's function as a tumor suppressor.

DEDICATION

With love, I dedicate my dissertation to Mom,

Dad, Amy, Kelly, Lori, Scott, Andre, and Marshall.

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problem. In the final months of preparing my dissertation, although he was tremendously busy as a new assistant professor in Milwaukee, he found time to return to East Lansing to help me complete this task.

TABLE OF CONTENTS

LIST OF TABLES

Chapter II

Appendix

LIST OF FIGURES

Chapter I

Chapter II

Appendix

ABBREVIATIONS

APAF1,	apoptotic protease activating factor-1
APC,	adenomatous polyposis coli
apoB,	apolipoprotein B
apoE,	apolipoprotein E
BCL-2,	B-cell lymphoma 2
bHLHZ,	basic helix-loop-helix zipper
BPDE,	benzo(a)pyrene-diol-epoxide
cdk,	cyclin-dependent kinases
cDNA,	complementary DNA
CUB,	Complement factor C1s/C1r, Urchin embryonic growth factor, Bone morphogenetic protein
DCC,	deleted in colorectal carcinoma
DISC,	death-inducing signaling complex
DPC4,	deleted in pancreatic carcinoma 4
EGF,	epidermal growth factor
EGFR,	epidermal growth factor receptor
ERK,	extracellular signal-regulated kinase
FAK,	focal adhesion kinase
FAP,	familial adenomatous polyposis
FH,	familial hypercholesterolemia
FISH,	fluorescent in situ hybridization
FZ,	frizzled

GAP, GTPase activating protein

GEF, GDP exchange factor

GPI, glycosylphosphatidyl-inositol

G-protein, guanine-nucleotide binding protein

GSK-3β, glycogen synthase kinase-3β

GTPase, guanosine triphosphate hydrolase

HAT, histone acetyltransferese

HDL, high-density lipoprotein

HNPCC, hereditary nonpolyposis colorectal cancer

IDL, intermediate-density lipoprotein

LDL, low-density lipoprotein

LDLR, low-density lipoprotein receptor

LDLRA, low-density lipoprotein receptor class A

LRP, LDLR-related protein

MAP, mitogen-activated protein

MAPK, mitogen-activated protein kinase

Max, Myc-associated factor X

MDM2, murine double minute clone 2

MEGF7, multiple epidermal growth factor-like domains protein 7

MEK, MAP/ERK kinase

MIBP, muscle integrin binding protein

MLCK, myosin light chain kinase

MMP, matrix metalloproteinase

MMR, DNA mismatch repair

NSCLC, non-small cell lung carcinoma

OPPG, osteoporosis-pseudoglioma

PI3K, phosphatidylinositol 3-kinase

PAI-1, plasminogen activator inhibitor type-1

PDGF, platelet-derived growth factor

PDGFR, platelet-derived growth factor receptor

PEX, hemopexin

PKA, protein kinase A

PKC, protein kinase C

PTB, phosphotyrosine binding

RACK1, receptor for activated protein C kinase

RB, retinoblastoma

SARA, SMAD anchor for receptor activation

SD, synthetic dropout

SH2, Src homology 2

SH3, Src homology 3

SOS, son of sevenless

TGF, transforming growth factor

TNF, tumor-necrosis factor

tPA, tissue plasminogen activator

TRRAP, transforming/transcription domain associated protein

TSP-1, thrombospondin-1

uPA, urokinase plasminogen activator

uPAR, urokinase-type plasminogen activator receptor

VEGF, vascular endothelial growth factor

VLDL, very low-density lipoprotein

VLDLR, very low-density lipoprotein receptor

INTRODUCTION

It is widely accepted that human tumors originate from a single cell that has acquired all of the properties necessary to become malignant, and replication of that cell gives rise to all of the cells of the tumor. Theories of carcinogenesis must explain how the "first" tumor cell acquired the appropriate genetic and/or epigenetic changes required to render it malignant, which for tumors in adults are estimated to be five or more. Although tumors arise in virtually all tissues of the human body and each type of tumor displays some unique characteristics, the basic steps responsible for the conversion of a normal cell into a malignant cell are believed to be common. In a recent review, Hanahan and Weinberg (2000) propose six essential phenotypic characteristics that must be acquired if a normal cell is to become a malignant cell: i) the ability to proliferate without mitogenic signals; ii) the ability to evade growth-inhibitory signals; iii) the ability to evade apoptosis; iv) the ability to proliferate limitlessly; v) the ability to initiate and to sustain angiogenesis; and vi) the ability to invade tissue and to metastasize. Each successive change thwarts an "anticancer defense mechanism" inherent in every cell. The first section of Chapter I reviews the current model of the molecular mechanisms of the multi-step process of tumorigenesis, focusing on the six characteristics that are required for a cell to become tumorigenic. The second section reviews the structure and function of several key members of the low-density lipoprotein receptor (LDLR) superfamily and the relationship of the protein encoded by the novel gene ST7, discovered in the Carcinogenesis Laboratory in 1997, to this superfamily.

Chapter II consists of a manuscript describing my work characterizing the ST7 protein and identifying proteins that interact with its cytoplasmic domain. In 1997, when the novel gene, designated *ST7,* was identified by Jing Qing using differential mRNA display, she recognized that the ST7 protein had the characteristics of a transmembrane protein, but at that time no other proteins with significant similarity to ST7 had been reported. Approximately one year later, Yamamoto and his associates, using degenerate oligonucleotides corresponding to the highly conserved region of the ligand-binding domains found in the proteins of the LDLR superfamily, discovered a novel LDLR-related gene, which they designated *LRP3* (Ishii et al., 1998). Our search of databases for proteins structurally related to ST7, after the sequence of LRP3 had been submitted, revealed a very strong similarity between LRP3 and ST7, enabling us to recognize that ST7, although it differed significantly from the LDLR prototype, could be a novel member of the LDLR superfamily. Although proteins of the LDLR superfamily are best known as endocytic receptors (reviewed by Krieger and Herz, 1994 and Hussain et al., 1999), recent studies show that the cytoplasmic domains of several LDLR superfamily proteins interact with a variety of adaptor and scaffold proteins implicated in signal transduction (reviewed by Li et al., 2001; Herz and Bock, 2002; van der Geer, 2002). To evaluate ST7's relationship to the proteins of the LDLR superfamily, I used proteomic tools including similarity searches, sequence alignments, pattern and profile searches, and post-translational modification prediction programs to analyze the functional motifs present in ST7. Such analyses showed that ST7 is, indeed, a novel

member of the LDLR superfamily and that like several other LDLR superfamily proteins, ST7's cytoplasmic domain contains several motifs implicated in endocytosis and/or signaling. To identify proteins that interact with ST7's cytoplasmic domain, I used the yeast two-hybrid system. These studies showed that ST7's cytoplasmic domain interacts with several proteins related to signal transduction and/or endocytosis, suggesting that ST7 functions in these pathways.

The appendix describes research I carried out to test the hypothesis that ST7 functions as a tumor suppressor. A tumorigenic cell line derived from a patient's fibrosarcoma, designated SHAC, was transfected with a plasmid encoding full-length ST7 or an ST7 protein with a severely truncated cytoplasmic domain, i.e., containing ST7's extracellular and transmembrane domains, but only the first 10 of the 343 amino acids that comprise its cytoplasmic domain. The tumorigenicity of independent transfectants expressing these proteins, as well as that of independent vector-control transfectants and non-ST7-expressing control transfectants, was assessed by subcutaneous injection of these various cell strains into athymic mice. Tumors formed at a significantly lower frequency in athymic mice injected with SHAC cells expressing full-length ST7 than in athymic mice injected with any of the control cell strains. Any tumors that did develop exhibited a much greater latency. These data support the hypothesis that ST7 is a tumor suppressor. The fact that expression of a truncated form of ST7 failed to inhibit the tumor-forming ability of SHAC cells strongly suggests that the cytoplasmic domain of ST7 plays an important role in the protein's function as

a tumor suppressor. These studies are still in progress. When the final experiments have been completed, a manuscript describing the results will be submitted to *Cancer Research*.

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CHAPTER I

LITERATURE REVIEW

I. A review of the molecular mechanisms of tumorigenesis

A. Six types of phenotypic changes typically involved in cancer

Although tumors arise in virtually all tissues of the human body and each type of tumor displays some unique characteristics, the basic steps responsible for the conversion of a normal cell into a malignant cell are believed to be common. In a recent review, Hanahan and Weinberg (2000) discuss the molecular mechanisms that give rise to cancer. They propose six essential phenotypic characteristics that must be acquired if a normal cell is to become a malignant cell: i) the ability to proliferate without mitogenic signals; ii) the ability to evade growth-inhibitory signals; iii) the ability to evade apoptosis; iv) the ability to proliferate limitlessly; v) the ability to initiate and to sustain angiogenesis; and vi) the ability to invade tissue and to metastasize. Each successive change thwarts an "anticancer defense mechanism" inherent in every cell Hanahan and Weinberg, 2000). A discussion of these six hallmarks of cancer follows.

1. The ability to proliferate without mitogenic signals

Normal human cells proliferate in response to mitogenic signals. Such signals include soluble growth factors, extracellular matrix components, and cell-cell interactions (Hanahan and Weinberg, 2000). Unregulated cellular proliferation, often accomplished by mutating oncogenes, is one hallmark of tumor cells. Several types of oncogenic alterations can result in deregulation of

growth pathways, allowing these cells to proliferate in the absence of mitogenic signals. Tumor cells can synthesize growth factors that act in an autocrine fashion to stimulate their growth. Mutations in growth factor receptors can cause constitutive activation of these receptors. Mitogenic pathways can also become constitutively active because of mutations in downstream molecules responsible for transmitting mitogenic signals from the growth factor receptor to the cell's nucleus.

a. The proto-oncogene *MYC*

The study of transforming retroviruses has provided insight into human cancer. Many cellular proto-oncogenes were identified because they are homologues of viral oncogenes. The *c-MYC* proto-oncogene was identified as the cellular homologue of the viral oncogene *v-MYC* present in the genome of the avian myelocytomatosis retrovirus (Vennstrom et al., 1982). The *c-MYC* proto-oncogene plays a causal role in many types of human tumors (reviewed by Facchini and Penn, 1998). For example, translocations between chromosome 8 and chromosomes 2, 14, or 22, which have been observed in Burkitt's lymphoma, position the *c-MYC* proto-oncogene in close proximity to immunoglobulin enhancer elements, resulting in up-regulation of *c-MYC* gene expression (Solomon et al., 1991). Additionally, deregulation of *c-MYC* expression has been observed in breast carcinomas, colorectal carcinomas, cervical carcinomas, small cell lung carcinomas, osteosarcomas, glioblastomas, and melanomas (reviewed by Pelengaris et al., 2002).

The *c-MYC* gene encodes a transcription factor in the basic helix-loop-

helix zipper (bHLHZ) family (Kato et al., 1990). Through its bHLHZ domain, Myc heterodimerizes with Max, another bHLHZ protein (Blackwood and Eisenman, 1991; Kato et al., 1992; Amati et al., 1993a, 1993b). Myc-Max heterodimers bind to specific DNA promoter sequences to activate the transcription of genes that stimulate cellular proliferation and growth and to repress the transcription of genes that inhibit terminal differentiation. The interaction of Myc-Max heterodimers with other proteins in the cell determines whether transcriptional activation or repression occurs. For example, Myc-Max heterodimers activate transcription of the *CYCLIN D2* gene by recruiting the transforming/transcription domain associated protein (TRRAP) to the *CYCLIN D2* promoter (Bouchard et al., 2001). TRRAP is a component of a chromatin remodeling complex containing histone acetyltransferase (HAT) activity (McMahon et al. 2000). Histone acetylation changes the chromatin conformation, enabling transcription factors to gain access to promoter sequences (reviewed by Peterson, 2002). This facilitates the activation of *CYCLIN D2* expression by Myc-Max heterodimers. Conversely, to inhibit the expression of $p15^{INK4B}$ and $p21^{WAF1}$, Myc-Max heterodimers sequester the transcription factor Miz1 (Staller et al., 2001; Herold et al. 2002). The interaction of Myc-Max with Miz1 prevents Miz1 from binding to p300, a co-activator required for Miz1 transactivation of the $p15^{INK4B}$ and $p21^{WAF1}$ promoters.

The Mad family of bHLHZ transcription repressor proteins antagonizes Myc function (Ayer et al., 1993; reviewed by Grandori et al., 2000). By binding to Max, Mad proteins prevent the formation of Myc-Max heterodimers. Mad-Max

heterodimers bind to the same DNA promoters as Myc-Max heterodimers (Lutz et al., 2002). Mad-Max heterodimers, however, recruit histone deacetylases to promoters, and subsequent histone deacetylation causes chromatin remodeling that represses transcription (Ayer et al., 1995; Schreiber-Agus et al., 1995).

Expression of the *c-MYC* gene is important for cell cycle progression, specifically for G1 to S progression (Steiner et al., 1995; Berns et al., 1997). Myc-Max heterodimers induce expression of several key cell cycle regulatory proteins including cyclin D2, cyclin dependent kinase 4 (cdk4), and CUL1 (reviewed by Pelengaris et al., 2002). Cyclin D2-cdk4 complexes bind to and sequester the cell cycle inhibitor $p27^{KIP1}$. The $p27^{KIP1}$ protein normally binds to and inhibits cyclin E-cdk2 complexes. Sequestration of $p27^{KIP1}$ frees cyclin E-cdk2 complexes to phosphorylate the cell cycle inhibitor pRb. Such phosphorylation of pRb causes it to release bound E2F family transcription factors, which then act to stimulate cell cycle progression. (The role of pRb in the cell cycle is discussed in detail below.) The CUL1 protein inhibits $p27^{KIP1}$ by targeting it for proteosomal degradation (O'Hagan et al., 2000).

b. The proto-oncogene *RAS*

Two of the three *RAS* proto-oncogenes, *H-RAS* and *K-RAS*, were identified as the cellular homologues of the viral oncogenes carried by the Harvey and Kirsten murine sarcoma viruses, respectively (Shih et al., 1979; Chang et al., 1982; Parada et al., 1982; Pulciani et al., 1982; Santos et al., 1982; Der et al., 1982). The *N-RAS* proto-oncogene was isolated from a neuroblastoma cell line (Shimizu et al., 1983). Mammalian cells ubiquitously

express at least one of these three Ras isoforms (reviewed by Macaluso et al., 2002). The likely importance of Ras proteins in tumorigenesis is demonstrated by the presence of *RAS* genes with activating mutations in at least 30% of human tumors (reviewed by Bos et al., 1989). Point mutations in codon 12, 13, 59, or 61 are most commonly observed in human tumors (Reddy et al., 1982; Taparowsky et al., 1982; Tong et al., 1989; Krengel et al., 1990; Macaluso et al., 2002). Such mutations disrupt the GTPase activity of the protein, producing a constitutively active Ras protein, i.e., GTP-bound Ras.

Each *RAS* proto-oncogene encodes a 21 kD protein that functions as a monomeric guanine-nucleotide binding protein (G-protein) (reviewed by Barbacid, 1987). As a monomeric G-protein, Ras binds to and hydrolyzes guanosine triphosphate (GTP) via an intrinsic guanosine triphosphate hydrolase (GTPase) activity (Shih et al., 1980; Gibbs et al., 1984; Temeles et al. 1985; de Vos et al., 1988). The hydrolysis of GTP yields an inactive GDP-bound protein. Two classes of proteins facilitate the cycling of Ras between its active, GTP-bound state and its inactive, GDP-bound state. GTPase activating proteins, or GAPs, stimulate GTPase activity, leading to the conversion of active, GTP-bound Ras to inactive, GDP-bound Ras; GDP exchange factors, or GEFs, assist in the dissociation of GDP from the inactive protein so that GTP can bind (reviewed by Takai et al., 2001). The ability of Ras to cycle between active and inactive states enables it to act as a molecular switch for the pathways it regulates. Post-translational modifications of the Ras protein target it to the plasma membrane (Hancock et al., 1990; reviewed by Newman and Magee, 1993 and Macaluso et

al., 2002). These modifications include the farnesylation and subsequent methylation of cysteine 186 and the palmitoylation of cysteine residues between amino acids 165-186.

Binding of growth factors to their cognate cellular receptors, which contain intracellular tyrosine kinase domains, leads to receptor dimerization and autophosphorylation at specific tyrosine residues (reviewed by Heldin et al., 1995). The receptor's phosphotyrosine residues serve as docks for cytoplasmic scaffold proteins. As their name suggests, the scaffold proteins provide additional binding sites for proteins required to transmit mitogenic signals (Pawson and Scott, 1997). One such scaffold protein is Grb2, which interacts with phosphotyrosines in the cytoplasmic tails of growth factor receptors via its Src homology 2 (SH2) domain (Lowenstein et al., 1992; Matuoka et al., 1993). The Grb2 protein also binds to proline residues in the Ras-specific GDP exchange factor (GEF) SOS through its Src homology 3 (SH3) domain (Chardin et al., 1993). This interaction places SOS in close proximity to its substrate, GDP-bound Ras. The interaction of SOS with GDP-bound Ras stimulates the dissociation of GDP from Ras, thereby enabling the Ras protein to bind to GTP and become active. GTPase activating proteins (GAPs) then associate with GTP-bound Ras to stimulate GTP hydrolysis. While in its active, GTP-bound state, Ras can bind to and stimulate its effectors. There are three main Ras effector pathways: 1) the RAF1/MEK/ERK pathway; 2) the PI3K/Akt pathway; and 3) the Ral-GDS/Ral GTPase/RIP1 pathway (reviewed by Takai et al., 2001; Macaluso et al., 2002). Activation of these pathways by Ras leads to the

expression of cellular genes that stimulate cell cycle progression, that promote cellular survival over apoptosis, and that regulate cytoskeletal organization. For example, activation of the RAF1/MEK/ERK pathway by Ras leads to transcription of the *CYCLIN D1* gene (reviewed by Robinson and Cobb, 1997). D-type cyclins interact with cyclin-dependent kinases (cdks) and inactivate the cell cycle inhibitor pRb, thereby stimulating cell cycle progression. (The role of pRb in the cell cycle is discussed in detail below.)

2. The ability to evade growth inhibitory signaling

Normal human cells possess numerous genes encoding proteins that function to protect the cells from inappropriate cellular proliferation. For example, when contact is made with other cells, signals to stop proliferation are transmitted. Damage to the genome initiates signaling cascades designed to halt the cell cycle, allowing time for DNA repair to occur before S-phase of the cell cycle. If cellular DNA repair mechanisms fail to adequately repair the damage, activation of programmed cell death (apoptosis) can occur. In order to gain the ability to proliferate uncontrollably and form tumors, cells must not only activate oncogenes but must also subvert these protective growth inhibitory pathways.

a. The tumor suppressor gene *RB*

Retinoblastoma is a cancer of the retina of the eye. There are two forms of retinoblastoma, familial and sporadic. The familial form of retinoblastoma is characterized by retinal tumors that occur in both eyes. Each eye may have one or more tumors. These tumors almost always develop before the age of five.

The sporadic form usually results in a single retinal tumor that develops later in life. Knudson (1971) proposed a "two-hit" model of tumorigenesis to explain the occurrence of both familial and sporadic retinoblastoma. According to this model, retinoblastoma arises because of loss of a protein required for negative regulation of retinal cell growth. To inactivate the function of this protein, mutations must occur in both alleles of the gene. Loss of the ability to produce functional protein results in the unregulated proliferation of the retinal cells with this defect. Knudson referred to these protective genes as anti-oncogenes; scientists now refer to them as tumor suppressor genes. The familial form of retinoblastoma involves the inheritance of a germline mutation in one allele and the subsequent acquisition of a somatic mutation in the other allele, whereas the sporadic form involves the acquisition of somatic mutations in both alleles. The probability that a person born with two wild-type alleles will acquire somatic mutations in both alleles to cause the development of retinoblastoma is very low because both mutations must occur in a single cell for a tumor to arise. However, the probability that a person born with a germline mutation in one allele will acquire a somatic mutation in the other allele in a single cell is significantly higher because every cell in the retina of this individual contains the first mutation. This explains why familial retinoblastoma occurs early in life and why the affected individuals typically have bilateral tumors. Moreover, individuals who inherit a germline mutation are predisposed to bone and soft tissue sarcomas later in life (reviewed by Nevins, 2001).

Studies of retinoblastoma led to the identification the first tumor

suppressor gene, designated the *retinoblastoma* (*RB*) gene (Friend et al., 1986; Fung et al., 1987; Lee et al., 1987). Although first associated with retinoblastoma, mutations in the *RB* gene have been observed in many human tumors including osteosarcomas, small cell lung carcinomas, and breast carcinomas (reviewed by Nevins, 2001). Moreover, the Rb pathway is altered in greater than 80% of human cancers (reviewed by Ortega et al., 2002). As predicted by Knudson, the *RB* gene encodes a protein that plays a critical role in negatively regulating cellular proliferation. Specifically, the Rb protein serves as a cell cycle gatekeeper by regulating the cell's transition from G1 phase of the cell cycle into S phase. Phosphorylation of Rb regulates its function. Unphosphorylated Rb binds to members of the E2F family of transcription factors, preventing these factors from activating transcription of E2F-responsive genes. Such genes encode proteins required for cell cycle progression (reviewed by Trimarchi and Lees, 2002). Therefore, E2F proteins must be active in order for the cell cycle to progress. Mitogenic signals lead to the activation of D-family cyclins and their associated kinases, cyclin-dependent kinases 4 and 6 (cdk4 and cdk6) (reviewed by Sears and Nevins, 2002). Activated cyclin D-cdk4/6 complexes phosphorylate Rb. Phosphorylation of Rb disrupts its interaction with the E2F proteins, freeing the E2F proteins to activate transcription. Rb inactivation is enhanced by expression of two E2F responsive genes, the *CYCLIN E* and *CDK2* genes. Cyclin E and cdk2 form a complex that further phosphorylates Rb. Tumor cells that have lost functional Rb protein exhibit unrepressed E2F activity, and this provides one mechanism to evade

growth inhibitory signaling.

b. The tumor suppressor gene *p53*

Another key protein involved in protecting cells from malignancy is the tumor suppressor p53. This protein was first identified in 1979 as a cellular protein with a high binding affinity for the SV40 large T antigen (Lane and Crawford, 1979; Linzer and Levine, 1979). Simultaneously, DeLeo et al. (1979) showed that the p53 protein is highly expressed in chemically-transformed sarcoma-derived cells. Although *p53* was first classified as an oncogene, subsequent work by several groups demonstrated that the original *p53* cDNA isolated encoded mutant p53 protein and that wild-type p53 strongly suppresses growth and inhibits transformation (Hinds et al., 1989; Finlay et al., 1989; Eliyahu et al., 1989; Baker et al., 1989).

Consistent with its potent ability to inhibit cell growth and to induce apoptosis, normal cells maintain a very low level of p53 protein (reviewed by Levine, 1997). The ubiquitin ligase MDM2 binds to p53 protein causing the ubiquitination of p53 and subsequent degradation of ubiquitinated p53 by the proteosome (Haupt et al., 1997; Kubbutat et al., 1997; Midgley and Lane, 1997). Forces that interrupt the MDM2-p53 interaction can lead to stabilization of the p53 protein. For example, DNA damage induces several protein kinases, including Chk1, Chk2, ATM, and ATR, that phosphorylate p53 to destabilize its interaction with MDM2, thereby preventing p53 degradation (reviewed by Vousden, 2002). Interaction of the ARF protein with MDM2 also results in the stabilization of p53 (Stott et al., 1998). Once stabilized, p53 translocates to the

nucleus where it can activate the transcription of genes that cause cell cycle arrest (e.g., *p21*$^{WAF1/Cip-1}$), apoptosis (e.g., *BAX*), and/or DNA repair (e.g., *GADD45*). In a developing tumor, p53 also regulates the transcription of inhibitors of angiogenesis (e.g., *TSP1*). Loss of functional p53 protein, therefore, can lead to the propagation of abnormal cells.

Mutations that cause the inactivation of the p53 protein have been observed in approximately 50% of human tumors (reviewed by Hollstein et al., 1994). In a recent review, Vousden and Lu (2002) note that most of the efforts to identify mutations in *p53* examined only the sequence of exons five through eight. Recent analyses of the entire *p53* coding sequence show that mutations occur in other regions, suggesting that the true frequency of *p53* mutations in human tumors may be significantly higher. Furthermore, even if human tumors contain wild-type p53 protein, its function can be lost because of amplifications of the *MDM2* gene or the related *MDMX* gene (Oliner et al., 1992; Riemenschneider et al., 1999). Amplification of these genes leads to a higher steady-state level of the proteins (Ramos et al., 2001). Increased MDM2 protein causes increased p53 degradation. The MDMX protein inhibits p53's function as a transcription factor, thereby preventing p53-mediated responses to cellular stresses (Shvarts et al., 1996; Finch et al., 2002; Sabbatini and McCormick, 2002).

3. The ability to evade apoptosis

Apoptosis, a well-organized and highly regulated process through which cells undergo programmed cell death, is characterized by the cell's autodigestion of its contents (reviewed by Igney and Krammer, 2002). Proteases digest

nuclear envelope proteins and cytoskeletal proteins, and nucleases digest the genomic material. Both lead to the formation of apoptotic bodies that are rapidly phagocytosed. Embryonic development relies upon apoptosis to shape the developing organism (Alberts et al., 1994). Apoptosis also provides a key cellular defense mechanism against tumorigenesis because it eliminates abnormal cells from the body's tissues. If abnormal cells become resistant to apoptosis, these cells continue to proliferate, which potentially leads to the acquisition of additional genetic changes that convert these cells into tumor cells.

There are two well-characterized apoptotic pathways, the extrinsic pathway and the intrinsic pathway (reviewed by Ashkenazi, 2002; Igney and Krammer, 2002). Death receptors, members of the tumor-necrosis factor (TNF) receptor superfamily, mediate apoptosis through the extrinsic pathway. These receptors contain a protein motif known as a death domain that interacts with intracellular death-domain containing adaptor proteins (Hofmann and Tschopp, 1995; Feinstein et al., 1995). A death-inducing signaling complex, or DISC, assembles at the receptor's death domain (Kischkel et al., 1995). The critical players in the DISC are the cysteine aspartyl-specific proteases known as caspases (reviewed by Shi, 2002). Activation of the initiator caspases 8 and 10 in the DISC leads to activation of executioner caspases (caspases 3, 6, and 7) that carry out apoptosis by cleaving death substrates.

Ill-defined death-inducing signals activate the intrinsic apoptotic pathway, which is mediated by the pro-apoptotic members of the BCL2 family of proteins (reviewed by Cory and Adams, 2002). These pro-apoptotic proteins can be

further divided into two subfamilies, the BAX family, which includes BAX, BAK and BOK, and the BH3 family, which includes proteins such as BID, BIM, BIK, and BAD. The pro-apoptotic BAX and BAK proteins function at the mitochondrial membrane, where they induce the release of cytochrome c, a key step in the intrinsic apoptotic pathway (Jurgensmeier et al, 1998; O'Connor et al., 1998; Korsmeyer et al., 2000). Subsequent to cytochrome c release, a complex containing cytochrome c, APAF1 (apoptotic protease activating factor-1), ATP, and procaspase 9 assembles in the cytosol (Li et al., 1997). This complex is known as the apoptosome. Cleavage of procaspase 9 to yield the active caspase occurs in the apoptosome, leading to the activation of executioner caspases, such as caspase 3, that then cleave death substrates (Li et al., 1997).

The BCL2 protein family also contains anti-apoptotic proteins, including BCL-2 and BCL-X_L, which antagonize the pro-apoptotic factors (reviewed by Cory and Adams, 2002). Cells receive specific survival signals, which include growth factors, cytokines, hormones, and adhesion molecules, and these signals activate cellular signal transduction pathways that cause the inhibition of pro-apoptotic factors. For example, activation of the PI3K/Akt signaling pathway by specific types of survival signals inhibits apoptosis (reviewed by Vivanco and Sawyers, 2002). Specifically, Datta et al. (1997) showed that Akt phosphorylates BAD, a pro-apoptotic protein of the BCL2 family, and this event suppresses apoptosis. As a pro-apoptotic protein, BAD dimerizes with the anti-apoptotic factor BCL-X_L to inhibit this factor (Yang et al., 1995; Ottilie et al., 1997; Kelekar et al., 1997). When phosphorylated by Akt, BAD no longer binds to BCL-X_L,

thereby freeing BCL-X_L to carry out its role as an anti-apoptotic protein. Finucane et al. (1999) demonstrated that BCL-X_L promotes survival through its ability to prevent BAX-induced mitochondrial release of cytochrome c.

Several mechanisms can enable tumor cells to evade apoptosis. Up-regulation of the expression of anti-apoptotic proteins favors cell survival. For example, BCL-2 is overexpressed in B-cell follicular lymphoma (Cleary et al., 1986; Tsujimoto and Croce, 1986). Conversely, down-regulation of the expression of pro-apoptotic proteins or the acquisition of inactivating mutations in genes encoding pro-apoptotic proteins also favors cell survival. For example, inactivating mutations in the pro-apoptotic *BAX* gene have been observed in human tumors (Rampino et al., 1997; Meijerink et al., 1998; Molenaar et al., 1998). The inactivation of tumor suppressor genes in combination with the activation of cellular proto-oncogenes in tumor cells can lead to resistance to apoptosis through the repression of pro-apoptotic signaling pathways and the stimulation of anti-apoptotic signaling pathways (reviewed by Igney and Krammer, 2002). For example, as previously noted, *p53* is a tumor suppressor gene that can initiate apoptosis. Inactivating mutations in *p53*, therefore, inhibit the cell's ability to activate apoptosis. Moreover, up-regulation of the PI3K pathway through constitutively active Ras or constitutively active growth factor receptors stimulates survival pathways, allowing tumor cells to evade apoptosis.

4. The ability to proliferate limitlessly (the acquisition of an infinite lifespan)

Human cells in culture have a finite lifespan. In 1961, Hayflick and

Moorhead were the first to demonstrate that human embryonic fibroblasts in culture divide only 40-60 times. Their studies of cultured human fibroblasts also demonstrated that the cells have an innate ability to count their cell divisions. For example, if human cells are frozen at cell division 20, the cells will begin cell division 21 when returned to culture. The cells remember their cell division history (reviewed in Hayflick, 2000).

At the end of a cell's replicative lifespan, growth is arrested and the cell enters a state known as replicative senescence. Senescent human fibroblasts are metabolically active but unable to divide (Hayflick and Moorhead, 1961). They have a distinct large, flat morphology and are usually multinucleated. Although the precise mechanism by which cells count cell divisions remains elusive, clues about the signals that drive a cell into senescence emerged with discovery of telomeres.

Naked chromosomal ends are susceptible to degradation, recombination, or end-to-end chromosome fusions, and these events can cause the loss of genetic material, genomic instability, or cell death (reviewed by Kim et al., 2002; Hackett and Greider, 2002). Moreover, loss of chromosomal DNA occurs with each cycle of DNA replication because the 3' ends of linear chromosomal DNA cannot be replicated completely, and this is referred to as the "end replication problem" (Watson, 1972; Olovnikov, 1973). Protection of vulnerable chromosomal DNA ends is accomplished by telomeres, which consist of simple hexameric DNA repeats and a complex of telomeric DNA binding proteins (Blackburn and Challoner, 1984; Shampay et al., 1984; Blackburn et al., 1989;

reviewed by Blackburn, 1991). In human cells, telomeres range from 10-15 kb in length (reviewed by Hackett and Greider, 2002). Erosion of telomeres occurs with each cycle of DNA replication. Loss of the repetitive telomeric DNA prevents the loss of adjacent DNA that contains genes. Telomeres can be maintained by the activity of telomerase, a reverse transcriptase that synthesizes new telomeric DNA (Greider and Blackburn, 1985, 1989; Shippen Lentz and Blackburn, 1989, 1990; Strahl and Blackburn, 1996; Lingner et al., 1997; Cech et al., 1997). Most adult somatic human cells, however, lack telomerase activity (Kim et al., 1994).

Senescent cells have short telomeres, leading to the attractive theory that telomeric length provides the cell division counting mechanism noted by Hayflick and Moorhead (reviewed in Hayflick, 2000). Several lines of evidence support this theory. First, Harley et al. (1990) showed that telomeres in normal human diploid fibroblasts shorten as the cells are cultured. Second, Allsopp et al. (1992) showed that replicative capacity in culture correlates with initial telomere length. Cells in culture with long telomeres can undergo more cell divisions than those in culture with short telomeres. Apparently, the telomeric structure itself conveys signals that count the number of cell divisions. A minimal length of telomeric DNA is required so that the complement of telomeric binding proteins can assemble and form a functional telomere. When telomeres reach this critical minimum length in normal cells, telomeric structure is disrupted, and the p53 and pRb pathways are activated to initiate senescence (reviewed by Kim et al., 2002). The exact mechanism for the activation of p53 and pRb as a result of

telomeric loss remains to be uncovered.

Normal human fibroblasts have a finite lifespan, and such cells have never been reported to become malignantly transformed. It is argued that the reason for this is that they do not undergo a sufficient number of cell replications to acquire all of the necessary phenotypic changes required to form tumors. In contrast, tumorigenic human fibroblasts have an infinite lifespan, suggesting that the acquisition of an infinite lifespan is a necessary prerequisite for tumor development. Loss of the p53 and pRb tumor suppressor pathways provides one mechanism for human fibroblasts to acquire an infinite lifespan (reviewed by Kim et al., 2002). When telomeres reach a minimal critical length in p53 and pRb deficient cells, genomic instability ensues. Such instability favors a genetic change that leads to stabilization of telomeres, thereby promoting survive. For example, induction of telomerase expression provides one way to stabilize telomeres. Approximately 85-90% of human tumor cells display telomerase activity (Kim et al., 1994; Ducray et la. 1999; Klingelhutz, 1999; Hahn and Meyerson, 2001; Kang and Park, 2001). The remaining human tumor cells lacking telomerase activity are also capable of maintaining telomeres through an alternative recombination-based mechanism (ALT) (Bryan et al., 1997; Dunham et al., 2002).

5. The ability to initiate and to sustain angiogenesis

Tumor tissue, like all tissue, requires an adequate blood supply. As tumor tissue expands, so must its blood supply. When the cells of a tumor lack necessary oxygen and nutrients and waste products accumulate, the cells die,

blocking further expansion of the tumor. Therefore, angiogenesis, which refers to the sprouting of new capillaries from existing blood vessels, must occur (Gilbert, 1997). Angiogenesis is a complex process that involves several steps (reviewed by Hanahan and Folkman, 1996; Webb and Vande Woude, 2000). Local degradation of the basement membrane surrounding the endothelial cells of pre-existing vessels occurs. The basement membrane, composed of extracellular matrix proteins including laminins, collagens, and proteoglycans, provides an acellular support for epithelial, mesenchymal, and endothelial cells (reviewed by Hood and Cheresh, 2002). The endothelial cells of the vessels then change shape and invade the surrounding stroma, forming a migrating column. Proliferating endothelial cells make up the leading edge of the migrating column. A region of differentiated endothelial cells follows the leading edge. The differentiated cells, which no longer proliferate, change shape and tightly adhere to each other to form the lumen of a new capillary tube. Fusion of the sprouting tubes results in the formation of loops that circulate blood to the surrounding tissue.

In the early 1970s, Folkman and colleagues provided the first evidence that tumor cells must recruit blood vessels in order to survive. Specifically, implantation of tumor fragments or tumor cells from culture at avascular sites in rabbit corneal tissue results in the growth of new capillaries into the tumor (Gimbrone et al., 1972). Inhibition of angiogenesis dramatically reduces the growth of the implanted tumor tissue or cells (Gimbrone et al., 1972). Additional studies showed that a lack of adequate vasculature causes tumor cells to

undergo necrosis and/or apoptosis (Brem et al., 1976; Holmgren et al., 1995; Parangi et al., 1996).

Studies of three transgenic mouse models of cancer (islet cell carcinoma, dermal fibrosarcoma, and epidermal squamous cell carcinoma) as well as studies of two human cancers (breast carcinoma and cervical carcinoma) provide additional evidence that one of the phenotypic requirements of a developing tumor is the activation of angiogenesis (Hanahan, 1985; Lacey et al., 1986; Kandel et al. 1991; Sippola-Thiele et al., 1989; Arbeit et al., 1994; Hurlin et al., 1995; Coussens et al., 1996; Weidner et al., 1991, 1992; Brown et al., 1995; Guidi et al., 1994, 1995; Smith-McCune and Weidner, 1994). In these types of cancer, histologically distinct intermediates mark the course of the conversion of a normal cell into a tumor cell. By measuring the blood vessel density and the level of certain immunological markers of angiogenesis (e.g. von Willebrand factor) in these intermediates, researchers determined that the early lesions are usually avascular, whereas the malignant tumors that arise are highly vascular. For example, the development of dermal fibrosarcomas in a transgenic mouse model is marked by at least three stages: mild fibromatosis, aggressive fibromatosis, and fibrosarcomas (Sippola-Thiele et al., 1989). Benign hyperproliferation of dermal fibroblasts residing below the epidermis characterizes both types of fibromatosis. Greater cell density, an increased mitotic index, and a general thickening of the dermal layer distinguish aggressive fibromatosis from mild fibromatosis (Kandel et al., 1991). Fibrosarcomas, the malignant lesions, protrude from the skin and have an even greater cell density

and an anaplastic character compared with benign fibromatoses. Using immunohistochemical staining of von Willebrand factor, a protein expressed specifically in endothelial cells, Kandel et al. (1991) measured the degree of vascularization in normal dermis, mild fibromatoses, aggressive fibromatoses, and fibrosarcomas. The normal dermal tissue and mild fibromatosis tissue show a low level of vascularization. In contrast, aggressive fibromatosis tissue and fibrosarcomas exhibit a high degree of vascularization, indicating that the activation of angiogenesis occurs during the transition from mild fibromatosis to aggressive fibromatosis. Such a change contributes to the evolution of a malignant tumor.

These studies led to the theory of an "angiogenic switch" (reviewed by Hanahan and Folkman, 1996). During tumor development, a change occurs in the cells that "switches on" angiogenesis, thereby converting an avascular tumor to an angiogenic tumor. Additional evidence, such as the studies of Bouck and colleagues presented below, indicates that the angiogenic switch is not quite as simple as "on" or "off". Rather, the formation of new blood vessels becomes increasingly prevalent during tumorigenesis (reviewed by Webb and Vande Woude, 2000). A balance between pro-angiogenic and anti-angiogenic factors actually controls the angiogenic switch. In normal tissue and avascular tumor tissue, anti-angiogenic factors outweigh pro-angiogenic factors, and the genesis of new capillaries does not occur. In angiogenic tumors, pro-angiogenic factors dominate, and new capillaries sprout. The studies of Bouck and colleagues, which defined the mechanism for the acquisition of an angiogenic phenotype by

Li-Fraumeni fibroblasts during their conversion to tumorigenicity, illustrate the balance model of the angiogenic switch (Dameron et al., 1994a and 1994b, Bouck et al., 1996; Volpert et al., 1997). Fibroblast cell lines derived from Li-Fraumeni patients contain one wild-type *p53* allele and one mutant *p53* allele. Passaging of these cells results in loss of the wild-type *p53* allele in a subset of the population. The cells lacking wild-type p53 protein acquire an infinite life-span. Introduction of the *H-RAS* oncogene into these cells followed by appropriate selection can yield tumorigenic cell lines (Bischoff et al., 1991). However, less than half of the Ras-transformed Li-Fraumeni fibroblast cell lines are tumorigenic. This indicates that additional changes must occur in the Ras-transformed Li-Fraumeni cell lines to produce tumorigenic cell lines (Bischoff et al., 1991). The angiogenic switch necessary in the Li-Fraumeni model of tumorigenesis occurs in two steps through two key mediators of angiogenesis, thrombospondin-1 (TSP-1) and vascular endothelial growth factor (VEGF). First, loss of wild-type p53 protein in these cells results in the loss of TSP-1 expression and secretion and in an increase in VEGF expression and secretion (Dameron et al., 1994a, 1994b; Volpert et al., 1997). Second, expression of oncogenic H-Ras protein in the p53 null Li-Fraumeni cells further increases VEGF expression and secretion. In this model system, the secretion of the anti-angiogenic factor TSP-1 decreases as the secretion of the pro-angiogenic factor VEGF increases (Volpert et al., 1997). Therefore, neovascularization occurs because the factors that stimulate angiogenesis override those that would inhibit it.

TSP-1 belongs to a multi-protein family that also includes

thrombospondin-2, -3, -4, and -5 (reviewed by de Fraipont et al., 2001). TSP-1 and TSP-2 constitute a TSP subfamily. Secreted as disulfide-bonded homotrimers, TSP-1 and TSP-2 associate with molecules in the extracellular matrix as well as with cellular receptors. Both TSP-1 and TSP-2 have been shown to be potent inhibitors of angiogenesis in numerous cell types (reviewed by Adams, 2001). Because TSP-1 has been studied in more detail than TSP-2, the discussion that follows will emphasize the current model for TSP-1's inhibition of angiogenesis. TSP-1 regulates several properties of endothelial cells including proliferation, motility, cell-cell interactions, and morphogenesis (reviewed by Cavallaro and Christofori, 2001). Jiménez et al. (1996) demonstrated that TSP-1 inhibits angiogenesis by causing endothelial cells to undergo apoptosis. Briefly, TSP-1 binds to the extracellular domain of the CD36 receptor. The interaction of TSP-1 and CD36 recruits the Src-family non-receptor tyrosine kinase Fyn to the cytoplasmic tail of CD36. The downstream consequence of the TSP-1-CD36-Fyn interaction is the activation of the caspase 3-like protein. The signaling intermediates connecting Fyn to the caspase 3-like protein have not been identified. Activation of the caspase 3-like protein leads to phosphorylation of p38 and translocation of phospho-p38 from the cytosol to the nucleus. Presumably, nuclear localized phospho-p38 stimulates the transcription of pro-apoptotic genes. Production of pro-apoptotic proteins then induces apoptosis. Subsequent to this study, Volpert et al. (2002) demonstrated that the pro-apoptotic Fas/Fas ligand pathway is required for TSP-1-mediated apoptosis in endothelial cells. Activation of p38 by the TSP-1 pathway leads to increased

expression of the Fas ligand (FasL), which binds to its death-inducing receptor Fas to activate the extrinsic apoptotic pathway. Interestingly, Volpert et al. (2002) also demonstrated that treatment of endothelial cells with pro-angiogenic factors, such as VEGF, increases the amount of Fas receptor on the cell surface, thereby sensitizing the angiogenic endothelial cells to apoptosis. In contrast, pro-angiogenic factors like VEGF also promote cell survival and induce the expression anti-apoptotic proteins. Again, the idea of a balance comes into play. When endothelial cells are exposed to a limiting amount of pro-angiogenic factors, apoptosis occurs. When pro-angiogenic factors increase to a critical amount, survival overrides apoptosis and new blood vessels form.

6. The ability to invade surrounding tissues and to metastasize

The metastasis of primary tumors accounts for 90% of cancer deaths (reviewed by Hanahan and Weinberg, 2000). Metastasis refers to the spread of tumor cells from their site of origin to new sites in the body (Alberts et al., 1994). For metastasis to occur, tumor cells must enter the circulatory system (reviewed by Hood and Cheresh, 2002 and Chambers et al., 2002). To gain access to the circulatory system, tumor cells infiltrate the basement membrane that surrounds the tumor itself and the endothelial cells of blood vessels. After the invading tumor cells successfully penetrate these basement membranes, the tumor cells enter the circulatory system and travel to the tissue where the secondary tumor will arise. At this secondary site, the tumor cells must again penetrate the basement membrane of the vasculature and of the target tissue. Once the tumor cells have successfully invaded the target tissue, the cells must initiate and

sustain growth to give rise to a secondary tumor. It is important to note that there is an intimate link between metastasis and angiogenesis. As discussed above, angiogenesis involves the local degradation of the basement membrane surrounding the endothelial cells of pre-existing vessels and the subsequent invasion of the surrounding tumor tissue by the endothelial cells. Because the vasculature in this angiogenic environment is compromised, tumor cells can more easily enter the circulatory system and metastasize.

Certain cancers seem to have preferred sites for metastasis (Weiss et al., 1986; Weiss, 1992; Radinsky et al., 1995; Radinsky and Ellis, 1996). For example, breast carcinomas often metastasize to bone, liver, brain, and lungs. Prostate cancer primarily metastasizes to bone. Colorectal carcinomas predominately metastasize to the liver. Two main ideas emerged to explain organ-specific metastases. The "seed and soil" model is attributed to Steven Paget (1889). This theory proposes that although cancer cells ("seeds") spread virtually everywhere throughout the body, only specific organs ("soil") provide adequate conditions for those specific types of tumor cells to develop into secondary tumors. The second model, proposed by James Ewing (1928), attributes that path of blood flow in the body as the reason for certain tumor types to metastasize to specific organs. In their review of metastasis, Chambers et al. (2002) explain that experimental evidence supports both theories. The pattern of blood flow in the body adequately explains only 66% of metastases; the remaining metastases may be explained by interactions between the tumor cells and the new environment.

The processes of invasion and metastasis are very complex and require the cooperation and coordination of many cellular signaling pathways. The tumor cells switch from a proliferative to a migratory program in order to move from the primary tumor site into the bloodstream. Once in the bloodstream, the cells activate survival pathways. At the secondary tumor site, the cells must then switch from a migratory to a proliferative program to establish the metastatic lesion. Cellular adhesion molecules, cytoskeletal proteins, and proteases play a critical role in this process.

a. Integrins, cellular adhesion and signaling proteins that play a critical role in invasion and metastasis

Heterodimeric cell surface glycoproteins known as integrins regulate a cell's interaction with the surrounding extracellular matrix (reviewed by Juliano, 2002). There are 18 known integrin α-subunits and 8 known integrin β-subunits expressed in human cells that form non-covalently heterodimers, each with unique ligand binding properties. Each α and β subunit is composed of a large extracellular domain, which contains ligand binding motifs, a transmembrane domain, and a relatively short cytoplasmic domain, which can interact with intracellular molecules (reviewed by Liu et al., 2000).

To promote a migratory phenotype, alterations in integrin expression patterns occur in tumor cells. Expression of integrins that promote migration in a specific cell type increases while expression of those that inhibit migration decreases. For example, invasive melanomas express a high level of the $\alpha_v\beta_3$ integrin at the invasive front of the tumor and in angiogenic blood vessels,

whereas the premalignant lesions and quiescent blood vessels express a low level of this integrin (Felding-Habermann et al., 1992; Brooks et al., 1994). Breast carcinoma cells decrease expression of specific integrin subunits, including α_1, α_3, α_6, β_1, and β_4 (reviewed by Mizejewski, 1999). Alternatively, reorganization of the cellular distribution of integrins to promote migration can occur. For example, in colon carcinoma cells, the laminin-binding integrin $\alpha_6\beta_4$ moves from stable cellular junctions known as hemidesmosomes to migratory regions of the cells, where it functions in the formation and stabilization filopodia and lamellae (Rabinovitz and Mercurio, 1997).

The interaction of integrins and extracellular matrix proteins causes integrin clustering and the formation of focal contacts or focal adhesions (reviewed by Juliano, 2002). Various intracellular proteins bind to the integrin cytoplasmic tail at focal adhesions to transduce signals (reviewed by Hynes, 2002). Most interestingly, integrins not only convey signals from the extracellular environment to the intracellular environment, known as outside-in signaling, but they also convey signals from the intracellular environment to the extracellular environment, known as inside-out signaling.

Outside-in signaling modulates the cell's response to extracellular stimuli. Intracellular proteins such as the actin binding proteins α-actinin, talin, and paxillin and the scaffold proteins RACK1 and Grb2 bind to integrin cytoplasmic domains in response to an extracellular matrix protein binding to integrin extracellular domains (Otey et al., 1990; Pavalko et al, 1991; Horwitz et al., 1986; Knezevic et al., 1996; Pfaff et al., 1998; Schaller et al., 1995; Tanaka et al., 1996;

Chen et al., 2000; Liu et al., 1999; Liu and Ginsberg, 2002; Liliental and Chang, 1998; Law et al., 1996). Kinases including focal adhesion kinase (FAK) and the Src family of non-receptor tyrosine kinases also interact with integrin cytoplasmic domains in response to an extracellular matrix protein binding to integrin extracellular domains (Schaller et al., 1992, 1999; Hildebrand et al. 1993; Chen et al., 2000). These interactions link integrins to the actin cytoskeleton and to kinases, two key mediators of cellular motility. For example, FAK associates with the cytoplasmic domain of integrins at focal adhesions and becomes activated by autophosphorylation of tyrosine 397 (reviewed by Parsons et al., 2002). Phosphorylated FAK can then activate several signaling cascades depending upon which intracellular proteins bind to it. One branch involves the recruitment of the scaffolding protein Grb2 to the focal adhesion. This leads to Ras activation and subsequent activation of the RAF1/MEK/ERK pathway. In addition to activating gene transcription, Erks directly regulate enzymes required for cellular motility. Erk phosphorylates myosin-light-chain kinase (MLCK), a kinase that regulates contractile force in cells (Klemke et al., 1997). Activation of myosin through MLCK generates force that pulls the cell forward toward new adhesive contacts and breaks old contacts.

Inside-out signaling modulates the cell's adhesiveness, the strength of the interaction between integrins and the extracellular matrix (Huttenlocher et al., 1996; Palecek et al., 1997). Proteins that bind to integrin cytoplasmic domains can affect integrin quaternary structure, thereby influencing the integrin's affinity and avidity for its ligands. An intermediate level of adhesiveness promotes

migration. A high level of adhesiveness immobilizes cells, whereas a low level of adhesiveness fails to provide an adequate amount of traction, rendering migration inefficient.

b. Matrix metalloproteinases, a family of proteases that plays a critical role in invasion and metastasis

To gain access to the circulatory system, tumor cells infiltrate the basement membranes surrounding the tumor itself and the endothelial vessel cells. The matrix metalloproteinase (MMP) family of proteases plays a critical role in the regulated degradation of basement membrane (reviewed by Nagese and Woessner, 1999, Egeblad and Werb, 2002; Hood and Cheresh, 2002). Specifically, two members of the family, MMP2 and MMP9, are closely linked to invasion and metastasis. MMP2 and MMP9 degrade type IV collagen, the predominant component of the basement membrane. Increased MMP protein has been detected on stromal cells in proximity to invasive melanoma cells, on metastatic breast carcinoma cells, and on invading angiogenic endothelial cells (Monteagudo et al., 1990; Pyke et al., 1992; Brooks et al., 1996; Deryugina et al., 1997). The fact that MMP protein is found not only on tumor cells but also on surrounding stromal and endothelial cells emphasizes the importance of the interplay of multiple cell types in the tumor microenvironment. Signals from surrounding cells can promote or inhibit tumor growth.

In addition to being a barrier to invasion and metastasis, the extracellular matrix also serves as a track along which the cells migrate (Hood and Cheresh, 2002). Therefore, the proteolytic processing of the basement membrane must be

tightly regulated to prevent over-degradation of the extracellular matrix track. MMPs are secreted by cells as inactive enzymes (pro-MMPs), and activation of pro-MMPs occurs on the cell surface (reviewed by Nagese, 1997). For example, the cell surface receptor membrane-type 1 MMP (MT1-MMP) contains a protease domain, and it cleaves the pro-peptide from pro-MMP2 to yield mature, active MMP2 (Murphy et al., 1999). Active MMP2 can then associate with the integrin αvβ3, thereby localizing active MMP2 at the invasive front of the tumor (Brooks et al. 1996; Deryugina et al., 2000; Hofmann et al., 2000). One consequence of MMP2 activity is the generation of MMP2 inhibitory fragments known as hemopexin (PEX) fragments, which bind to MMP2 preventing MMP2-αvβ3 interactions (Brooks et al., 1998). Therefore, a negative feedback loop provides one mechanism to regulate MMP2-mediated proteolysis of the extracellular matrix.

B. The Multi-Step Model of Tumorigenesis

1. Epidemiological evidence

It is widely accepted that cancer develops via a multi-step process, which involves a series of genetic changes that cause a normal cell to evolve into a cancer cell. Using a mathematical model to estimate the number of changes required to cause human carcinomas, Peto (1977) concluded that a very large number of rate-limiting cellular changes is not necessarily required to convert an epithelial cell into a carcinoma cell. In fact, he estimated that approximately 6 changes are required. Subsequently, Renan (1993) extended the work of Peto. He analyzed 28 types of human malignancies and found that approximately 7-12

genetic changes are required for human tumors to develop. The presence of intermediates between normal cells and malignant cells, as is seen in colon cancer, further supports the multi-step model of tumorigenesis (Kinzler and Vogelstein, 1996). Exposure to carcinogens does not lead to the immediate occurrence of cancer. For example, individuals who smoke cigarettes develop lung cancer only after decades of exposure to the carcinogens in cigarette smoke (Peto, 1977). Moreover, the incidence of cancer in a population increases with age (Dix, 1989). Both observations support the contention that cancer develops via a multi-step process, which involves a series of genetic changes that occur over time to convert a normal cell into a cancer cell.

2. Colorectal tumorigenesis

Because the process of tumorigenesis in colorectal epithelium is marked by distinct morphological changes, colorectal cancer provides an excellent model for studying the multi-step nature of tumorigenesis. Normal colon epithelia, dysplastic adenomas (benign tumors), and colon carcinomas can be distinguished and isolated to determine a genetic profile for each type of tissue. Through molecular analyses of tissues representing each stage of colorectal tumorigenesis, researchers demonstrated that at least seven genetic events must occur to give rise to colorectal carcinomas (reviewed by Kinzler and Vogelstein, 1996).

Study of familial adenomatous polyposis (FAP), an autosomal dominant genetic disorder that predisposes patients to colorectal cancer, led to the discovery of one key tumor suppressor gene involved in preventing colorectal

cancer (Kinzler and Vogelstein, 1996). FAP patients develop hundreds to thousands of colorectal tumors known as adenomatous polyps (Gardner, 1951, 1972). A single polyp, which is benign, poses no immediate danger to the patient's life. The sheer number of polyps that develop, however, guarantees that a cell in at least one benign polyp will accrue additional genetic changes to progress to malignancy. In view of this situation, colorectal cancer develops in virtually 100% of FAP patients unless the colon is removed, and such diagnoses occur approximately 25 years earlier than in individuals who develop sporadic colorectal cancer (reviewed by Fearon and Jones, 1992; Kinzler and Vogelstein, 1996; Marsh and Zori, 2002).

FAP patients inherit a germline inactivating mutation in one allele of the *adenomatous polyposis coli* (*APC*) gene (Groden et al., 1991; Nishisho et al., 1991). The acquisition of an inactivating somatic mutation in the other *APC* allele results in the loss of functional APC protein, which causes dysplasia in colorectal epithelium (Kinzler and Vogelstein, 1996). The APC protein exerts its tumor suppressive power by negatively regulating a component of the Wnt signaling pathway, thereby inhibiting this proliferation promoting pathway (reviewed by Moon et al., 2002). (The Wnt pathway is reviewed in Section II.) The APC protein forms a complex with axin and glycogen synthase kinase-3β (GSK-3β) that binds to β-catenin. The formation of a quaternary complex of β-catenin/APC/axin/GSK-3β enables GSK-3β to phosphorylate β-catenin. Such phosphorylation targets β-catenin for proteosomal degradation (reviewed by Bienz, 2002). Inactivating mutations in the *APC* gene, which result in loss of

functional APC protein, have been observed in greater than 80% of all colorectal cancers, supporting the argument that APC is an important gatekeeper in colorectal epithelium (Kinzler and Vogelstein, 1996). Moreover, loss of functional APC protein appears to be an early event in colorectal tumorigenesis because such loss has been detected in dysplastic aberrant crypt foci (ACF), the earliest observable colorectal neoplastic lesions (Jen et al., 1994).

Activating mutations of the proto-oncogene *K-RAS* have been detected in adenomas, which occur later in the development of colorectal cancer (Kinzler and Vogelstein, 1996). It is believed that ACFs give rise to adenomas. As discussed above, the Ras family of small G-proteins activates cellular proliferation. Constitutive activation of Ras small G-proteins through activating mutations can enable cells to proliferate in the absence of mitogenic signals. Such a change likely plays a causal role in the progression of an ACF to an adenoma.

Loss of heterozygosity at chromosomes 18q and 17p occurs even later in the progression of colorectal tumorigenesis (Kinzler and Vogelstein, 1996). These chromosomal aberrations have been detected in large adenomas and in carcinomas. Two putative tumor suppressor genes have been identified at the 18q locus: the *deleted in colorectal carcinoma* (*DCC*) gene and the *deleted in pancreatic carcinoma* (*DPC4/SMAD4*) gene (Fearon et al., 1990 and Hahn et al., 1996). The *DCC* gene encodes a protein containing immunoglobulin superfamily repeats (reviewed by Graziano et al., 2002; Calvert and Frucht, 2002). Such domains may indicate a role for DCC in cell to cell or cell to matrix interactions.

Figure 1. The multi-step and clonal model of carcinogenesis: colon cancer. The multi-step and clonal model of carcinogenesis predicts that tumors originate from a single cell that has acquired all of the necessary genetic changes to be able to produce a malignant tumor. All cells of the tumor are the progeny of this first malignant cell. This diagram illustrates how this model applies to human colon cancer. Activating mutations in colon cancer-related oncogenes (e.g., *RAS*) and inactivating mutations in colon cancer-related tumor suppressor genes (e.g., *APC, DCC,/DPC4,* and *p53*) provide a growth advantage to the cell, thereby enabling clonal expansion. Each arrow indicates a single cell that acquired a genetic change (e.g., loss of APC); each triangle represents a large population of colon epithelial cells that arose through clonal expansion of the cell that has acquired one or more colon cancer-related mutation(s). The histologically distinct types of colon tissues that emerge during this process are indicated below the triangles. (Adapted from Kinzler and Vogelstein, 1996)

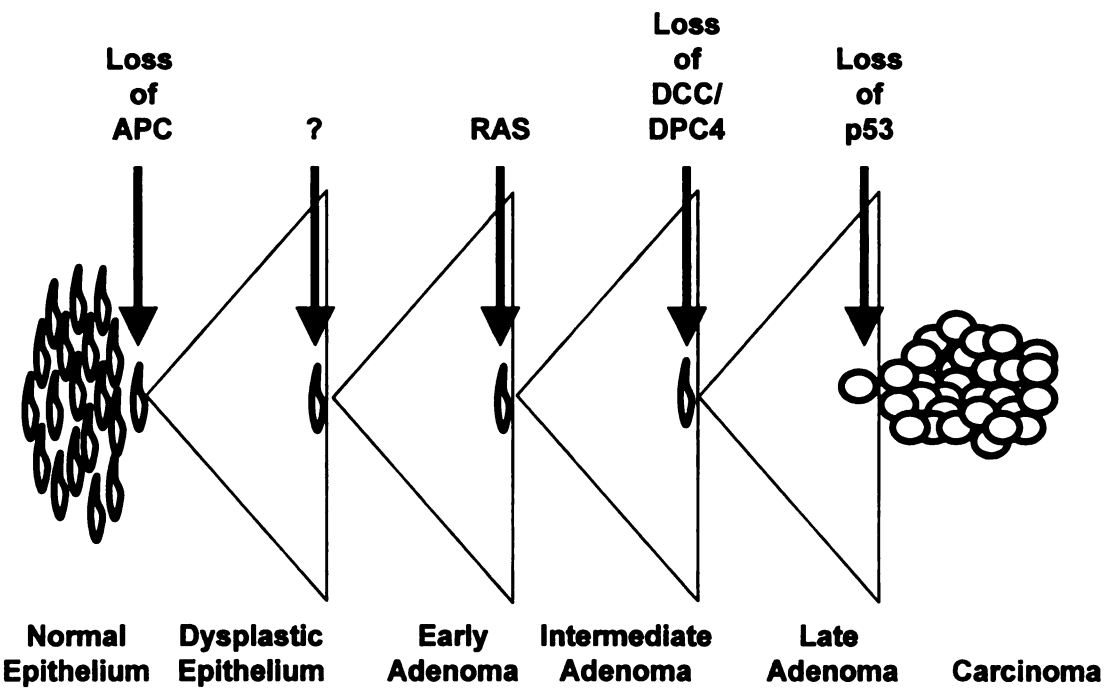
Loss of APC
?
RAS
Loss of DCC/ DPC4
Loss of p53
Normal Epithelium
Dysplastic Epithelium
Early Adenoma
Intermediate Adenoma
Late Adenoma
Carcinoma

Figure 1

Mehlen et al. (1998) and Chen et al. (1999) report that *DCC* may play a role in inducing apoptosis. The *DPC4* gene encodes the SMAD4 protein, a key mediator of the TGF-beta signaling cascade. This signaling pathway acts as a negative regulator of cellular proliferation in colon epithelia. SMAD4 heterodimerizes with receptor-activated SMADs, and these heterodimers translocate to the nucleus to regulate transcription of TGF-beta responsive genes (reviewed by Weinstein et al., 2000). The specific genes regulated depend upon which receptor-activated SMAD interacts with SMAD4. Loss of functional SMAD4 protein prevents transcription of TGF-responsive genes, which encode proteins to repress cellular proliferation. The gene implicated at the chromosome 17p locus is the tumor suppressor gene *p53*. Inactivating mutations in the *p53* gene, which results in loss of functional p53 protein, have been observed in more than 50% of colorectal cancers (reviewed by Markowitz et al., 2002). As discussed above, because of its potent ability to inhibit cell growth and to induce apoptosis, p53 is a key protein involved in protecting cells from malignancy.

Studies of hereditary nonpolyposis colorectal cancer (HNPCC), another disease that predisposes individuals to colon cancer, shed additional light onto the process of colorectal tumorigenesis. Although HNPCC patients develop adenomas at a rate similar to non-affected individuals, these adenomas almost assuredly progress to malignancy (reviewed by Lynch et al., 1993, 2000). The accelerated rate of tumorigenesis evident in HNPCC patients occurs because of loss of function of proteins involved in DNA mismatch repair (MMR). Individuals affected by HNPCC inherit an inactivating mutation in one allele of a MMR gene.

Germline mutations have been observed in five different MMR genes: *hMSH2, hMSH6, hMLH1, hPMS1*, and *hPMS2* (Leach et al., 1993; Fishel et al., 1993; Bronner et al., 1994; Papadopoulos et al., 1994; Nicolaides et al., 1994; Miyaki et al., 1997; Akiyama et al. 1997). The acquisition of a somatic mutation in the other allele yields a cell that cannot perform MMR. As in FAP patients, colorectal cancer diagnoses occur much earlier in HNPCC patients compared with individuals who develop sporadic colorectal cancer (Kinzler and Vogelstein, 1996). Because DNA repair is compromised, mutations can quickly accumulate in the colorectal epithelial cells of the adenomas. Mutations that occur in the genes involved in colorectal tumorigenesis lead to the conversion of adenomas to carcinomas. HNPCC illustrates how deficiency in DNA repair can accelerate the tumorigenesis process.

3. MSU-1 lineage as a model for tumorigenesis

a. Generation and characterization of the MSU-1 lineage

To study the multi-step process of tumorigenesis in human fibroblasts, the Carcinogenesis Laboratory created the MSU-1 cell lineage as a model system (Morgan et al., 1991). The MSU-1 lineage contains non-tumorigenic, diploid, finite lifespan human fibroblasts through tumorigenic, nearly diploid, infinite life span human fibroblasts (Figure 2). To establish the MSU-1 lineage, normal human fibroblasts derived from the foreskin of a neonate were put into culture. This cell line, designated LG1, was transfected with plasmid containing both the viral oncogene v-*MYC* and the drug resistance marker neomycin (Morgan et al., 1991). Drug resistant, clonal populations were propagated, and virtually all of

Figure 2. The MSU-1 human fibroblast lineage. To establish the MSU-1 lineage, normal human fibroblasts derived from the foreskin of a neonate, designated LG1, were transfected with plasmid containing the viral oncogene v-*MYC*. Clonal populations expressing v-Myc were propagated, and virtually all of these populations eventually entered a senescent state and died. A few cells, probably a clonal population, escaped senescence and continued to proliferate. This surviving infinite life span cell strain was designated MSU-1.0. Subsequent studies by our laboratory showed that telomerase is active in the MSU-1.0 cell strain, whereas telomerase is inactive in its parental cell line LG1. During propagation of the MSU-1.0 cell strain, a faster growing variant cell strain arose and overgrew the culture. This infinite life span variant cell strain was designated MSU-1.1. Karyotype analysis of the MSU-1.1 cell strain showed that it contains 45 chromosomes including two marker chromosomes. In addition to an infinite lifespan, the MSU-1.1 cell strain is also partially growth factor independent. When MSU-1.0 or MSU-1.1 cells are injected in athymic mice, neither forms tumors. However, carcinogen treatment or oncogene transfection of MSU-1.1 cells followed by the isolation of focus-derived MSU-1.1 cells can produce cells that are tumorigenic in athymic mice. In this figure, each arrow indicates a single cell that acquired genetic change or changes (e.g., expression of v-Myc, activation of telomerase, or the presence of two marker chromosomes); each triangle represents a large population of human fibroblasts that arose through clonal expansion of the cell that acquired one or more genetic changes.

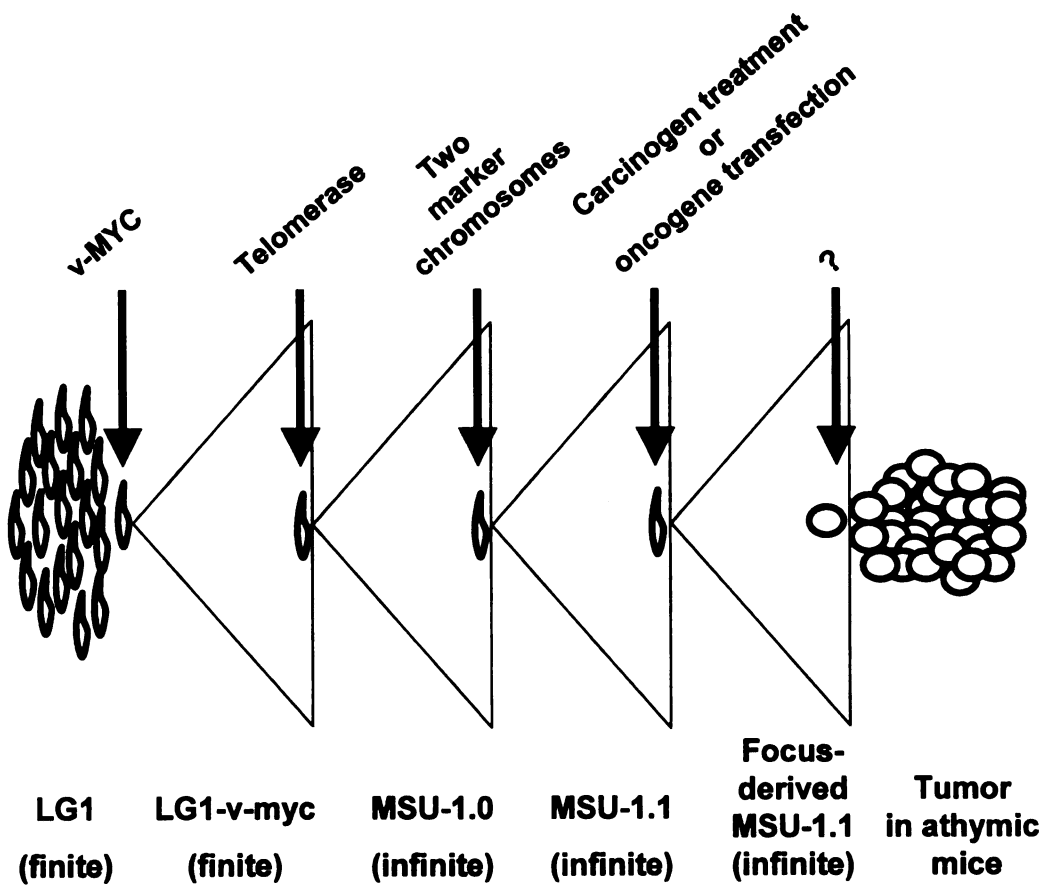
v-MYC
Telomerase
Two marker chromosomes
Carcinogen treatment or oncogene transfection
?
LG1 (finite)
LG1-v-myc (finite)
MSU-1.0 (infinite)
MSU-1.1 (infinite)
Focus-derived MSU-1.1 (infinite)
Tumor in athymic mice

Figure 2

these populations eventually entered a senescent state and died. A few cells, probably a clonal population, escaped senescence and continued to proliferate. The surviving infinite life span cell strain was designated MSU-1.0. Karyotype analysis of MSU-1.0 demonstrated that the cell strain is diploid and has no visible chromosomal anomalies.

Transformed human cells often behave differently in culture than their non-transformed counterparts. Properties such as an infinite lifespan, the loss of contact inhibited growth, the loss of growth factor dependence, the loss of anchorage-dependent growth, and the ability to form tumors in an appropriate host distinguish transformed cells from non-transformed cells (reviewed by Pelengaris et al., 2002). Although the MSU-1.0 cell strain has an infinite lifespan, it does not display the other properties of transformation. It is contact inhibited, growth factor dependent, and anchorage dependent. It does not form tumors when subcutaneously injected into athymic mice.

The fact that only one v-*MYC* expressing cell clone escaped senescence to yield an infinite life span cell strain indicates that expression of the *v-MYC* oncogene alone was insufficient to cause immortalization. Rather, at least one additional genetic change was required to produce the immortalized human fibroblast cell strain MSU-1.0. Subsequent studies by our laboratory show that telomerase is active in the MSU-1.0 cell strain, whereas telomerase is inactive in its parental cell line LG1. Additional studies have demonstrated that expression of the transcription factor Sp1 is approximately 2-3 fold higher in MSU-1.1 cells compared with LG1 cells (Z. Lou, unpublished studies). Since both Myc and Sp1

function as transcription factors for the telomerase gene, the up-regulation of Sp1 together with Myc expression may be responsible for the immortalization of a single clone of v-*MYC* expressing LG1 cells (Kyo et al., 2000).

During propagation of the MSU-1.0 cell strain, a faster growing variant cell strain arose and overgrew the culture. This infinite life span variant cell strain was designated MSU-1.1. Karyotype analysis of the MSU-1.1 cell strain showed that it contains 45 chromosomes including two marker chromosomes. In addition to an infinite lifespan, the MSU-1.1 cell strain is also partially growth factor independent. MSU-1.1 does not display the other markers of transformation; it is anchorage dependent, contact inhibited, and does not form tumors when subcutaneously injected into athymic mice. However, carcinogen treatment (gamma irradiation, UV irradiation, benzo(a)pyrene-diol-epoxide, methyl-nitrosourea, or ethyl-nitrosourea) or oncogene transfection (*H-RAS* or *N-RAS)* of MSU-1.1 followed by the appropriate selection method can produce cells that form tumors in athymic mice (Fry et al., 1986; Hurlin et al., 1989; Yang et al., 1992; Lin et al., 1995; O'Reilly et al., 1998; Boley et al., 2000). Attempts to generate tumorigenic MSU-1.0 derivatives using carcinogens or oncogenes have consistently failed, indicating that the genetic changes that converted MSU-1.0 into MSU-1.1 are critical in the multi-step process of tumorigenesis. Thus, the MSU-1 lineage provides a set of isogenic human fibroblast cell lines/strains at varying steps of the tumorigenesis pathway. These cell lines/strains can be utilized to dissect the molecular mechanisms of carcinogenesis.

b. Using the MSU-1 lineage to study the multi-step process of tumorigenesis in human fibroblasts

The work of Qing et al. (1997, 1999) of this laboratory demonstrates the usefulness of the MSU-1 lineage in studying the multi-step process of tumorigenesis. Because the MSU-1 lineage contains isogenic cell lines/strains, the gene expression pattern of non-tumorigenic MSU-1.1 cells can be compared with the gene expression pattern of a tumorigenic MSU-1.1 derivative. Such comparisons provide information about the genetic changes required to convert a non-tumorigenic MSU-1.1 cell into a tumorigenic cell line. Genes with up-regulated expression in the tumorigenic cell line represent potential oncogenes; genes with down-regulated expression in the tumorigenic cell line represent potential tumor suppressor genes. To carry out such a comparison, Qing et al. (1997, 1999) used differential mRNA display. The gene expression profiles of two cell strains/lines of the MSU-1 lineage, MSU-1.1 and L210 6A/SB1, were compared. MSU-1.1 is an infinite lifespan, nearly diploid human fibroblast cell strain. As discussed above, oncogene transfection or carcinogen treatment of MSU-1.1 followed by appropriate selection can produce cells that form tumors in athymic mice. The cell line L210 6A/SB1 is one such derivative. To generate the L210 6A/SB1 cell line, MSU-1.1 cells were treated with the carcinogen benzo(a)pyrene-diol-epoxide (BPDE), and BPDE-transformed cells were selected using a focus assay. The tumorigenic potential of the focus-derived cell strains was assayed by subcutaneous injection of the focus-derived cells into athymic mice. Tumors that formed in the mice were removed and cultured to

yield tumor-derived MSU-1.1 cell lines. The tumorigenic MSU-1.1 derivative cell line L210 6A/SB1 was derived from a single tumor formed in an athymic mouse by a single focus-derived cell strain. Through the comparison of MSU-1.1 cells with L210 6A/SB1 cells, Qing et al. (1997, 1999) identified two cDNAs strongly down-regulated in the tumorigenic cell line L210 6A/SB1. One cDNA isolated encodes the fibulin-1D extracellular matrix protein. The other cDNA isolated encodes a novel protein designated ST7.

Fluorescent in situ hybridization (FISH) analysis demonstrated that the *ST7* gene is located on the q arm of human chromosome 8 (8q22.2-23.1) (Qing et al., 1999). Examination of the tissue distribution of *ST7* expression by Northern blotting analysis showed that it is widely expressed in human organs (Qing et al., 1999). *ST7* mRNA is most abundant in heart and skeletal muscle, less abundant in brain, lung, pancreas and placenta, and non-detectable in liver and kidney.

To determine if loss of *ST7* expression is a general characteristic of tumorigenic cells, Qing et al. (1999) performed Northern and Western blotting analyses of a set of 31 tumor-derived cell lines consisting of 15 cell lines derived from patients' tumors (sarcomas and carcinomas) and 16 cell lines derived from tumors formed in athymic mice by malignantly transformed MSU-1.1 cells; 92% of the former and 38% of the latter express very low or non-detectable ST7. Based on this data, Qing et al. (1999) concluded that ST7 may play an important role in the transformation of human fibroblasts.

When the novel gene *ST7* was first identified, it was recognized that it had

the characteristics of a transmembrane protein, but at that time no other proteins with significant similarity to ST7 had been reported. Approximately one year later, Yamamoto and his associates, using degenerate oligonucleotides corresponding to the highly conserved region of the ligand-binding domains found in the proteins of the LDLR superfamily, discovered a novel LDLR-related gene, which they designated as the *LRP3* gene (Ishii et al., 1998). Our search of the databases for proteins structurally related to ST7, after the sequence of LRP3 had been submitted, revealed a very strong similarity between LRP3 and ST7, enabling us to recognize that ST7, although it differed significantly from the LDLR prototype, was also a novel member of the LDLR superfamily. In 2000, Sugiyama et al. identified the murine LDLR-related protein 9 (LRP9), another protein homologous to both ST7 and LRP3. The identification of LRP9 supported the classification of ST7 as a novel member of the LDLR superfamily and suggested that ST7, LRP3 and LRP9 constitute a subfamily of the LDLR superfamily. In light of our finding that ST7 is an LDLR-related protein, Section II of this literature review contains a discussion of the structure and function of several key LDLR superfamily members. Section II also discusses the relationship of ST7 to LDLR-related proteins.

II. The LDLR superfamily proteins and their relationship to ST7

A. The LDLR Superfamily

The low-density lipoprotein receptor superfamily is composed of 14 proteins. The superfamily can be divided into core members and more distantly related subfamilies. The seven core members of the superfamily are: **1)** the low-density lipoprotein receptor (LDLR) (Yamamoto et al., 1984); **2)** the very low-density lipoprotein receptor (VLDLR) (Takahashi et al., 1992); **3)** the apolipoprotein E receptor 2 (ApoER2) (Kim et al., 1996; Novak et al., 1996); **4)** the low-density lipoprotein receptor-related protein 1 (LRP1), which is also referred to as the α_2-macroglobulin receptor (Herz et al., 1988; Strickland et al., 1990); **5)** the low density lipoprotein receptor-related protein 2 (LRP2), which is also referred to as megalin (Saito et al., 1994); **6)** the low-density lipoprotein receptor-related protein 1B (LRP1B) (Liu et al., 2000); and **7)** the multiple epidermal growth factor-like domains protein 7 (MEGF7), which is also referred to as *human* LRP4 (Nakayama et al., 1998). The low-density lipoprotein receptor-related protein 5 (LRP5) (Dong et al., 1998; Hey et al., 1998) and the low-density lipoprotein receptor-related protein 6 (LRP6) (Brown et al., 1998) comprise one LDLR subfamily. Another subfamily contains ST7, LRP3, and *murine* LRP9. Finally, LR11/SORLA, which is also referred to as *human* LRP9, (Jacobsen et al., 1996; Morwald et al., 1997) and corin, which is also referred to as *murine* LRP4, (Tomita et al., 1998; Yan et al., 1999) are two more distantly related members of the LDLR superfamily.

Classically, members of the LDLR superfamily such as LDLR, VLDLR,

ApoER2, LRP1, and LRP2 have been defined as endocytic receptors for a plethora of diverse ligands, including lipoproteins and proteases (reviewed by Strickland et al., 1995; Hussain et al., 1999; Howell and Herz, 2001). Recent studies, however, reveal a new role for these proteins, namely as signal transducers (reviewed by Li et al., 2001a; Herz and Bock, 2002; van der Geer, 2002).

1. LDLR

LDLR, the prototype receptor of the superfamily, has been studied since the early 1970s, when Michael S. Brown and Joseph L. Goldstein undertook the task of determining the molecular mechanism underlying the autosomal dominant genetic disorder familial hypercholesterolemia (FH). Patients suffering from FH have an increased concentration of plasma cholesterol and are susceptible to heart attacks very early in life (reviewed by Brown and Goldstein, 1986). Cholesterol exists in the bloodstream in a complex with transport plasma lipoproteins. There are four major classes of plasma lipoproteins: low-density lipoprotein (LDL), very low-density lipoprotein (VLDL), intermediate-density lipoprotein (IDL), and high-density lipoprotein (HDL) (Brown and Goldstein, 1986). Of these four lipoproteins, LDL and HDL transport the majority of cholesterol (Brown and Goldstein, 1986). In 1974, Brown and Goldstein provided the first evidence for the existence of plasma lipoprotein receptors on the cell surface. Specifically, they demonstrated that ^{125}I-LDL binds to normal fibroblasts but does not bind to FH fibroblasts, indicating that the FH fibroblasts lack a functional LDL receptor (LDLR) (Brown and Goldstein, 1974a and 1974b).

Subsequent studies on the fate of the ^{125}I-LDL bound to the cell surface revealed that LDLR mediates uptake of LDL by cells via a process termed receptor-mediated endocytosis (Brown and Goldstein, 1986). Using fibroblasts derived from an FH patient that could bind to ^{125}I-LDL but could not internalize ^{125}I-LDL, they determined that LDLR internalization requires the clustering of receptors in coated pits (Anderson et al., 1976; 1977a; 1977b). Brown and Goldstein received the Nobel Prize in Physiology or Medicine in 1985 for their pioneering work in unraveling the role LDL and LDLR in cholesterol homeostasis.

In 1984, Yamamoto et al. identified the human *LDLR* cDNA. Examination of the tissue distribution of *LDLR* expression in various adult human tissues by Northern blotting analysis shows that it is most abundant in liver and adrenal tissue (reviewed by Hussain et al., 1999). *LDLR* is expressed at a lower level in heart, placenta, lung, kidney, ovary, testis, intestine, brain, and skeletal muscle. Expression is not detectable in adipose tissue. The human *LDLR* gene is located on the p arm of chromosome 19 (19p13.1-13.3) (Lindgren et al., 1985). The protein contains six important structural features: 1) a signal sequence; 2) cysteine-rich, complement-like domains known as low-density lipoprotein receptor class A (LDLRA) domains; 3) an epidermal growth factor (EGF) precursor homology domain; 4) an O-linked glycosylation domain; 5) a single transmembrane domain; and 6) a cytoplasmic domain containing a signal to direct endocytosis (Figure 3) (reviewed by Brown and Goldstein, 1986; Krieger and Herz, 1994; Hussain et al., 1999; Herz and Bock, 2002).

Proteins destined for the transport through the endoplasmic reticulum to

Figure 3. Structures of representative proteins of the LDLR superfamily (adapted from Strickland et al., 2002). The types of domains in these proteins and the organization of these domains divide the superfamily into at least four subfamilies: 1) LDLR, VLDLR, and ApoER2; 2) LRP1, LRP1B, and LRP2; 3) LRP5 and LRP6; 4) ST7, LRP3, and murine LRP9. The cytoplasmic domains of these proteins contain motifs implicated in endocytosis and/or signal transduction, which are not shown. (Images in this dissertation are presented in color.)

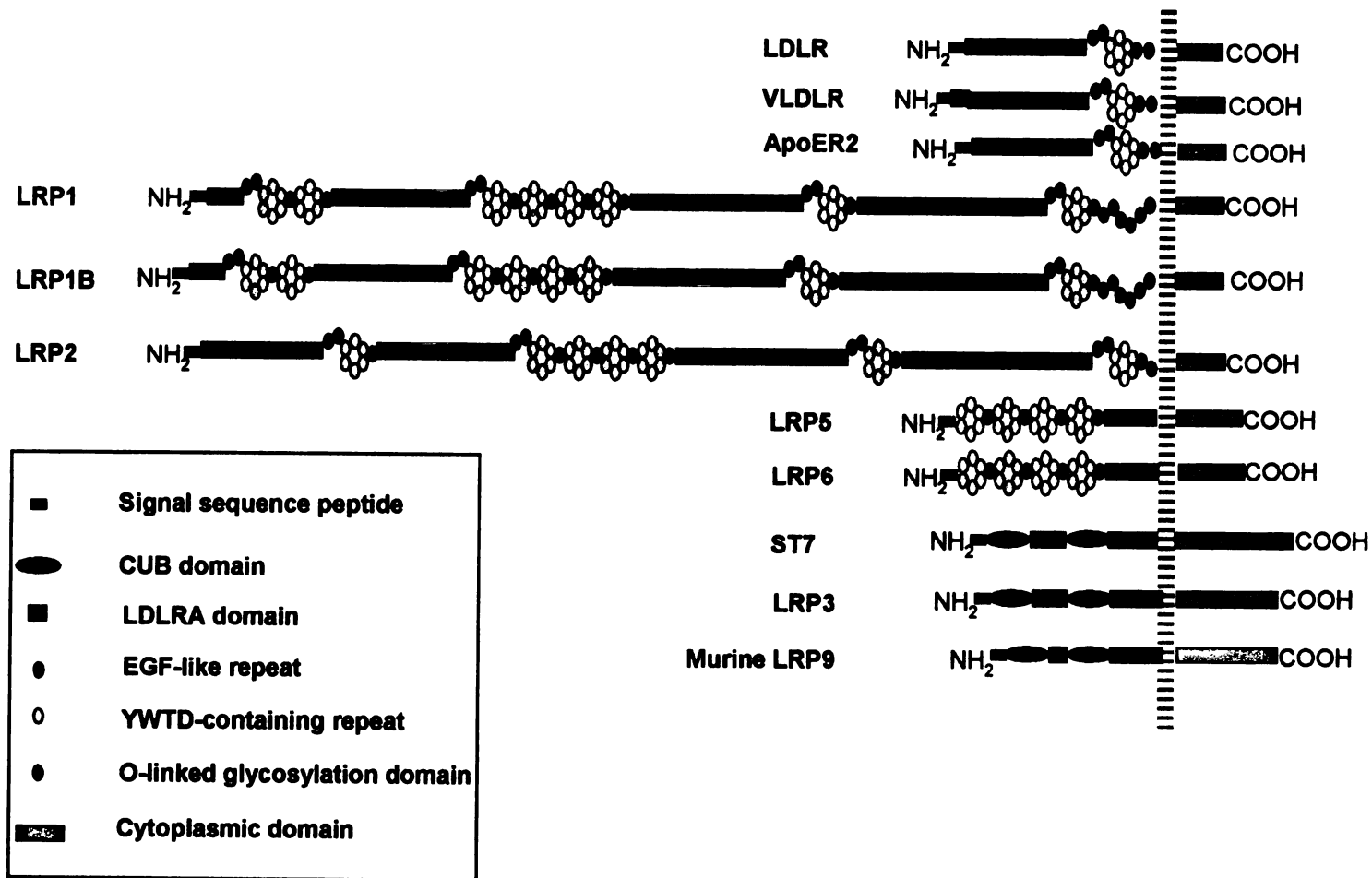
LDLR
VLDLR
ApoER2
LRP1
LRP1B
LRP2
LRP5
LRP6
ST7
LRP3
Murine LRP9
NH2
COOH
Signal sequence peptide
CUB domain
LDLRA domain
EGF-like repeat
YWTD-containing repeat
O-linked glycosylation domain
Cytoplasmic domain

Figure 3

the Golgi apparatus, lysosomes, cell membrane, or extracellular space contain N-terminal signal sequences (reviewed by Claros et al., 1997). These sequences consist of 15-25 residues characterized by a short, positively charged N-terminal region, a hydrophobic core, and a more polar C-terminal region. A cleavage site often follows the signal sequence, and an enzyme known as a signal peptidase cleaves the signal sequence peptide to yield a mature protein. In the case of LDLR, the first 21 amino acids of the protein provide the signal sequence, which is cleaved from the protein on its path from the endoplasmic reticulum to the plasma membrane (Brown and Goldstein, 1986).

LDLR contains seven LDLRA ligand binding domains at its N-terminus, arranged as a cluster of 4 repeats and a cluster of 3 repeats separated by a short linker (Yamamoto et al., 1984). Each LDLRA domain is composed of approximately 40 residues with two distinct features. First, six conserved cysteine residues form three intramolecular disulfide bonds; second, a conserved triad of serine, aspartic acid, and glutamic acid resides at the C-terminus of each domain (Yamamato et al., 1984; Daly et al., 1995). In LDLR and other members of the LDLR superfamily, the LDLRA domains function in ligand binding (Yamamoto et al., 1984; Südhof et al., 1985a, 1985b). For example, these domains in LDLR bind to apolipoprotein E (apoE) and apolipoprotein B-100 (apoB-100) containing lipoprotein particles (Esser et al., 1988; Russell et al., 1989). van Driel et al. (1987) showed that the binding of these ligands to LDLR is Ca^{2+}-dependent. Daly et al. (1995a) determined the first three-dimensional structure of an LDLRA domain. Their work indicates that LDLRA domains fold to

form a β-hairpin structure followed by a series of β turns and that the conserved serine, aspartic acid and glutamic acid residues at the C-terminus of the domain, as well as additional acidic residues, are clustered on one face of the protein. Subsequently, the three-dimensional structures of several more LDLRA domains were determined, both from LDLR and from the LDLR-related protein 1 (LRP1) (Daly et al., 1995b; Fass et al., 1997; Huang et al., 1999; North et al., 2000; Simonovic et al., 2001). All additional structures agreed with that of Daly et al. (1995a). It is thought all LDLRA domains fold in the same general manner, but that each individual LDLRA domain has a unique surface with which ligands interact. These interactions may rely on the acidic clusters, on accessible hydrophobic residues, or on the combination of acidic and hydrophobic residues (Huang et al., 1999). These unique surfaces most likely provide domain specificity. For example, LDLR utilizes different combinations of its seven ligand binding domains to interact with apoE or apoB-100 lipoprotein particles. LDLRA domains 3-7 are required for apoB-100 binding, whereas LDLRA domain 5 is required for apoE binding (Esser et al., 1988; Russell et al., 1989).

EGF precursor homology domains consist of three EGF-type cysteine rich repeats and YWTD repeats that constitute a YWTD domain (reviewed by Krieger and Herz, 1994). The EGF precursor homology domain of LDLR functions in the acid-dependent dissociation of ligands from internalized LDLR and in recycling of the receptor (Davis et al., 1987). The EGF-type cysteine repeats span approximately 40 residues and contain six conserved cysteine residues that form three intramolecular disulfide bonds. Two of the three EGF-type cysteine repeats

(A and B) are separated from the third repeat (C) via the YWTD domain (Krieger and Herz, 1994). Repeats A and B bind calcium (Malby et al., 2001). The three-dimensional structure of the A and B repeats from LDLR has been determined. Saha et al. (2001) showed that these repeats have an elongated, rod-like arrangement. In an analysis of the YWTD repeats from a number of proteins including LDLR, Springer (1998) redefined the boundaries of the YWTD repeat regions. Previously, researchers reported YWTD repeats occur in clusters of five. Springer defined a YWTD domain, which consists of six contiguous YWTD repeats. He proposed that these repeats are not autonomous and that they must fold together to form a single domain with a six-bladed β-propellor structure. Jeon et al. (2001) determined the three-dimensional structure of the YWTD domain in LDLR and confirmed that this domain indeed folds as Springer (1998) suggested.

The extracellular domain of LDLR functions to bind to ligands; the intracellular domain directs endocytosis (reviewed by Brown and Goldstein, 1986; Krieger and Herz, 1994; Hussain et al., 1999). In 1990, Chen et al. reported that the sequence NPVY found in the cytoplasmic domain of LDLR is required for clathrin-mediated endocytosis of the low density lipoprotein receptor (LDLR). This sequence was more generally defined as NPXY since the residue found at position three could be any amino acid. Further work indicated that a larger six-residue sequence, FDNPVY, is actually required by LDLR for internalization (Collawn et al., 1991). In a 1993 review, Trowbridge et al. specify a more general tyrosine-based internalization motif, namely aromatic-X-X-X-X-

aromatic, with arginine and proline perhaps preferred at the third and fourth positions, respectively (aromatic-X-N-P-X-aromatic). (For the sake of simplicity, LDLR-type tyrosine-based endocytic signal will be referred to as an NPXY motif.) Davis et al. (1986) identified a point mutation in LDLR isolated from fibroblasts of a patient suffering from familial hypercholesterolemia. This mutation alters the LDLR endocytic signal, converting the critical tyrosine residue of the signal to cysteine (FDNPVY to FDNPVC). They demonstrated that the replacement of this tyrosine by cysteine significantly decreased the internalization of ^{125}I-LDL.

In summary, LDLR binds to lipoprotein cholesterol transporters in the bloodstream through its interaction with either apoE or apoB-100 and then internalizes the bound lipoproteins (reviewed by Nykjaer and Willnow, 2002). Uptake of these lipoprotein particles from the bloodstream provides cells with the cholesterol necessary to maintain membrane integrity and to synthesis steroid hormones (Brown and Goldstein, 1986). Defects in LDLR function cause aberrations in cholesterol metabolism and cholesterol homeostasis. In addition, Trommsdorff et al. (1998) demonstrated that the cytosolic adaptor proteins Dab1 and Fe65 bind to the NPXY motif in the cytoplasmic domain of LDLR. No functional significance has been uncovered for this interaction. LDLR has not been implicated as a receptor that directly transduces signals.

2. VLDLR and ApoER2

In a search for LDLR related proteins, Takahashi et al. (1992) identified the rabbit VLDLR protein. Briefly, they used low stringency conditions to screen an LDLR-subtracted rabbit heart cDNA library with a probe derived from the

LDLR sequence. Expression of one of the isolated cDNAs in an LDLR-deficient Chinese hamster ovary (CHO) cell line enabled the cDNA-expressing cells to bind to and internalize apoE-containing lipoproteins, VLDL, β-VLDL, and intermediate density lipoprotein (IDL) but not LDL. Examination of the distribution of *VLDLR* expression in various adult rabbit tissues by Northern blotting analysis shows that it is most abundant in heart, muscle, and adipose tissue and that it is also expressed at a lower level in spleen, lung, brain, kidney, adrenal tissue, testis, and small intestine. In contrast to LDLR, VLDLR is barely detectable in liver. In 1994, both Sakai et al. and Oka et al. identified the human *VLDLR* cDNA. Sakai et al. (1994) isolated two VLDLR isoforms. These researchers found that the pattern of *VLDLR* expression in human tissues matches the pattern of *VLDLR* expression in rabbit tissue. The human *VLDLR* gene is located on the p arm of chromosome 9 (9p24) (Oka et al., 1994). The human and rabbit VLDLR proteins are virtually identical. Moreover, the structure of the VLDLR protein is remarkably similar to that of the LDLR protein (Figure 3). VLDLR contains eight LDLRA domains at its N-terminus, arranged as a cluster of 5 repeats and a cluster 3 repeats separated by a short linker (reviewed by Hussain et al., 1999). As described above, LDLR contains only seven LDLRA domains at its N-terminus. The presence or absence of the O-linked glycosylation motif distinguishes the two VLDLR isoforms (Sakai et al., 1994). Both isoforms bind VLDLR ligands, so the loss of this motif does not appear to affect VLDLR function (Hussain et al., 1999).

In 1996, Kim et al. identified the human *ApoER2* cDNA by screening a

human placenta cDNA library using PCR with degenerate primers corresponding to the highly conserved internalization signal found in LDLR and VLDLR. Expression of the *ApoER2* cDNA in an LDLR-deficient CHO cells enables the cells to bind to and internalize apoE-enriched β-VLDL efficiently. These studies also show that ApoER2 binds to LDL, but with low affinity. Examination of the distribution of *ApoER2* expression in various adult human tissues by Northern blotting analysis shows that it is mainly expressed in brain and in placenta (Kim et al., 1996). A low level of expression is detectable in ovary and testis (reviewed by Hussain et al., 1999). Expression is undetectable in heart, lung, liver, skeletal muscle, kidney, and pancreas. The human *ApoER2* gene is located on the p arm of chromosome 1 (1p34) (Kim et al., 1997). The *ApoER2* cDNA encodes a protein very similar to LDLR and VLDLR. The ApoER2 protein contains seven LDLRA domains at its N-terminus, arranged as clusters of 5 repeats and 2 repeats separated by a linker (Figure 3).

The similarity between structures of VLDLR, ApoER2, and LDLR and the fact that VLDLR and ApoER2 bind to and internalize lipoproteins suggests that VLDLR and ApoER2 may function similarly to LDLR, namely in lipoprotein metabolism (Takahashi et al., 1992; Oka et al., 1994; Kim et al., 1996). More recently, however, the VLDR and ApoER2 proteins have been implicated in an important signal transduction pathway that regulates brain development (reviewed by Herz, 2002; Rice and Curran, 2001). The first clue that LDLR superfamily members function in the regulation of brain development was provided by Trommsdorff et al. (1998), who demonstrated that the cytosolic

adaptor protein mDab1 binds to the NPXY sequence found in the cytoplasmic domains of both LDLR and LRP. Previously, Howell et al. (1997) showed that Dab1 plays a critical role in brain development. The *dab1* knock-out mouse exhibits abnormal cellular layering in the cerebral cortex, hippocampus, and cerebellum. This phenotype very closely matches that of the *reeler* mouse, implicating Dab1 as a player in the Reelin pathway. The *reeler* mouse, which arose spontaneously in a population of "snowy-bellied" mice, lacks the large extracellular protein Reelin (D'Arcangelo et al., 1995; reviewed by Rice and Curran, 2001). These knock-out mice showed that Reelin and Dab1 function in the same pathway in the developing brain, but the receptor connecting the extracellular Reelin protein to the intracellular Dab1 adaptor protein was not known. The fact that Dab1 binds to the NPXY sequence in LDLR superfamily members made these proteins interesting candidates for the Reelin receptor. Finally, characterization of the *vldlr/apoer2* double knock-out mouse showed that its phenotype is virtually indistinguishable from that of the *reeler* mouse, suggesting that VLDLR and ApoER2 serve as receptors for Reelin (Trommsdorff et al., 1999). Further studies show that both Reelin and Dab1 bind to VLDR and ApoER2 (Trommsdorff et al., 1999; D'Arcangelo et al., 1999; Hiesberger et al., 1999). Moreover, Reelin must interact with VLDLR and ApoER2 for tyrosine phosphorylation of Dab1 to occur (D'Arcangelo et al., 1999; Hiesberger et al., 1999). Phosphorylation of Dab1 is required to transmit Reelin signals (Howell et al., 2000).

In their recent review, Rice and Curran (2001) synthesize the available

data regarding the Reelin signaling pathway and outline the following model for the role of VLDLR and ApoER2 in this pathway. Reelin binds to the extracellular domains of VLDLR and ApoER2. Such binding has two major consequences, the internalization of Reelin and the activation of a tyrosine kinase signaling cascade resulting in the phosphorylation of Dab1 protein bound to the cytoplasmic domain of the receptors. ApoE inhibits this signaling cascade, presumably by binding to VLDLR and ApoER2 thereby preventing Reelin binding to the receptors. The tyrosine kinase required for Dab1 phosphorylation has not yet been identified. Neither VLDLR nor ApoER2 possess tyrosine kinase activity. Endocytosis of the receptor complex might play a key role in this cascade by shuttling Dab1 from the membrane into the cytosol where a tyrosine kinase can act on it. This theory is intriguing because it links the classical function of LDLR superfamily proteins, namely endocytosis, with a novel function, namely signal transduction. Alternatively, VLDLR and ApoER2 may interact with another cell surface receptor that itself has tyrosine kinase activity or that recruits a tyrosine kinase.

3. LRP1 and LRP1B

The goal of Herz et al. (1988) was to identify additional receptors for apoE-containing lipoproteins. Because Yamamoto et al. (1984) showed that the conserved acidic residues in the LDLRA ligand-binding domains mediate the interaction of LDLR and apoE, Herz et al. (1988) designed oligonucleotides complementary to these highly conserved acidic regions in LDLR and screened a mouse leukocyte library to isolate cDNAs containing such conserved regions.

One partial cDNA isolated from the mouse library encodes eight LDLRA domains. They used this cDNA as an initial probe for cDNA walking in a human liver cDNA library and isolated eight cDNAs covering approximately 15 kB. Together, these cDNAs encode a 4544 residue protein designated LRP1. Subsequent sequencing of the α_2-macroglobulin receptor protein revealed that it is identical to LRP1 (Strickland et al., 1990). Examination of the expression pattern of *LRP1* in various adult human tissues demonstrated that LRP1 is most abundant in liver, brain, and lung, moderately abundant in adrenal tissue, and least abundant in intestine, kidney, placenta, ovary, and testis (reviewed by Hussain et al., 1999). The human *LRP1* gene is located on the q arm of chromosome 12 (12q13.1-14.3) (Myklebost et al., 1989; Hilliker et al., 1992).

Like other members of the LDLR superfamily, LRP1 contains five key structural elements: 1) a signal sequence; 2) cysteine-rich, complement-like domains known as low-density lipoprotein receptor class A (LDLRA) domains; 3) epidermal growth factor (EGF) precursor homology domains; 4) a single transmembrane domain; and 5) a cytoplasmic domain containing a signal to direct endocytosis (Figure 3) (reviewed by Krieger and Herz, 1994). Unlike LDLR, VLDLR, and ApoER2, LRP1 does not contain an O-linked glycosylation domain. LRP1 is approximately five times larger than the LDLR, VLDLR, and ApoER2 proteins. It is synthesized as a single polypeptide chain of 600 kD. Cleavage of this polypeptide into two subunits, a 515 kD subunit and a 85 kD subunit, occurs in the Golgi apparatus, presumably by the protease furin. At the cell surface, the two LRP1 subunits associate by strong noncovalent interactions.

The large 515 kD subunit contains the majority of the protein's extracellular domain; the smaller 85 kD subunit mainly contains the protein's transmembrane and cytosolic domains.

The extracellular region of LRP1 contains 31 LDLRA ligand-binding domains, 22 EGF-type cysteine-rich repeats, and 8 YWTD domains (Figure 3) (Herz et al., 1988). The LDLRA domains are arranged in four clusters containing 2, 8, 10, and 11 LDLRA repeats. Twelve of the 22 EGF-type cysteine-rich repeats, together with four of the eight YWTD domains, constitute four EGF-precursor homology domains. A single EGF-precursor homology domain follows each cluster of LDLRA domains. Four EGF-type cysteine-rich repeats and the four remaining YWTD domains form incomplete EGF-precursor homology domains, each comprised of one YWTD domain followed by one EGF-type cysteine rich repeat. A single incomplete EGF-precursor homology domain immediately precedes the second LDLRA cluster, and three incomplete EGF-precursor homology domains immediately precede the third LDLRA cluster. The remaining six EGF-type cysteine-rich repeats link the extracellular domain to the protein's transmembrane domain. The protein's cytoplasmic domain contains two copies of the NPXY endocytic signal (Herz et al., 1988). The LRP1 cytoplasmic tail also harbors two additional types of internalization signals, a single YXXØ internalization motif and two dileucine motifs (Li et al., 2000). The function of these internalization motifs is discussed below.

Although first identified as an apoE-binding receptor, subsequent studies of LRP1 revealed that its extracellular domain recognizes greater than 30

different ligands (reviewed by Herz and Strickland, 2001; Strickland et al. 2002). These ligands include lipoproteins, proteinases, proteinase-inhibitor complexes, extracellular matrix proteins, bacterial toxins, and viruses. Most LRP1 ligands interact with the second and fourth LDLRA domain clusters (Moestrup et al. 1993; Nykjaer et al., 1994; Horn et al., 1997; Neels et al., 1999). The three-dimensional structures of two individual LDLRA domains located in the second LDLRA cluster of in LRP provide insights into the mechanism by which one protein interacts with so many different ligands (Huang et al., 1999; Dolmer et al., 2000). In general, the primary structure of each individual LDLRA domain is highly variable, with the exception of the six conserved cysteine residues and the conserved acidic residues at the C-terminus of each domain. The conserved residues enable LDLRA domains to fold similarly. The highly variable nature of the other residues in each LDLRA domain provides each domain with a unique surface with which ligands interact. For example, each domain has a unique charge density that dictates ligand binding (Huang et al., 1999; Dolmer et al, 2000). Recently, Mikhailenko et al. (2001) demonstrated that LDLRA clusters may cooperate in ligand binding. Specifically, binding of α_2-macroglobulin requires both the first and second LDLRA domain clusters.

It has been well-established that LRP1 acts as an endocytic receptor for its many ligands (reviewed by Krieger and Herz, 1994; Hussain et al., 1999). Ligand binding leads to internalization of the LRP1-ligand complex through clathrin-coated pits (Czekay et al., 2001). Upon internalization, a decrease in endosomal pH occurs, and the bound ligand dissociates. The receptor is then

recycled to the cell surface and the ligand is degraded in the lysosome. In contrast to LDLR in which the NPXY signal directs LDLR-mediated endocytosis, the single YXXØ motif in the LRP cytoplasmic tail acts as the dominant signal for LRP1-mediated endocytosis (Li et al., 2000). One of the two dileucine motifs also functions to a lesser degree in directing endocytosis. Subsequently, Li et al. (2001b) also demonstrated that protein kinase A (PKA) mediates phosphorylation of a serine residue in the LRP1 cytoplasmic domain. Mutation of this serine residue to either alanine or threonine abolishes LRP1 phosphorylation by PKA. Moreover, these LRP1 mutants exhibit a reduced rate of endocytosis and less efficient delivery of ligands for degradation, suggesting that PKA-mediated phosphorylation of LRP1 regulates endocytosis.

Recent evidence suggests that LRP1 not only functions in endocytosis but also in signal transduction (Trommsdorff et al., 1998; Gotthardt et al., 2000; Barnes et al., 2001; Boucher et al., 2002; Loukinova et al., 2002; Lutz et al., 2002). Trommsdorff et al. (1998) and Gotthardt et al. (2000) demonstrated that the cytoplasmic tail of LRP1 binds to multiple adaptor and scaffold proteins. These investigators utilized the yeast two-hybrid assay system, GST-fusion protein pull-down assays, and co-immunoprecipitation to discover and confirm these interactions. Trommsdorff et al. (1998) showed that the cytosolic adaptor proteins Dab1 and Fe65 bind to the NPXY motif in the cytoplasmic domain of LRP. Gotthardt et al. (2000) detected eleven proteins that interact with the cytoplasmic tail of LRP1 (e.g., JIP-1, JIP-2, ICAP-1, PSD-95). These LRP-1 interacting proteins are known to be involved in cellular processes such as "the

regulation of synaptic transmission, activation and modulation of MAP kinase pathways, local organization of the cytoskeleton, cell adhesion, and endocytosis" (Gotthardt et al., 2000).

Barnes et al. (2001) demonstrated that LRP1 is tyrosine-phosphorylated in v-Src transformed fibroblasts and that tyrosine-phosphorylated LRP1 interacts with the cytosolic adaptor protein Shc. They propose that LRP1 provides a link between the Src and Ras pathways in v-Src transformed fibroblasts. Specifically, myristylation of the non-receptor tyrosine kinase Src targets it to insert in the plasma membrane. This event localizes Src in proximity to the LRP1 cytoplasmic domain, and Src phosphorylates LRP1. Tyrosine-phosphorylated LRP1 then recruits Shc to the membrane by binding to Shc's phosphotyrosine binding (PTB) domain. At the membrane, Src can phosphorylate Shc. Phosphorylated Shc binds to the scaffolding protein Grb2, which recruits SOS leading to the activation of the Ras pathway.

Finally, Boucher et al. (2002) and Loukinova et al. (2002) showed that LRP1 serves as a co-receptor for the growth factor PDGF beta. Specifically, these studies showed that LRP1 directly interacts with PDGF beta and that treatment of fibroblasts with PDGF beta induces phosphorylation of tyrosine residues in the cytoplasmic domain of LRP1. Moreover, only LRP1 molecules localized in caveolae are phosphorylated upon PDGF treatment. Caveolae are invaginations of the plasma membrane that are rich in cholesterol, GPI-anchored proteins, and several receptor and nonreceptor tyrosine kinases (reviewed by Pelkman and Helenius, 2002). These membrane structures are involved in

mediating endocytosis through a clathrin-independent mechanism. Moreover, the fact that so many signaling molecules reside in caveolae suggests that these structures may also function in regulating signal transduction. In addition to LRP1, both PDGF receptors (PDGFR) and Src family kinases can be localized to caveolae (Boucher et al., 2002). These studies of LRP1 phosphorylation further demonstrated a requirement for the kinase activity of the PDGFR because PDGFR-specific kinase inhibitors block LRP1 phosphorylation. However, wortmannin, an inhibitor of PI3K, also completely blocks LRP1 phosphorylation, suggesting that the PDGFR kinase does not directly phosphorylate LRP1. Instead, Boucher et al. (2002) proposed that a PDGFR/PI3K activated tyrosine kinase phosphorylates LRP1. The Src family kinases fit this profile. The work of Barnes et al. (2001) described immediately above supports the conclusion that Src family kinases phosphorylate LRP1. Moreover, Loukinova et al. (2002) showed that a Src family-specific kinase inhibitor blocks LRP1 phosphorylation. Taken together, these studies suggest the following model for LRP1's interaction with the PDGF beta pathway. Homodimers of the PDGF beta growth factor bind to the PDGFR causing receptor dimerization and autophosphorylation of tyrosines in the receptor's cytoplasmic tail. Activation of PDGFR leads to activation of Src family kinases co-localized with PDGFR at caveolae. Because LRP1 also binds to PDGF beta homodimers and both PDGFR and LRP1 reside in caveolae, PDGFR and LRP1 co-localize at the cell surface. This brings LRP1 in close proximity to active Src family kinases, which phosphorylate LRP1. Subsequently, phosphorylated LRP1 recruits Shc to the membrane. This leads

to activation of the Shc and Shc-mediated signaling pathways. Therefore, the localization of these signaling molecules to caveolae directly contributes to this pathway, suggesting an intimate link between signaling and endocytosis.

Using representational difference analysis (RDA), a technique for comparing complex but highly related genomes to discern losses or gains in chromosomal DNA, Lisitsyn et al. (1995) identified seven loci in the human genome that appear to be homozygously deleted in a variety of human tumor cell lines. One such homozygously deleted locus observed in both a renal cancer cell line and a bladder cancer cell line mapped to chromosome 2q21.2. Liu et al. (2000) proposed that chromosome 2q21.2 contains a candidate tumor suppressor gene. Using exon trapping analysis and 5' and 3' RACE, they identified another novel member of the LDLR superfamily, *LRP1B* (initially designated *LRP1-DIT*). Liu et al. (2000a, 2000b) then examined a panel of non-small cell lung carcinoma (NSCLC) cell lines for alterations in the *LRP1B* gene. They reported inactivation of the *LRP1B* gene in approximately 45% of the cell lines studied. Inactivation of the *LRP1B* gene can occurs through homozygous deletions within the *LRP1B* gene. Point mutations in the *LRP1B* alleles were also observed. Finally, some cell lines expressed abnormal truncated *LRP1B* transcripts. In contrast, none of the small cell lung cancer cell lines studied had any observable defects in the *LRP1B* sequence, and only one such line contained abnormal *LRP1B* transcripts. These findings strongly suggest that inactivation of *LRP1B* plays an important in the development of non-small cell lung cancer, but not the development of small cell lung cancer. Most recently,

Langbein et al. (2002) also documented alterations of the *LRP1B* gene in high grade urothelial cancer.

LRP1 and LRP1B share 59% amino acid identity (Liu et al., 2000b). Moreover, the LRP1 and LRP1B proteins have virtually identical structures (Figure 3). LRP1B contains one additional LDLRA domain in its fourth cluster, and it has a small sequence insertion in its cytoplasmic domain between the two NPXY domains. The expression pattern of *LRP1B* differs from that of *LRP1*. As described above, *LRP1* expression is most abundant liver, lung, and brain (reviewed by Hussain et al., 1999); *LRP1B* expression is most abundant in thyroid gland, salivary gland, and brain (Liu et al., 2001). *LRP1B* transcripts are also detectable by RT-PCR in heart, kidney, lung, liver, and bladder, although expression in these tissues is low.

Because of the strong structural similarity between LRP1 and LRP1B, Liu et al. (2001) proposed that LRP1B binds to LRP1 ligands. Therefore, they assessed the ability of three LRP1 ligands, urokinase plasminogen activator (uPA), tissue plasminogen activator (tPA), and plasminogen activator inhibitor type-1 (PAI-1), to bind to LRP1B. Both uPA and tPA are serine proteases, and PAI-1 is a serine protease inhibitor that targets uPA and tPA (reviewed by Andreasen et al., 2000). As discussed above, LRP1 contains two major ligand binding sites, the second and fourth LDLRA clusters. Because previous work demonstrated that an LRP1 minireceptor containing the fourth LDLRA cluster, the transmembrane domain, and the cytoplasmic tail binds to and internalizes ligands, Liu et al. (2001) constructed a similar LRP1B minireceptor, containing its

fourth LDRLA cluster, transmembrane domain, and cytoplasmic tail. *LRP1*-null CHO cells expressing the LRP1B minireceptor bind to and internalize uPA, tPA, and PAI-1, however, the kinetics of ligand endocytosis differs between LRP1 and LRP1B. Specifically, LRP1B internalizes ligands much more slowly than LRP1.

The finding that LRP1B serves as a receptor for uPA and PAI-1 raises the possibility that LRP1B regulates the uPA system. It is well established that the uPA system plays a key role in the invasion and metastasis of tumor cells as well as in angiogenesis (reviewed by Dano et al., 1999; Andreasen et al., 2000). To understand the role LRP1B plays in regulating this system, it is first necessary to review how LRP1 regulates the uPA system. Much work has shown that LRP1 mediates the function of the uPA system (Nykjaer et al., 1992; Kounnas et al., 1993; Conese et al., 1995; Nykjaer et al., 1997; Weaver et al., 1997; Czekay et al., 2001; also reviewed in Herz and Strickland, 2001). Three recent reviews, two by Andreasen et al. (1997, 2000) and one by Herz and Strickland (2001), explain the uPA proteolytic system and its interaction with LRP1. Briefly, uPA is synthesized as a single chain zymogen (pro-uPA) and proteolysis of pro-uPA yields an active two-chain enzyme (uPA). Interaction of uPA with its receptor uPAR, a 55-kDa glycosylphosphatidyl-inositol (GPI) -anchored cell surface protein, enhances the conversion of pro-uPA to uPA by concentrating pro-uPA on the cell surface. Receptor-bound uPA then cleaves plasminogen to produce the active protease plasmin, which degrades many components of the extracellular matrix and may be involved in activating matrix metalloproteinases (MMPs). The serine proteinase inhibitor PAI-1 binds to receptor-bound uPA. A

conformational change occurs in PAI-1 upon binding to uPA, revealing a high affinity binding site for LRP1. Interaction of the PAI-1:uPA:uPAR complex with LRP1 triggers rapid endocytosis of the complex. uPAR and LRP1 are recycled, and the uPA and PAI-1 molecules are degraded. In summary, LRP1 functions to regenerate unoccupied uPAR on the cell surface. Unoccupied uPAR then binds to pro-uPA to begin the proteolytic cascade again. Therefore, the presence of LRP1 on the cell surface promotes uPA-mediated cellular responses, such as cellular motility.

In contrast, the presence of LRP1B on the cell surface inhibits the uPA proteolytic cascade (Liu et al., 2002). CHO cells expressing an LRP1B minireceptor exhibit a 90% reduction in endocytosis of PAI-1:uPA:uPAR complexes compared with CHO cells expressing a comparable LRP1 minireceptor. Moreover, the LRP1B-expressing cells display a decreased rate of cellular migration compared with the LRP1-expressing cells. Therefore, accumulation of LRP1B:PAI-1:uPA:uPAR complexes on the cell surface inhibits the regeneration of unoccupied uPAR, the activation of plasminogen by uPA, and cellular migration. Liu et al. (2002) propose that LRP1B functions a tumor suppressor by negatively regulating proteolytic cascades necessary for invasion and metastasis. Therefore, loss of LRP1B protein would contribute to the regeneration of free uPAR because PAI-1:uPA:uPAR complexes would be free to bind to another receptor with faster endocytic kinetics. Ultimately, this causes a higher level of uPA activity on the surface of cells lacking LRP1B. As discussed above, uPA activity leads to degradation of the extracellular matrix and activation

of MMPs. Such activity contributes to tumor invasion and metastasis.

4. LRP5 and LRP6

In their search for candidate genes in an insulin-dependent diabetes mellitus locus (IDDM4) at chromosome 11q13, Hey et al. (1998) identified a cDNA encoding the LDLR-related protein 5 (LRP5). Specifically, they constructed a contig containing the IDDM4 locus and used high-throughput sequence analysis to identify genes within this region. Simultaneously, Dong et al. (1998) identified the *LRP5* cDNA in their screen for novel genes expressed in human osteoblasts. Examination of the tissue distribution of *LRP5* expression in various adult human tissues by Northern blotting analysis shows that it most abundant in liver, pancreas, prostate, placenta, and small intestine (Hey et al., 1998). It is less abundant in heart, lung, skeletal muscle, kidney, spleen, thymus, testis, and colon. A very low level of *LRP5* mRNA is detectable in brain and leukocytes.

Brown et al. (1998) used a region of the *LRP5* nucleotide sequence to screen a mouse liver cDNA library for *LRP5*-related sequences and isolated a cDNA encoding a part of the novel LDLR-related protein LRP6. To isolate the human *LRP6* cDNA, they used the mouse *LRP6* cDNA as probe to screen a human kidney cDNA library. Examination of the tissue distribution of *LRP6* expression in various adult human tissues by Northern blotting analysis shows that it is most abundant in heart, brain, placenta, lung, kidney, pancreas, spleen, testis, and ovary. It is expressed at a lower level in liver, skeletal muscle, prostate, and colon. *LRP6* mRNA is undetectable in thymus, small intestine, and

leukocytes. The human *LRP6* gene is located on the p arm of chromosome 12 (12p11.2-13.324) (Brown et al., 1998).

The LRP5 and LRP6 proteins share 82% amino acid similarity, as determined by the protein-protein BLAST program (www.ncbi.nlm.nih.gov). Most importantly, these proteins consist of the same types of functional motifs arranged in an identical configuration (Figure 3). The N-terminus of each protein contains a putative signal sequence to direct the protein to the plasma membrane, residues 1-25 in LRP5 and 1-19 in LRP6. These signal peptides are predicted to be cleaved from the mature protein. Each mature protein consists of four key structural features linking these LRPs to the LDLR superfamily: 1) components of EGF-precursor homology domains; 2) LDLRA ligand-binding domains; 3) a single transmembrane domain; 4) a cytoplasmic tail with a signal to direct endocytosis (Hey et al., 1998; Brown et al., 1998). The arrangement of these conserved protein domains in LRP5 and LRP6, however, differs from that of the core members of the LDLR superfamily (Figure 3). The N-terminus of both LRP5 and LRP6 contain four YWTD domains and four EGF-type cysteine rich repeats. A single EGF-type cysteine rich repeat follows each YWTD domain. Therefore, the extracellular domain of LRP5 and LRP6 contains four incomplete EGF-precursor homology domains. Three LDLRA ligand-binding domains follow the incomplete EGF-precursor homology domains.

The cytoplasmic tails of LRP5 and LRP6 are highly conserved between these two proteins, but are much less similar to the other LDLR family members. The serine and proline rich cytoplasmic tails of LRP5 and LRP6 lack the

conserved NPXY internalization sequence found in other LDLR family members. These proteins, however, contain two other motifs implicated in directing receptor-mediated endocytosis (Hey et al., 1998; Brown et al, 1998). Both LRP5 and LRP6 contain a tyrosine-based YXXØ internalization motif, which was first identified as the signal required for clathrin-mediated endocytosis of the transferrin receptor and the cation independent mannose 6-phosphate receptor (Jing et al., 1990; Collawn et al., 1990;Canfield et al., 1991; Lobel et al., 1989; Jadot et al., 1992). In addition to this tyrosine-based motif, LRP5 and LRP6 contain a dileucine motif, a category of sorting signals distinct from the tyrosine-based motifs. It has been reported that dileucine motifs play two roles in protein sorting (Trowbridge et al., 1993; Rapoport et al., 1998). First, dileucine motifs are recognized at the plasma membrane and direct clathrin-mediated endocytosis. Second, dileucine motifs are recognized in the trans-Golgi network (TGN) and direct protein sorting from the TGN to lysosomes or secretory vesicles.

The unique arrangement of the extracellular domains in LRP5 and LRP6 as well as their unique cytoplasmic tails indicates that these proteins constitute a subfamily of the LDLR superfamily. No studies have been done to demonstrate that LRP5 and LRP6 function in receptor-mediated endocytosis. However, several groups recently demonstrated that LRP5 and LRP6 act as essential players in the Wnt signaling pathway (Wehrli et al., 2000; Tamai et al., 2000; Pinson et al., 2000; Mao et al., 2001). In their studies of the Wingless (Wg) pathway, the *Drosophila* counterpart of the Wnt pathway, Wehrli et al. (2000)

isolated the *arrow* gene and determined that its gene product is essential for all Wg signaling pathways. The arrow protein is homologous to the human and murine LRP5 and LRP6 proteins. Tamai et al. (2000) showed that ectopic expression of the human LRP6 protein in *Xenopus* embryos induces expression of Wnt-responsive genes, leading to dorsal axis duplication and neural crest formation. They also demonstrated that LRP6 binds to Wnt-1 and that the LRP6-Wnt complex associates with Frizzled (Fz). There are at least ten Fz proteins that constitute a family of receptors for Wnt glycoproteins (reviewed by Wodarz and Nusse, 1998). The findings of Tamai et al. (2000) suggest that LRP5 and LRP6, together with Fz, act as co-receptors for Wnt family glycoproteins. Pinson et al. (2000) provided additional evidence supporting the evolutionarily conserved role of LRP5 and LRP6 as Wnt co-receptors. Specifically, they isolated mice with a disrupted *LRP6* gene and found that these mice, which lack functional LRP6 protein, die at birth because of severe developmental defects, including malformation of the skeleton, limbs, eyes, central nervous system, and urogenital tract. The phenotype of *LRP6* knock-out mice mimics the phenotype of *Wnt* null mice (Pinson et al., 2000). *LRP6* null mice, however, do not demonstrate all of the phenotypes of *Wnt* null mice, and the defects observed in the *LRP6* null mice are generally less severe than those observed in *Wnt* null mice. The presence of LRP5 may partially compensate for the lack of LRP6 in these mice. *LRP5* null mice exhibit a different phenotype compared with *LRP6* null mice. These mice are viable and fertile but display defects in eye vascularization and in skeletal development (Kato et al., 2002). Interestingly, *LRP5* heterozygote mice also

displayed a mild defect in skeletal development. The activity of the Wnt-responsive transcription factor Lef1 in osteoblasts derived from *LRP5* null mice was lower than in osteoblasts derived from normal mice (Kato et al., 2002). This suggests that LRP5 acts to mediate Wnt signaling in osteoblasts.

Mutations in the human *LRP5* gene result in defects in bone density and eye development (Gong et al., 2001). Individuals homozygous for inactivating mutations in the LRP5 gene suffer from the autosomal recessive genetic disorder osteoporosis-pseudoglioma (OPPG) syndrome. Children born with OPPG syndrome have a very low bone density and are prone to bone fractures and skeletal and ocular deformities. Similar to the *LRP5* heterozygous mice, OPPG carriers (individuals with one mutant *LRP5* allele and one wild-type *LRP5* allele) exhibit a lower bone density than non-carriers. Gong et al. (2001) also provide evidence of a functional interaction between LRP5 and Wnt family glycoproteins. Recently, two other groups reported that LRP5 mutations cause the autosomal dominant high-bone-mass (HBM) trait (Little et al., 2002; Boyden et al., 2002).

Although the studies described above show that LRP5 and LRP6 act as co-receptors for Wnt glycoproteins, they do not explain how these co-receptors transduce Wnt signals. Specifically, studies of the Wnt signaling pathway show that the binding of Wnt to its co-receptors leads to the stabilization of β-catenin protein, the translocation of stabilized β-catenin protein from the cytosol to the nucleus, the activation of the TCF/LEF family of transcription factors by β-catenin protein, and the transcription of TCF/LEF responsive genes (reviewed by Wodarz and Nusse, 1998; Polakis, 2000; Zorn, 2001). An important breakthrough in

understanding how these co-receptors transduce Wnt signals comes from the work of Mao et al. (2001), who demonstrated that LRP5 binds to the scaffolding protein axin, thereby promoting β-catenin stabilization. Axin contains binding sites for three important intracellular Wnt pathway proteins: β-catenin, glycogen synthase kinase-3β (GSK-3β), and APC (Nakamura et al., 1998; Kishida et al., 1998). In unstimulated cells, a complex of axin, β-catenin, GSK-3β, and APC forms (reviewed by Bienz, 2002; Moon et al., 2002). GSK-3β phosphorylates β-catenin, which targets β-catenin for ubiquitination and subsequent proteosomal degradation. Mao et al. (2001) proposed that the binding of Wnt to LRP5 causes axin to translocate from the cytosol, where it promotes the degradation of β-catenin, to the cell membrane, where it no longer promotes the degradation of β-catenin. Therefore, β-catenin accumulates in the cytosol. This leads to the translocation of β-catenin from the cytosol to the nucleus and subsequent activation of Wnt-responsive genes. They also showed that localization of axin at the membrane promotes the destabilization and degradation of axin.

Up-regulated activation of gene transcription by β-catenin occurs in human cancer cells through disruption of the Wnt signaling pathway. Several genes in the Wnt pathway have been shown to be proto-oncogenes (β-*CATENIN*) or tumor suppressor genes (*APC, AXIN*). Monoallelic activating mutations in the *β-CATENIN* gene have been observed in colorectal tumors, gastric tumors, hepatocellular carcinomas, thyroid tumors, endometrial ovarian tumors, prostate tumors, and melanomas (reviewed by Polakis, 2000). These mutations usually affect the APC-interaction domain of β-catenin such that mutant β-catenin can

not interact with APC (Morin et al., 1997; Rubinfeld et al. 1997). Therefore, it is not targeted for proteosomal degradation. As described above, biallelic inactivating mutations in the *APC* gene have been observed in greater than 80% of all colorectal cancers, supporting the argument that APC is an important gatekeeper in colorectal epithelium (Kinzler and Vogelstein, 1996). Biallelic inactivating mutations in the *axin* gene have been observed in human hepatocellular carcinomas (Satoh et al., 2000). It will not be surprising if mutations in *LRP5* and *LRP6* are observed in human tumors. Although mutations in *FZ* have not been documented in human tumors, overexpression of *FZ* has been observed in human esophageal tumor cells but not in matched normal cells (Tanaka et al., 1998). Such overexpression leads to increased nuclear localization of β-catenin and a concomitant increase in transcription of Wnt-responsive genes.

5. LRP3 and LRP9, two ST7-related proteins

When the novel gene *ST7* was first identified, it was recognized that it had the characteristics of a transmembrane protein, but at that time no other proteins with significant similarity to ST7 had been reported. Approximately one year later, Yamamoto and his associates discovered a novel protein with a high degree of similarity to ST7 (Ishii et al., 1998). To isolate novel members of the LDLR superfamily, they designed oligonucleotides corresponding to a highly conserved region of the ligand-binding domains found in the LDLR superfamily proteins and used these oligonucleotides to amplify novel LDLR-like cDNAs from a rat liver cDNA library. One cDNA isolated contained DNA sequences encoding

LDLR ligand-binding domains, and it was designated LDLR-related protein 3 (LRP3). Using the rat *LRP3* cDNA as a probe, they then isolated the human *LRP3* cDNA from a library constructed from the human liver cell line HepG2. Using FISH analysis, Ishii et al. (1998) determined that the *LRP3* gene is located on the q arm of human chromosome 19 (19q12-13.2). Examination of the tissue distribution of *LRP3* expression by Northern blotting analysis shows that it is highly expressed skeletal muscle, ovaries, heart, brain, liver, pancreas, prostate, and small intestine and weakly expressed in testis, colon, and leukocytes. A trace amount of *LRP3* transcript is detectable in placenta, lung, kidney, spleen, and thymus. Finally, Ishii et al. (1998) reported that LRP3 protein fails to bind to β-VLDL and the receptor associated protein (RAP), two proteins that bind to other members of the LDLR superfamily. Therefore, they concluded that LRP3 may function in a pathway other than lipoprotein metabolism. No further studies describing LRP3 have been reported.

In 2000, Sugiyama et al. identified the murine LDLR-related protein 9 (LRP9), a protein homologous to both ST7 and LRP3. To isolate novel members of the LDLR superfamily, they used a novel signal sequence trap method to screen a mouse lymphocyte cDNA library. This procedure takes advantage of the fact that cell membrane receptors contain signal peptides in their protein sequences that direct the receptors to their final destination in the plasma membrane. Briefly, cDNA fragments from the mouse lymphocyte cDNA library were fused to a constitutively active, signal-sequence deficient *mpl* cytokine receptor. These hybrid cDNAs were expressed in a murine interleukin-3-

dependent pro-B cell line. The presence of an in-frame signal sequence in the lymphocyte cDNA fragment causes targeting of the constitutively active *mpl* cytokine receptor to the plasma membrane. Cells with the constitutively active *mpl* cytokine receptor on the cell surface can grow in the absence of interleukin-3; cells lacking the constitutively active *mpl* cytokine receptor on the cell surface undergo apoptosis. Therefore, surviving cells contained lymphocyte cDNAs that encode signal sequence peptides. Once recovered from the surviving pro-B cells, the cDNA fragments were used to isolate the full-length cDNAs of interest, including *LRP9*. Examination of the tissue distribution of *LRP9* expression in various adult murine tissues by Northern blotting analysis shows that it is highly expressed in heart, lung, liver, and kidney, weakly expressed in brain and spleen, and not expressed in skeletal muscle and testis (Sugiyama et al., 2000). Examination of the tissue distribution of *LRP9* expression in various adult human tissues by Northern blotting analysis using the mouse *LRP9* probe shows a slightly different expression pattern (Sugiyama et al., 2000). The highest expression of *LRP9* transcripts in the human tissues examined occurs in the kidney. Skeletal muscle and heart tissue have slightly lower levels of *LRP9* mRNA. Trace amounts of *LRP9* mRNA are detectable in leukocyte, lung, placenta, small intestine, liver, spleen, thymus, and colon. *LRP9* mRNA is undetectable in brain. Sugiyama et al. (2000) also demonstrated that apoE-enriched β-VLDL stimulates cholesteryl ester formation in LDLR-deficient CHO cells expressing HA-tagged LRP9. Based on this observation, they concluded that LRP9 mediates the cellular uptake of apoE-enriched β-VLDL. They did not,

however, provide evidence of a direct interaction between apoE-enriched β-VLDL and LRP9. Finally, they used radiation hybrid mapping to localize the human *LRP9* gene to the q arm of chromosome 14 (14q11.2). The human homologue of the murine *LRP9* cDNA has not yet been isolated, and no further studies describing LRP9 have been reported. (The protein referred to as human LRP9 in the literature is not the homologue of the murine protein. It is the LR11/SORLA protein.)

The protein structures of LRP3 and LRP9 are highly conserved. LRP3 and LRP9 consist of the same types of functional motifs arranged in an identical configuration (Figure 3). Similar to other LDLR superfamily members, the N-terminus of each protein contains a putative signal sequence to direct the protein to the plasma membrane, residues 1-30 in LRP3 and residues 1-17 in LRP9 (Ishii et al., 1998; Sugiyama et al., 2000). These signal peptides are predicted to be cleaved from the protein. The extracellular domain of each mature protein consists of two key structural features. First, like other LDLR superfamily members, LRP3 and LRP9 contain LDLRA ligand-binding domains. There are five LDLRA domains present in LRP3 and four present in LRP9. In LRP3, these domains are arranged in two clusters, one containing two domains and the other containing three domains. In LRP9, the first cluster contains only one domain and the second cluster contains three domains. Second, unlike other LDLR superfamily proteins, LRP3 and LRP9 contain two Complement factor C1s/C1r, Urchin embryonic growth factor, Bone morphogenetic protein (CUB) domains. One CUB domain precedes each LDRLA cluster. Each CUB domain is

composed of approximately 110 residues with four conserved cysteine residues that form two intramolecular disulfide bonds. These domains are found in a wide variety of unrelated proteins and are believed to participate in protein-protein interactions (reviewed by Bork and Beckmann, 1993; Christensen and Birn, 2002). The three-dimensional structure of the CUB domain present in bovine acidic seminal fluid protein (aSFP), a member of the spermadhesin protein family, has been determined to be a barrel-like structure with two five-stranded β-sheets joined by surface exposed β-turns (Romao et al., 1997). CUB domain containing proteins have been shown to form dimers. For example, the three dimensional structure of a heterodimer of two porcine seminal plasma spermadhesins, PSP-I and PSP-II, shows that these proteins interact through the CUB domain present in each (Varela et al., 1997). Similar to other LDLR family members, a single transmembrane domain follows the extracellular domain of LRP3 and LRP9, and the cytoplasmic tails of LRP3 and LRP9 contain several putative signals to direct endocytosis. Specifically, these proteins contain at least one NPXY-like sequence, a YXXØ sequence, and at least one dileucine motif. The cytoplasmic tails of these proteins are also notably rich in serine, threonine, and proline residues.

B. The relationship between ST7 and the LDLR superfamily

The identification of LRP3 and LRP9 enabled our group to classify ST7 as a novel member of the LDLR superfamily. (Chapter II of this dissertation contains a detailed analysis of the ST7 protein structure.) The ST7 protein shares 66% amino acid similarity with LRP3 and 46% amino acid similarity with LRP9, as

determined by the protein-protein BLAST program (www.ncbi.nlm.nih.gov). The N-termini of ST7, LRP3 and LRP9 contain the same types of functional domains, namely LDLRA domains and CUB domains, arranged in an identical configuration (Figure 3). The juxtamembrane regions of the ST7, LRP3, and LRP9 cytoplasmic domains are also highly conserved. The other members of the LDLR superfamily are more distantly related to the ST7 subfamily proteins (reviewed by Howell and Herz, 2001). Specifically, the extracellular domains of the other LDLR superfamily proteins contain EGF-precursor homology domains, which are not present in the ST7 subfamily proteins, but lack CUB domains, which are present in the ST7 subfamily proteins. There are, however, three features that link the ST7 subfamily to all other members of the LDLR superfamily. First, all proteins contain signal sequences directing transport to the cell membrane. Second, all proteins contain LDLRA ligand-binding clusters. Finally, all proteins contain sequences implicated in receptor-mediated endocytosis.

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Chapter II

ST7 is a novel member of the low-density lipoprotein receptor (LDLR) superfamily with a cytoplasmic tail that interacts with proteins related to signal transduction pathways*

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[2] Manuscript in preparation, M.A. Battle, S. O'Reilly, V.M. Maher, and J. J. McCormick. Expression of ST7 protein inhibits the tumor-forming ability of a human fibrosarcoma-derived cell line.

[3] The abbreviations used are: LDLR, low-density lipoprotein receptor; LRP, lipoprotein receptor-related protein; LDLRA, low-density lipoprotein receptor type A; CUB, Complement factor C1s/C1r, Urchin embryonic growth factor, Bone morphogenetic protein; RACK1, receptor for activated protein kinase C; SARA, SMAD anchor for receptor activation; MIBP, muscle integrin binding protein; SNAPIN, SNARE-associated protein.

ABSTRACT

Previous studies identified the novel putative tumor suppressor ST7. Subsequent discovery of two proteins closely-related to ST7 strongly suggests that ST7 is a novel member of the low-density lipoprotein receptor (LDLR) superfamily. It has been proposed that ST7 and its two related proteins constitute an LDLR subfamily. Although proteins of the LDLR superfamily are best known as endocytic receptors, recent studies of several such proteins demonstrate that their cytoplasmic domains interact with a variety of adaptor and scaffold proteins implicated in signal transduction pathways. To evaluate ST7's relationship to the proteins of the LDLR superfamily, we used proteomic tools to analyze the functional motifs present in ST7. The data confirm that ST7 is, indeed, a member of an LDLR subfamily, which also contains LDLR-related protein 3 (LRP3) and murine LRP9. We also found that ST7's cytoplasmic domain contains several motifs implicated in endocytosis and signaling. Use of the yeast-two hybrid system to identify proteins that associate with ST7's cytoplasmic domain revealed that ST7's cytoplasmic domain, like those of other proteins in the LDLR superfamily, interacts with proteins related to signal transduction and/or endocytosis, strongly suggesting that ST7 functions in these two pathways.

INTRODUCTION

It is generally accepted that each human cancer is clonal in origin, i.e., begins with a single cell that has acquired all of the properties necessary to form a tumor. Every cell of the tumor is the progeny of this first cell. Theories of carcinogenesis must explain how the "first" tumor cell acquired the appropriate genetic and/or epigenetic changes required to render it malignant, which for tumors in adults are estimated to be five or more. To try to define the number and nature of such changes, McCormick and Maher (1996) developed a model system in which normal human fibroblasts in culture can be transformed into malignant fibroblasts by acquiring a series of genetic changes, each conferring a growth advantage that allows sequential clonal expansion.

In 1997, using differential mRNA display to compare an infinite life span, non-tumorigenic human fibroblast cell strain, designated MSU-1.1, to a carcinogen-transformed, malignant MSU-1.1 derivative, McCormick and his colleagues identified a novel gene, which they designated *ST7* (Qing et al., 1999). Compared to the level of expression in the parental cell strain, MSU1.1, *ST7* was found to be strongly down-regulated in the tumorigenic cell line. Additional malignant cell lines also were found to have a low level of ST7, suggesting that *ST7* is a novel tumor suppressor gene. Recent studies from this laboratory demonstrated that overexpression of ST7 protein inhibited the tumor-forming ability of a human fibrosarcoma-derived cell line, whereas overexpression of a truncated ST7 protein that lacked most of the C-terminus failed to do so[2]. These latter data strongly suggest that the C-terminus of ST7

plays a critical role in the protein's function as a tumor suppressor.

When the novel gene *ST7* was first identified, it was recognized that it had the characteristics of a transmembrane protein, but at that time no other proteins with significant similarity to ST7 had been reported. Approximately one year later, Yamamoto and his associates, using degenerate oligonucleotides corresponding to the highly conserved region of the ligand-binding domains found in the proteins of the LDLR[3] superfamily, discovered a novel LDLR-related gene, which they designated as the *LRP3* gene (Ishii et al., 1998). Our search of the databases for proteins structurally related to ST7, after the sequence of LRP3 had been submitted, revealed a very strong similarity between LRP3 and ST7, enabling us to recognize that ST7, although it differed significantly from the LDLR prototype, was also a novel member of the LDLR superfamily. Although proteins of the LDLR superfamily are best known as endocytic receptors (see Krieger and Herz, 1994; Hussain et al., 1999; Strickland et al., 2002 for review), recent studies show that the cytoplasmic domains of LRP1, LRP2, LRP5, LRP6, LDLR, VLDLR, and ApoER2 interact with a variety of adaptor and scaffold proteins implicated in signal transduction (see Li et al., 2001; Herz and Bock, 2002; van der Geer, 2002 for review).

To evaluate ST7's relationship to the proteins of the LDLR superfamily, we used proteomic tools including similarity searches, sequence alignments, pattern and profile searches, and post-translational modification prediction programs to analyze the functional motifs present in ST7. Such analyses showed that ST7 is, indeed, a novel member of the LDLR superfamily and that similar to other LDLR

proteins, ST7's cytoplasmic domain contains several motifs implicated in endocytosis and/or signaling. To identify intracellular proteins that interact with ST7's cytoplasmic domain, we used the yeast two-hybrid system. We found that ST7's cytoplasmic domain interacts with several proteins related to signal transduction and/or endocytosis, suggesting that ST7 functions in these pathways.

EXPERIMENTAL PROCEDURES

Materials—The sources of specific materials used are: restriction enzymes and T4 DNA ligase, New England Biolabs or Invitrogen; shrimp alkaline phosphatase, Promega; *Pfu* polymerase, Stratagene; oligonucleotides, the Michigan State University Macromolecular Structure, Sequencing, and Synthesis Facility; yeast culture medium, Clontech; and antibiotics, Sigma or Roche Molecular Biochemicals.

Cell Culture—The HEK 293-T cells were cultured in high-glucose Dulbecco's modified Eagle's medium containing 10% (v/v) fetal calf serum (HyClone), 100 units/ml penicillin, and 100 µg/ml streptomycin in a 37°C humidified incubator with 5% CO_2 in air.

Isolation of ST7 cDNA—To isolate a full-length *ST7* cDNA, the GeneTrapper™ cDNA Positive Selection System (Invitrogen) and SuperScript™ human heart cDNA library (Invitrogen) were used according to the manufacturer's directions. Briefly, single-stranded phagemid DNA was generated by digesting double-stranded phagemid DNA (5 µg) with bacteriophage f1 gene II protein (a site-specific F1 endonuclease) and *E.coli* exonuclease III. A biotinylated ST7-specific probe (5'-TTGCTCTTGCTTTTCCTCGCTGGGG-3') was hybridized with single-stranded DNA for 1h at 37°C. Following hybridization, streptavidin paramagnetic beads were used to capture *ST7* cDNA-probe complexes. After several washes, single-stranded DNA bound to the beads was eluted. To produce double-stranded DNA, captured DNA was repaired using the provided repair enzyme

and the same unbiotinylated *ST7*-specific oligonucleotide used in the capture procedure. Repaired DNA was electroporated into ultracompetent ElectroMax DH10B *E. coli* (Invitrogen) and plated on LB agar plates containing 100 µg/ml ampicillin. DNA isolated from several colonies was screened for the presence of a full-length *ST7* cDNA insert (~3.0 Kb) by digesting the DNA with several restriction endonucleases and sequencing the 5' and 3' ends of the DNA. The phagemid containing *ST7* was designated pCMV-SPORT-ST7.

DNA sequencing—The sequence of DNA samples was determined in one of three ways: manually determined using the dideoxy chain termination method with a TAQuence® cycle sequencing kit (US Biochemicals) or Fidelity DNA sequencing kit (Oncor), using primers radiolabeled by T4 polynucleotide kinase and γ-^{32}P dATP; automatically sequenced using a Visible Genetics Gene Clipper sequencer with either a ThermoSequenase Cy 5.5 terminator cycle sequencing kit or a ThermoSequenase primer (Cy 5.0 or Cy 5.5) cycle sequencing kit (Amersham); or sequenced by the Michigan State University Genomics Technology Support Facility.

Yeast two-hybrid assay— The MATCHMAKER GAL4 Two-Hybrid System 3 (Clontech) was used. The ST7 cytoplasmic bait plasmids were constructed using the pGBKT7 bait vector. To generate the bait plasmids, the appropriate region of ST7's cytoplasmic domain was amplified from pCMV-SPORT-ST7 using *Pfu* polymerase and the appropriate oligonucleotides. $ST7_{cyto1}$ contains residues 498-840; $ST7_{cyto2}$, residues 498-667; $ST7_{cyto3}$, residues 668-840; $ST7_{cyto4}$, residues 498-652; $ST7_{cyto5}$, residues 498-560; and $ST7_{cyto6}$, residues

561-667. The purified PCR products (QIAquick spin PCR purification kit, Qiagen) were digested with restriction endonucleases *EcoRI* and *XhoI* and subcloned into *EcoRI* and *SalI* sites of the bait vector pGBKT7. The *S. cerevisiae* strain AH109, which contains the *HIS3, ADE2*, and *MEL1* reporter genes, was transformed with the bait plasmid using the lithium acetate method described in the MATCHMAKER instruction manual (Clontech). Transformants containing the bait plasmid were selected by plating the cells on Minimal Synthetic Dropout (SD) agar lacking tryptophan (SD/-trp). A MATCHMAKER skeletal muscle cDNA library (Clontech) was amplified following the standard semi-solid amplification procedure described in the MATCHMAKER instruction manual, and plasmid DNA was prepared using the QIAGEN plasmid giga kit. To detect ST7-interacting proteins, AH109-$ST7_{cyto1}$ cells were transformed with the amplified library. Transformants were plated onto medium-stringency SD/-leu/-trp/-his agar. After incubation at 30°C for several days, colonies were streaked onto SD/-leu/-trp and SD/-leu/-trp/-his agar plates. These yeast colonies were subjected to a higher stringency test, i.e., they were replica plated onto SD/-leu/-trp/-his/-ade agar plates. The colonies judged to grow well at high stringency conditions were re-streaked for further analysis. Plasmid DNA was isolated from yeast colonies using the Zymoprep yeast plasmid miniprep kit (Zymo Research). Prey plasmids were rescued by transforming *E. coli* DH5α with the plasmid DNA isolated from yeast and by selecting these transformants on LB agar containing ampicillin at 100 μg/ml. The prey plasmids isolated from *E. coli* (QIAprep spin plasmid DNA miniprep kit, Qiagen) were sequenced using automated DNA sequencing

technology (Visible Genetics). To map various binding domains in ST7, bait plasmids encoding truncated forms of the ST7 cytoplasmic domain were engineered, and AH109 strains expressing the truncated bait proteins were transformed with the pACT2 prey plasmids isolated in the original yeast-two hybrid screen. As a negative control, these strains were also transformed with a prey plasmid encoding the SV40 large T-antigen. Transformants were plated on quadruple drop-out agar (SD/-leu/-trp/-his/-ade). Growth on this medium indicated that the truncated $ST7_{cyto}$ protein interacted with the prey protein of interest.

Oligonucleotides—Primer sequences (5'-3') used to construct the plasmids for the yeast two hybrid screen and for co-immunoprecipitation studies follow. Restriction sites used for cloning are in boldface type, stop codons are in italics, and template sequences are underlined: 5' primer for $ST7_{cyto1}$, $ST7_{cyto2}$, $ST7_{cyto4}$, and $ST7_{cyto5}$, CG**GAATTC**AAGCTTTATTCTCTGAGA; 3' primer for $ST7_{cyto1}$ and $ST7cyto_3$, CG**CTCGAG***CTA*ACAAAGTAACAAAGCCTC; 5' primer for $ST7_{cyto3}$, CG**GAA TTC**GCGACAGTAGGAGCATGT; 3' primer for $ST7_{cyto2}$ and $ST7_{cyto6}$, CG**CTC GAG***TTA*TTCCACTGCCGTTGTGGG; 3' primer for $ST7_{cyto4}$, CG**CTCGAG***TTA*GTGAGTATGATTTCT; 3' primer for $ST7_{cyto5}$, CG**CTCGAG-***TTA*CCTCAGATTTTCCAA; 5' primer for $ST7_{cyto6}$, CG**GAATTC**CCTAGC-GGTACGATCT. Primer sequences (5'-3') for subcloning of HA-tagged partial prey proteins into pcDNA6-V5/His follow: 5' primer for HA-$RACK1_p$, HA-$MIBP_p$, HA-$SARA_p$, HA-α-actinin-2_P, HA-$SNAPIN_P$, and HA-myotilin, AA**GCGGCCGC-**GCCACCATGGCTTACCCA; 3' primer for HA-$RACK1_p$, HA-$MIBP_p$, HA-$SARA_p$,

HA-α-actinin-2_P, HA-$SNAPIN_P$, and HA-myotilin, CG**ACTAGT**GAGATGGTGC-ACGATGCACAGTTG. Primer sequences for cloning full-length myotilin cDNA into the prey plasmid pACT2: 5' primer, CG**GAATTC**TAAGCATGTT-TAACTACGAACGT; 3' primer, CG**CTCGAG***TTA*AAGTTCTTCACTTTCATAG. Primer sequences for isolating full-length RACK1 cDNA: 5' primer, AA**GATATC**-CATGACTGAGCAGATGACC; 3' primer, AA**GTCGAC***CTA*GCGTGTGCC-AATGGTCAC; Primer sequences for isolating full-length MIBP cDNA: 5' primer, AA**GATATC**CATGAAGCTCATCGTGGGC. Primer sequences for constructing pcDNA6-ST7-V5/His, pcDNA6-$ST7_{tr1}$-V5/His, and pcDNA6-$ST7_{tr2}$-V5/His: 5' primer, which hybridizes to pCMV-SPORT vector sequence upstream of an *EcoRI* site and the *ST7* start codon, TAGGTGACACTATAGAAGG-TACGCCTGCAG; 3' for ST7-V5/His, CG**GAATTC**TCGTACCAAACA-AAGTAACAAAGC; 3' primer for $ST7_{tr1}$-V5/His, CG**CTCGAG**TCTTTCAAAC-ATTCTCAG; 3' primer for $ST7_{tr2}$-V5/His, CG**CTCGAG**CTTACAAGT-ACATCCCAA.

Construction of mammalian expression plasmids—cDNAs encoding the prey proteins of interest were PCR amplified from the appropriate pACT2 prey plasmids using *Pfu* polymerase. The 5' primer used hybridizes with the pACT2 HA epitope sequence located upstream of the cDNA insertion site; the 3' primer used hybridizes with sequence on pACT2 downstream of the cDNA insertion site. Therefore, each PCR products contains the entire partial prey cDNA with an N-terminal HA-tag. The GAL4 activation domain was not amplified in this procedure. Most PCR products (QIAquick spin PCR purification kit, Qiagen)

were digested with *SpeI* and subcloned the *EcoRV* and *XbaI* sites of the mammalian expression vector pcDNA6-V5/His (Invitrogen). One PCR product was left undigested, and this blunt-ended insert was subcloned into the *EcoRV* site of pcDNA6-V5/His. The primers were designed to contain a stop codon at the 3' end of each PCR product so that the proteins would not be V5/His tagged. Any full-length cDNA inserts isolated from the yeast library were used to generate an expression plasmid containing the entire open reading from with an N-terminal HA tag. First, the entire open reading frame was amplified from the pACT2 plasmid containing the ORF using *Pfu* polymerase. The purified PCR product was digested with *EcoRI* and *XhoI* and subcloned into the corresponding sites in pACT2. The HA-tagged, full-length prey protein was amplified from this plasmid and subcloned into pcDNA6-V5/His in the same manner as the others. Additional full-length cDNAs were amplified from the MATCHMAKER skeletal muscle cDNA library using *Pfu* polymerase and the appropriate primers. The purified PCR products were digested with *EcoRV* and *SalI* and subcloned into the *EcoRV* and *XhoI* sites of pcDNA6-HA. To construct pcDNA6-HA, the HA epitope was excised from pcDNA6-HA-MIBP$_P$ using *EcoRI* and subcloned into the *EcoRI* site of pcDNA6-V5/His. Again, because the primers were designed to contain the endogenous stop codons, these proteins were not tagged by V5/His. To construct mammalian expression plasmids containing full-length V5/His tagged ST7 or truncated V5/His tagged ST7, the appropriate region of ST7 was amplified from pCMV-SPORT-ST7 using *Pfu* polymerase and the appropriate oligonucleotides. The purified PCR products (QIAquick spin PCR purification kit,

Qiagen) were digested with restriction endonucleases *EcoRI* (ST7) or with *EcoRI* and *XhoI* ($ST7_{tr1}$ and $ST7_{tr2}$) and subcloned into *EcoRI* or *EcoRI* and *XhoI* sites pcDNA6-V5/His A (Invitrogen). Elimination of the endogenous stop codons caused the proteins encoded by these cDNAs to have C-terminal V5 and His epitope tags.

Co-immunoprecipitation of proteins from transiently transfected 293-T cells—293-T cells (1.5×10^6) were plated in 60-mm diameter tissue culture dishes one day prior to transfection. Cells were transfected with a total of 10 µg of plasmid DNA using LipofectAMINE (Invitrogen) following the manufacturer's instructions. Forty-eight hours after transfection, the cells were washed once in cold PBS and collected in cold lysis buffer (50 mM Tris-HCl, pH 7.2, 150 mM NaCl, 0.5% NP-40, 50 mM NaF, 1 mM Na_3VO_4, 1 mM benzamidine, 1 mM phenylmethylsulfonyl fluoride, 25 µg/ml aprotinin, and 25 µg /ml leupeptin). The cells were incubated in the lysis buffer for 30 min on ice with periodic gentle vortexing. The protein extracts were clarified by centrifugation (25,000 x g) for 30 min at 4°C. The supernatants were transferred to fresh tubes and centrifuged (25,000 x g) for an additional hour 4°C. Total protein concentration was determined using the Coomassie protein assay reagent (Pierce) following the manufacturer's instructions. For immunoprecipitation, protein extracts (250-500 µg) were pre-cleared using normal rabbit serum (0.25 µg) (Santa Cruz Biotechnology) and protein A-agarose beads (20 µl) (Santa Cruz Biotechnology) for 30 min at 4°C with end-over-end mixing. The pre-cleared supernatants were incubated with 2 µg rabbit polyclonal anti-HA antibody (Y-11, Santa Cruz

Biotechnology) for 2 h at 4°C with end-over-end mixing. A control reaction was carried out for each protein pair in which no HA antibody was included in the immunoprecipitation reaction. Protein A-agarose beads (20 µl) were added, and the reactions were incubated for an additional hour at 4°C with end-over-end mixing. The pellets were washed three times with a buffer containing 25 mM Tris-HCl, pH 7.4, 30 mM $MgCl_2$, 40 mM NaCl, 1.0% NP-40, and once with a buffer containing 25 mM Tris-HCl, pH 7.4, 30 mM $MgCl_2$, 40 mM NaCl. Following the washes, the agarose pellets were resuspended in 40 µl of 1X Laemelli SDS-PAGE sample buffer. Denatured immunoprecipitated proteins were separated by SDS-PAGE and transferred to Immobilon-P membrane (Millipore). Such membranes were blocked for 2 h with Tris-buffered saline containing 0.1% Tween-20 (TBST) and 5% non-fat milk. Blocked membranes were incubated with a monoclonal V5 antibody (Invitrogen) and a monoclonal HA antibody (F-7, Santa Cruz Biotechnology) diluted in the blocking solution for at least 2 h at room temperature and with the appropriate HRP-conjugated secondary antibody (Santa Cruz Biotechnology or Sigma) diluted in blocking solution for 1 h at room temperature. SuperSignal West Pico chemiluminescent substrate (Pierce) was used according to the manufacturer's instructions to detect proteins.

RESULTS

Isolation of ST7 cDNA from a human heart muscle cDNA library—Because the *ST7* cDNA fragments originally isolated had been obtained from multiple cDNA libraries using PCR with relatively low fidelity DNA polymerases, there was a possibility that they contained PCR generated mutations. Therefore, we isolated *ST7* cDNA from a human heart cDNA library using the GeneTrapper cDNA Positive Selection System. Comparison of this DNA sequence to those of Qing et al. (1999) revealed that this *ST7* cDNA is the shorter of the two known *ST7* isoforms. It lacks 57 nucleotides at its 5' end (nucleotides 79 -136 of GenBank accession number NM_013437). To determine if this isoform was the product of alternative splicing of the transcript, we used the human genome BLAST program to search the human genomic DNA database for the *ST7* sequence. The *ST7* gene was localized to an approximately 100 Kb region human chromosome 8q (Locus Link ID 29967), in agreement with the fluorescent in situ hybridization data of Qing et al. (1999) showing ST7's location to be chromosome 8q22.2-23.1. This sequence contains seven putative exons (Table I). The 57 nucleotides missing in the short isoform correspond to the second exon, strongly suggesting that the short isoform is a product of alternative splicing of the transcript.

ST7 is a novel member of the LDLR family of proteins—The similarity between ST7 and LRP3 strongly suggested that ST7 is a novel member of the LDLR superfamily. Comparison of residues 27-604 of ST7 with residues 42-627 of LRP3 using BLAST revealed that the two proteins are 50% identical and 66%

Table I

Structure of the ST7 gene

Exon	Intron – exon boundaries		Exon size (bp)	Exon – intron boundaries		Intron size (kb)
1			79	GGGGTGTACG	gtaagtgtcc	55.9
2	ttaattttag	GAAATGGTGC	57	GTGTCAACTG	gtaagtcatt	22.8
3	tatcttccag	CTTGTGGAGA	136	TACTATAAGG	gtaattctac	9.4
4	ttttttcag	TTTTCAGGAT	203	TATTTTCAG	gtgtgttttt	12.4
5	atcttcacag	GGAAATCTGA	1105	TTGAAAGAAG	gtcagtatca	1.8
6	ctctcaaaag	ATCATTTGAA	133	ACCTAATCAG	gtatattgca	3.5
7	atttttgtag	GCTTCTGTTT	866	ACTTTGTTAG		

similar. After this research had been carried out, Sugiyama et al. (2000) reported the discovery a murine LDLR-related protein, LRP9, which closely resembles ST7 and LRP3. They suggested that LRP9, LRP3, and ST7 constitute a novel subfamily of the LDLR superfamily. Comparison of ST7 with residues 28-698 of murine LRP9 showed that the proteins are 35% identical and 46% similar. The most striking characteristics common to all three proteins are: 1) their extracellular domains contain the same types of functional domains arranged in an identical configuration (Figure 1); 2) the juxtamembrane regions of their cytoplasmic domains are highly conserved (Figure 2A), strongly suggesting that this region is functionally important; and 3) their cytoplasmic tails contain putative signals for endocytosis and sequences that may participate in signal transduction. These highly related features are strong evidence for considering that ST7, LRP3, and LRP9 comprise an LDLR subfamily.

Analysis of the ST7 protein—Qing et al. (1999) predicted that *ST7* encodes a type I single-pass transmembrane protein. To gain additional information regarding ST7's protein structure, its sequence was further analyzed using proteomic analysis tools available through the ExPASy Molecular Biology Server (www.expasy.ch) The protein's putative extracellular domain, residues 1 to 473, contains a signal sequence peptide motif (residues 1-27) as determined by the SignalP prediction program (Nielsen et al., 1997). Such motifs are characteristic of proteins targeted to the plasma membrane (Claros et al., 1997). This region also contains five LDLRA domains arranged in two clusters and two CUB domains, as determined using ScanProsite (Gattiker et al., 2002) (Figure 1).

Figure 1. **Structures of representative proteins of the LDLR superfamily** (adapted from Strickland et al., 2002). The types of domains in these proteins and the organization of these domains divide the superfamily into at least four subfamilies: 1) LDLR, VLDLR, and ApoER2; 2) LRP1, LRP1B, and LRP2; 3) LRP5 and LRP6; 4) ST7, LRP3, and murine LRP9. The cytoplasmic domains of these proteins contain motifs implicated in endocytosis and/or signal transduction, which are not shown. (Images in this dissertation are presented in color.)

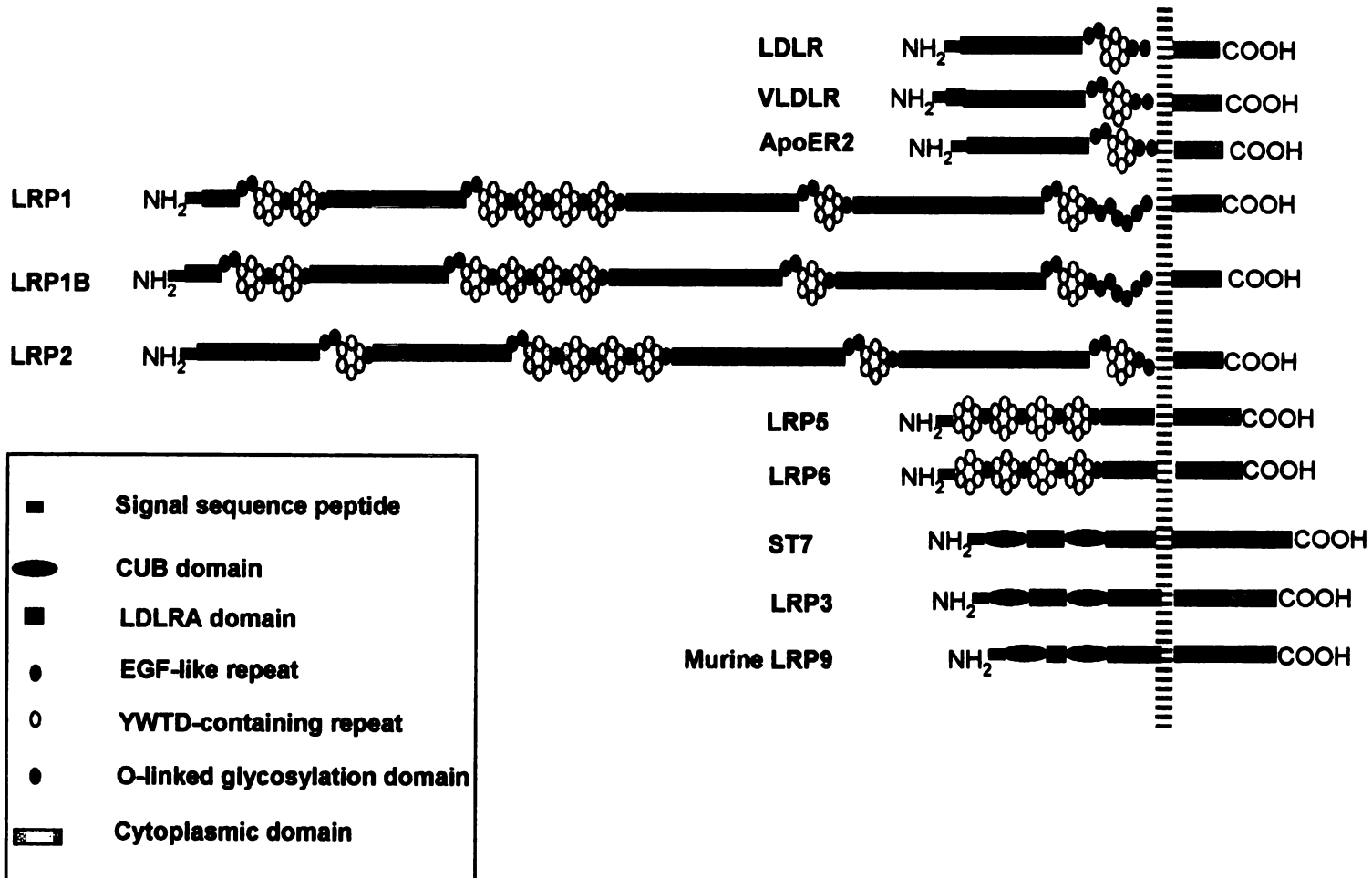
LDLR
VLDLR
ApoER2
LRP1
LRP1B
LRP2
LRP5
LRP6
ST7
LRP3
Murine LRP9
NH2
COOH
Signal sequence peptide
CUB domain
LDLRA domain
EGF-like repeat
YWTD-containing repeat
O-linked glycosylation domain
Cytoplasmic domain

Figure 1

Figure 2. **The cytoplasmic domain of ST7 (residues 498-840).** A. Alignment of the cytoplasmic domains of ST7, LRP3, and murine LRP9. Regions of identity in these proteins are red, and regions of similarity are blue. The consensus sequence is shown beneath the alignment in black. Uppercase letters designate amino acids conserved in all three proteins. Lowercase letters designate amino acids conserved in two of the three proteins. Note the high degree of sequence conservation in the juxtamembrane region (498-560 of ST7) and in the extreme carboxy terminal region of these proteins. B. Putative endocytic and signaling motifs identified in ST7's cytoplasmic domain. The YXXØ internalization motif is indicated by a double-underline. The NPXY-like internalization motif is shown by a dotted underline. A single underline denotes the three dileucine motifs. Potential phosphorylation sites are boxed. The four PDZ domain binding motifs are shown in boldface italic font. A line is drawn above the WW domain binding motif. (Images in this dissertation are presented in color.)

A

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ST7         498 KLYSLRMFERRSFETQLSRVEAELLRREAPPSYGQLIAQGLIPPVEDFPVCSPNQASVLE
LRP3        521 KLYSLRTQEYRAFETQMTRLEAEFVRREAPPSYGQLIAQGLIPPVEDFPVYSASQASVLQ
LRP9        446 KLYAIRTQEYSIFAP-LSPMEAEIVQQQAPPSYGQLIAQGAIPPVEDFPTENPNDNSVLG
consensus       KLYslRtqEyr FetqlsRvEAElvrreAPPSYGQLIAQGlIPPVEDFPv spnqaSVL

ST7         558 NLR--LAVRSQLG-FTSVRLPMAGRSSNIWNRIFNFARSRHSGSLALVSADGDEVVPSQS
LRP3        581 NLR--TAMRRQMRRHASRRGPSRRRLGRLWNRLFHRPRAPR-GQIPLLTAAR----PSQ-
LRP9        525 NLRSLLQILRQDMTPGGTSGGRRRQRGRSVRRLVRRLR--RWGLLPRTNT------PAR-
consensus       NLR  lavrrQl   as rgp rrr griwnRlfhr R  r G lplvsa      Psq

ST7         615 TSREPERNHTHRSLFSVESDDTDTENERRDMAGASGGVAAPLPQKVPPTTAVEATVGACA
LRP3        633 ---------------TVLGDGFLQP--------APG--AAPD----PPAPLMD--TG---
LRP9        576 ------------------APETRSQ-------------VTPS----VPSEALDDSTG---
consensus                   sv gddt t         a g  aaP     pPt avd ttG

ST7         675 SSSTQSTRGGHADNGRDVTSVEPPSVSPARHQLTSALSRMTQGLRWVRFTLGRSSSLSQN
LRP3        659 -----STR---AAGDR------PPS-AP-----------------------GRAPEVGPS
LRP9        598 ----HACEGG-AVGGQDGEQ-APP--LP-----------------------IKTPIPTPS
consensus           strgg A ggrd     PPs  P                        grsp lsps

ST7         735 QSPLRQLDNGVSGREDDDDVEMLIPISDGSSDFDVNDCSRPLLDLASDQGQGLRQPYNAT
LRP3        681 GPPLP------SGLRDPE---CR-PV-----DKDRKVCREPLADGPAP-ADAPREPCSAQ
LRP9        627 TLPALA-----TVSETPG------PL------PSVPVESS-LLSGVVQVLRGRLLP-SLW
consensus         Pl        sg edpd       Pi     d dv vcs pLldg    g g r P sa

ST7         795 NPG------VRPSNRDGPCERCGIVHTAQIPDTCLEVTLKNETSDDEALLLC
LRP3        725 DPHP---QVSTASSTLGPHSPEPLGVCRNPPPPCSPML---EASDDEALLVC
LRP9        668 SPGPTWTQTGTHTTVLSPEDEDDVLLLP-LAEPEVWVV---EA-EDEPLLA-
consensus       Pgp   q  t ss lgP e e ivv  qipdpcl vl   EasdDEaLLlc
```

Figure 2

B

498 KLYSLRMFER RSFETQLSRV EAELLRREAP PSYGQLIAQG LIPPVEDFPV

548 CSPNQASVLE NLRLAVRSQL GFTSVRLPMA GRSSNIWNRI FNFARSRHSG

598 SLALVSADGD EVVPSQSTSR EPERNHTHRS LFSVESDDTD TENERRDMAG

648 ASGGVAAPLP QKVPPTTAVE ATVGACASSS TQSTRGGHAD NGRDVTSVEP

698 PSVSPARHQL TSALSRMTQG LRWVRFTLGR SSSLSQNQSP LRQLDNGVSG

748 REDDDDVEML IPISDGSSDF DVNDCSRPLL DLASDQGQGL RQPYNATNPG

798 VRPSNRDGPC ERCGIVHTAQ IPDTCLEVTL KNETSDDEAL LLC

Figure 2 (cont'd)

The first LDLRA cluster contains two domains (146-182 and 195-236) and the second cluster contains three domains (355-392, 393-430, and 431-467). Each LDLRA domains consists of ~40 residues, with six conserved cysteine residues that form three intramolecular disulfide bonds and with conserved serine, aspartic acid, and glutamic acid residues at the domain's C-terminus (Yamamoto et al., 1984). All of the members of the LDLR superfamily contain LDLRA domain repeats (reviewed by Hussain et al., 1999; Strickland et al., 2002). In many of these proteins, such domains have been demonstrated to function as ligand binding sites (Yamamoto et al., 1984). A single CUB domain (residues 28-140 and 240-353) precedes each LDLRA domain cluster (Figure 1). CUB domains are composed of ~110 amino acids with four conserved cysteine residues that form two intramolecular disulfide bonds and are believed to participate in protein-protein interactions (reviewed by Bork and Beckmann, 1993; Christensen and Birn, 2002). Exon 2 of *ST7*, which is lacking in the isoform used in our studies, encodes protein sequence between the signal sequence peptide and the first CUB domain. Therefore, loss of this exon's sequence does not affect any of the functional domains present in ST7's extracellular domain. The other ST7 isoform simply contains 19 additional amino acids immediately upstream of the first CUB domain.

The putative extracellular domain of ST7 is followed by a single hydrophobic transmembrane helix flanked by positively charged residues (residues 474-497) (Qing et al., 1999). The C-terminal cytoplasmic tail of the protein (residues 498-840) contains several sequence motifs related to

endocytosis (Figure 2B). These endocytic signaling motifs include an YXXØ motif (YGQL, 530-533), a NPXY-like motif (EDFPVC, 543-548), and three potential dileucine repeats (521-522, 776-777, and 837-839). In these consensus sequences, X represents virtually any amino acid and Ø represents any amino acid with a bulky hydrophobic group (see Trowbridge et al., 1993 for a review of endocytic signals). Several putative signaling motifs were also found. Four PDZ domain binding motifs, (S/T)XV (SRV, 515-517; TSV, 570-572; TAV, 664-666; and TSV, 693-995), and a WW-domain binding motif, PPXY (PPSY, 527-530), are present (Figure 2B). Moreover, the cytoplasmic domain of ST7 is notably rich in proline, serine, and threonine residues. Proline-rich regions are often involved in protein-protein interactions (Kay et al., 2000). Because the phosphorylation of residues in the tails of receptors can regulate their interactions with intracellular proteins, we analyzed ST7's cytoplasmic tail for putative serine, threonine, and tyrosine phosphorylation sites using NetPhos 2.0 prediction software (Blom et al., 1999), available at the ExPASy webserver. Twenty-five serines and seven threonines were predicted to be potential sites of phosphorylation (based on a score of ~0.5 or greater) (Figure 2B). Six of the 32 putative phosphorylation sites, three serines and three threonines, are located within the consensus sequence for phosphorylation by PKC (Table II). Eight sites, five serines and three threonines, are located within the consensus sequence for phosphorylation by PKA (Table II). Serine and threonine residues constituting three of the four PDZ domain-binding motifs, as well as the serine residue present in the WW domain-binding motif, were also predicted to be

Table II

Putative PKC and PKA phosphorylation sites in ST7's cytoplasmic domain

Position	Sequence	Kinase
509	FERRSFETQ	PKA
581	AGRSSNIWN	PKA
596	RSRHSGSLA	PKA
615	PSQSTSREP	PKC
624	ERNHTHRSL	PKA, PKC
680	SSTQSTRGG	PKC
693	GRDVTSVEP	PKA
724	WVRFTLGRS	PKA
730	GRSSSLSQN	PKA
746	DNGVSGRED	PKC
801	GVRPSNRDG	PKA, PKC
826	CLEVTLKNE	PKC

potential phosphorylation sites. The three tyrosines present in ST7's cytoplasmic domain were not predicted to be phosphorylated. However, one of these tyrosine residues is shared by the WW domain-binding motif and the YXXØ internalization motif (*PPS*-Y-GQL), and several groups have reported phosphorylation of tyrosines in the context of either motif (Klingmuller et al., 1996; Sotgia et al., 2001; Macias et al., 2002).

Identification of proteins that interact with the cytoplasmic domain of the ST7 protein—Because tumorigenicity studies conducted in this laboratory suggest a functional role for ST7's cytoplasmic tail in inhibiting tumor growth[2] and because our analysis of ST7's cytoplasmic domain revealed several potential endocytic and signal transduction motifs, we used the yeast-hybrid system to identify proteins that interact with the ST7's cytoplasmic C-terminus (described under "Experimental Procedures"). Of 57 cDNAs isolated, 30 different cDNAs were identified. Six cDNAs, encoding proteins of the greatest interest, were chosen for further studies. These are: 1) RACK1, a WD domain-containing protein implicated as a key cellular scaffold protein that interacts with multiple cellular receptors (Ron et al., 1994, 1999; Chang et al., 2001); 2) MIBP, an integrin binding protein believed to participate in signal transduction pathways regulating myogenesis (Li et al., 1999); 3) SARA, a FYVE domain-containing cellular scaffold protein that shuttles SMAD2 and SMAD3 to the cytoplasmic domains of TGFβ receptors (Tsukazaki et al., 1998); 4) α-actinin-2, a cytoskeletal protein thought to link plasma membrane receptors to the cytoskeleton (Galliano et al., 2000); 5) myotilin, a novel cytoskeletal protein that

interacts with α-actinin-2. Defects in myotilin cause limb-girdle muscular dystrophy type 1A (Salmikangas et al., 1999; Hauser et al., 2000, 2002); and 6) SNAPIN, a novel binding partner for the SNAP-25 component of the SNARE vesicular transport complex (Ilardi et al., 1999). To verify that these cDNAs encode the proteins responsible for the original interactions observed in our yeast two-hybrid screen, AH109 yeast cells containing the $ST7_{cyto1}$ bait plasmid were transformed with each of the six prey plasmids, and the transformants were plated on selection medium. Each of the proteins tested interacted with the cytoplasmic domain of ST7, as detected by reporter gene activity.

Polymerase chain reaction (PCR) was used to subclone each of the six prey cDNAs into the mammalian expression vector pcDNA6-V5/His. An N-terminal HA-epitope tag was included to facilitate detection of the proteins, and a stop codon was included at the 3' end of each cDNA to exclude the V5 and His epitope tags from the resulting proteins. Table III indicates the cDNAs isolated from yeast. Those encoding only a portion of the prey protein are designated by a subscript "p". Because three of the four myotilin cDNAs isolated from yeast contained the entire open reading frame and some 5' upstream untranslated sequence, the entire myotilin open reading frame was subcloned into the expression vector. Sequencing of each insert to verify the fidelity of the PCR reactions revealed that we had isolated the longer isoform of the *MIBP* cDNA (GenBank accession number AK001663), which contains a 132 nucleotide in-frame insertion between nucleotides 328 and 329 of the short *MIBP* coding sequence (GenBank accession number NM_014446). The sequences of the two

Table III

Identification of proteins that interact with the cytoplasmic domain of ST7

To identify ST7-interacting proteins, the complete ST7 cytoplasmic domain was used as bait in a yeast two-hybrid screen of a human skeletal muscle cDNA library. The majority of positive cDNAs identified encode a partial prey protein, designated by a subscript "p". Column four lists the region of each prey protein encoded by isolated cDNAs. Three of the four myotilin prey plasmids isolated contained a cDNA that includes the entire open reading frame and some of the 5' untranslated sequence (5'UTR)

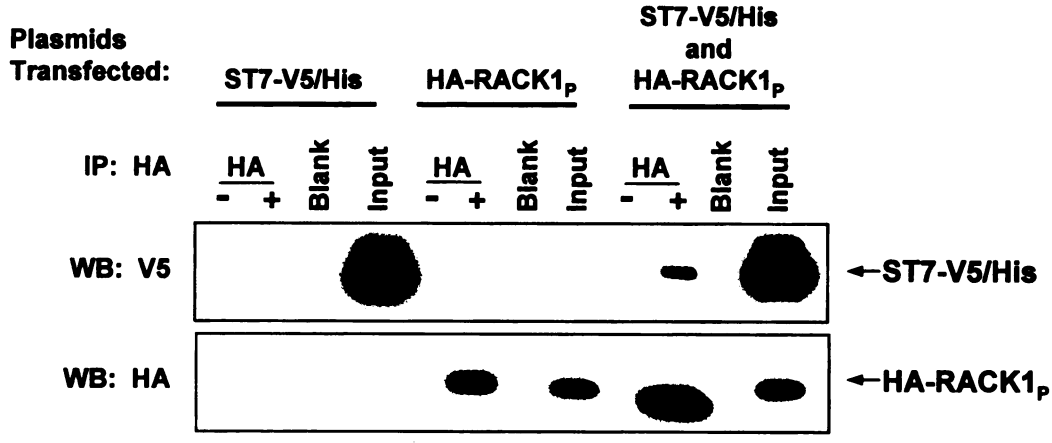
Name	Clones isolated	Independent clones isolated	Prey protein size	Function/pathway
$MIBP_p$	1	1	92-COOH	Signal transduction
$RACK1_p$	2	2	65-COOH 106-COOH	Signal transduction
$SARA_p$	1	1	731-COOH	Signal transduction
Myotilin	4	2	5'UTR-COOH 250-COOH	Actin cytoskeleton
α-Actinin-2_p	1	1	307-COOH	Actin cytoskeleton
$SNAPIN_p$	2	1	17-COOH	Synaptic transmission

MIBP proteins are identical except for an additional 44 residues in the longer form. Sequencing also demonstrated that a variant *SARA* cDNA had been isolated. To date, three *SARA* transcript variants have been observed and are reported in GenBank. Our partial cDNA, which lacks the 81 nucleotides of exon 14, represents a fourth *SARA* transcript variant.

Confirmation of the protein-protein interactions within human cells— Protein-protein interactions identified using the yeast two-hybrid assay are only considered valid if verified by other biochemical methods. Therefore, we used co-immunoprecipitation to determine whether the six prey proteins identified interact with ST7's cytoplasmic domain in human cells. 293-T cells were transiently co-transfected with plasmids encoding an HA-tagged partial prey protein and ST7-V5/His protein, and the HA-tagged prey protein was immunoprecipitated from lysates of these cells. The immunoprecipitation products were analyzed by Western blotting with a monoclonal V5 antibody to detect ST7-V5/His and a monoclonal HA antibody to detect the HA-tagged prey proteins. The ST7-V5/His protein was co-immunoprecipitated with HA-RACK1$_P$ (Figure 3A), HA-MIBP$_P$ (Figure 3B), and HA-SARA$_P$ (Figure 3C), indicating an interaction between these protein pairs. ST7-V5/His failed to co-immunoprecipitate with HA-α-actinin2$_P$, HA-SNAPIN$_P$, and HA-myotilin. An example is shown in Figure 3D. Failure to detect an interaction between these three prey proteins and ST7-V5/His by co-immunoprecipitation does not exclude these as ST7-interacting proteins because the co-immunoprecipitation approach is less sensitive than the yeast two-hybrid assay.

Figure 3. **ST7 interacts with RACK1$_P$, MIBP$_P$, and SARA$_P$ in human cells**. A. Western blotting analysis of HA immunoprecipitation reactions from 293-T cells transiently transfected with ST7-V5/His, HA-RACK$_P$, or both ST7-V5/His and HA-RACK$_P$. The upper panel was probed with anti-V5 antibody; the lower panel was probed with anti-HA antibody. Lanes designated with a minus (-) sign contain negative control IP reactions, i.e., immunoprecipitation reactions without HA antibody; lanes designated with a plus (+) sign contain immunoprecipitation reactions with anti-HA; lanes designated as input contain 25 µg of the lysate used in the immunoprecipitation reactions; lanes designated as blank were not loaded. B. Same as A except cells were transfected with ST7-V5/His, HA-MIBP$_P$, or both ST7-V5/His and HA-MIBP$_P$; C. Same as A except cells were transfected with ST7-V5/His, HA-SARA$_P$, or both ST7-V5/His and HA-SARA$_P$; D. Same as A except cells were transfected with both ST7-V5/His and HA-α-actinin-2$_P$.

A


Plasmids Transfected:
ST7-V5/His
HA-RACK1P
ST7-V5/His and HA-RACK1P
IP: HA
HA
- +
Blank
Input
WB: V5
WB: HA
←ST7-V5/His
←HA-RACK1P

B

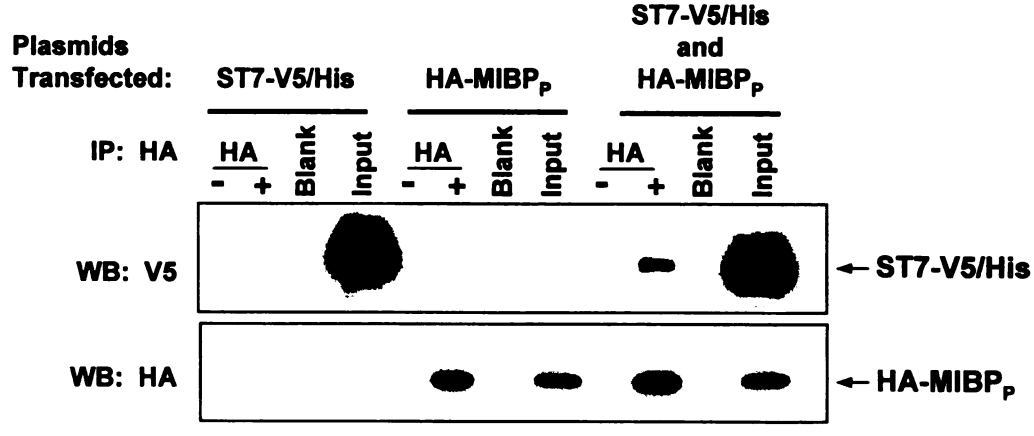
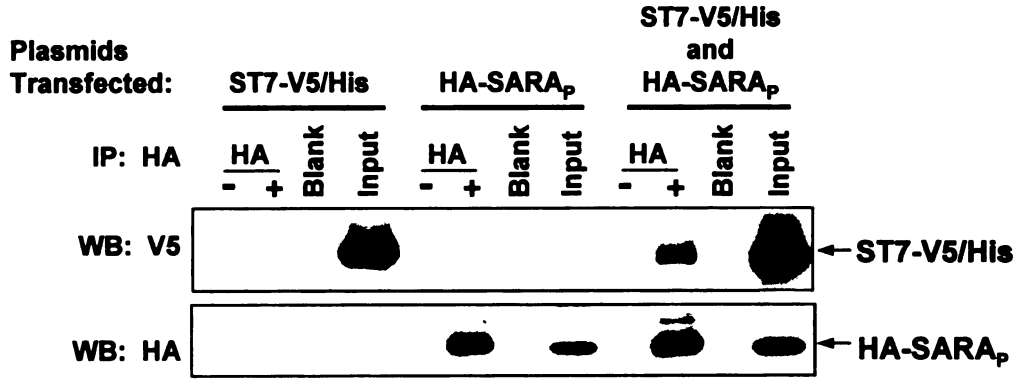

Plasmids Transfected:
ST7-V5/His
HA-MIBPP
ST7-V5/His and HA-MIBPP
IP: HA
HA
- +
Blank
Input
WB: V5
WB: HA
← ST7-V5/His
← HA-MIBPP

Figure 3

C



D

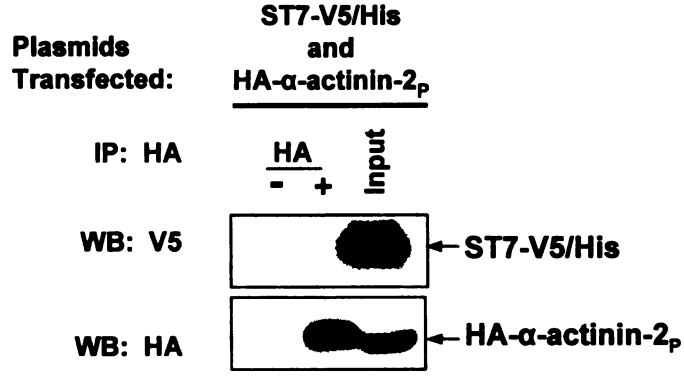
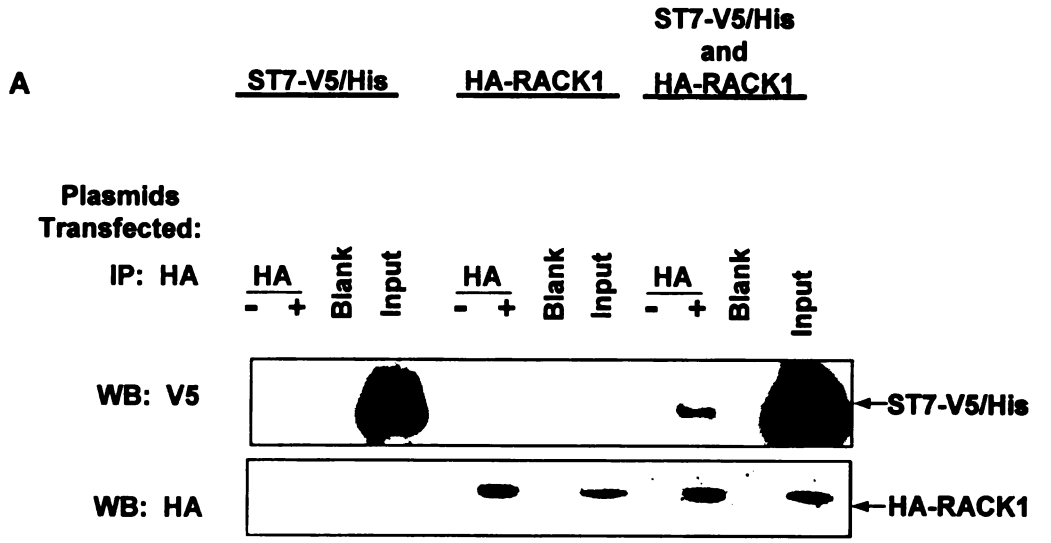


Figure 3 (cont'd)

The interaction of these proteins with ST7 may simply have been missed as the result of being weaker or transient. Whether full-length RACK1 and MIBP proteins interact with ST7 was determined as follows. cDNAs encoding full-length RACK1 and MIBP were isolated from the MATCHMAKER skeletal muscle cDNA library using PCR with appropriate oligonucleotides and subcloned into pcDNA6-HA. The endogenous stop codon was retained in each full-length cDNA so that the resulting proteins would not be tagged by the V5 and His epitopes. We sequenced the *HA-RACK1* and *HA-MIBP* cDNA inserts and verified that the PCR reactions were faithful. 293-T cells were transiently co-transfected with plasmids encoding HA-RACK1 or HA-MIBP and ST7-V5/His, and the HA-RACK1 or HA-MIBP protein was immunoprecipitated from lysates of these cells. The immunoprecipitation products were analyzed by Western blotting with a monoclonal V5 antibody and a monoclonal HA antibody. ST7-V5/His was co-immunoprecipitated with both HA-RACK1 (Figure 4A) and HA-MIBP (Figure 4B), indicating that the full-length RACK1 and MIBP proteins interact with ST7. The ability of full-length SARA to interact with ST7 has not yet been determined because using PCR, we were unable to isolate a cDNA containing the full-length variant *SARA*.

Identification of the RACK1, MIBP, and SARA binding domains within ST7's cytoplasmic domain—To identify the specific region within ST7's cytoplasmic domain required for its interaction with RACK1, MIBP, and SARA, we used the yeast two-hybrid assay with truncated forms of the ST7 cytoplasmic domain as bait (Figure 5). AH109 strains expressing the truncated $ST7_{cyto}$ bait

Figure 4. **ST7 interacts with full-length RACK1 and MIBP in human cells.** A. Western blotting analysis of HA immunoprecipitation reactions from 293-T cells transiently transfected with ST7-V5/His, HA-RACK, or both ST7-V5/His and HA-RACK. The upper panel was probed with anti-V5 antibody; the lower panel was probed with anti-HA antibody. Lanes designated with a minus (-) sign contain negative control IP reactions, i.e., immunoprecipitation reactions without HA antibody; lanes designated with a plus (+) sign contain immunoprecipitation reactions with anti-HA; lanes designated as input contain 25 μg of the lysate used in the immunoprecipitation reactions; lanes designated as blank were not loaded. B. Same as A except cells were transfected with ST7-V5/His, HA-MIBP, or both ST7-V5/His and HA-MIBP.


A
ST7-V5/His
HA-RACK1
ST7-V5/His and HA-RACK1
Plasmids Transfected:
IP: HA
HA
- +
Blank
Input
WB: V5
ST7-V5/His
WB: HA
HA-RACK1

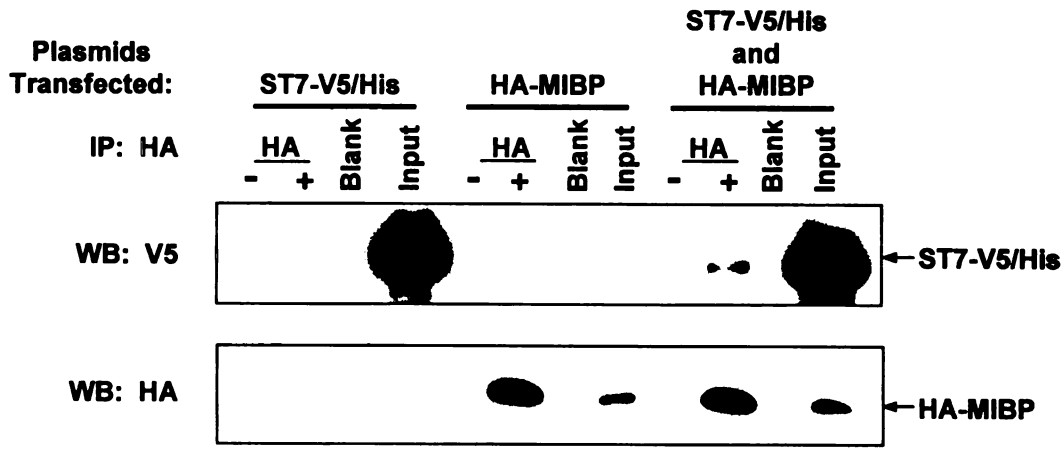

B
Plasmids Transfected:
ST7-V5/His
HA-MIBP
ST7-V5/His and HA-MIBP
IP: HA
HA
- +
Blank
Input
WB: V5
ST7-V5/His
WB: HA
HA-MIBP

Figure 4

Figure 5. **Illustration of the truncated ST7 cytoplasmic domain bait proteins used to determine the location of the MIBP, SARA, and RACK1 binding sites in ST7's cytoplasmic domain.** The appropriate bait-containing AH109 strains were transformed with an $MIBP_p$, $SARA_p$, or $RACK1_p$ prey plasmid. As a negative control, the bait-containing AH109 strains were also transformed with a prey plasmid encoding the SV40 large T-antigen, which does not bind to ST7. Transformants were plated on the most stringent medium, SD/-trp/-leu/-his/-ade. Growth on this medium indicated that the bait and prey proteins tested were able to interact within yeast cells.

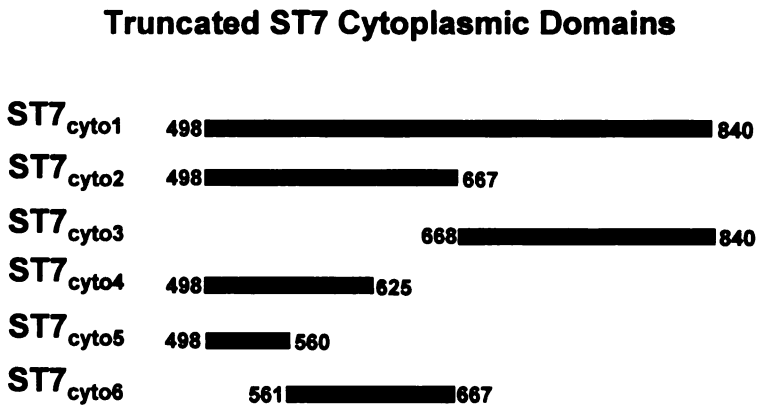

Truncated ST7 Cytoplasmic Domains
ST7cyto1
498
840
ST7cyto2
498
667
ST7cyto3
668
840
ST7cyto4
498
625
ST7cyto5
498
560
ST7cyto6
561
667

Figure 5

proteins were transformed with the pACT2 prey plasmids isolated in the original yeast-two hybrid screen. As a negative control, we transformed these AH109 strains with a prey plasmid encoding the SV40 large T-antigen, which does not interact with ST7. Transformants were plated on selective agar (SD/-leu/-trp/-his/-ade). Failure of the transformants to grow indicated that the truncated $ST7_{cyto}$ protein encoded by the bait plasmid failed to bind to the prey protein. Table IV summarizes the results of these studies. As previously determined, the $ST7_{cyto1}$ bait protein (residues 498-840) was able to interact with RACK1, MIBP, and SARA, but not with the negative control SV40 large T-antigen. The $ST7_{cyto1}$ bait was divided into an N-terminal $ST7_{cyto2}$ fragment (residues 498-667) and a C-terminal $ST7_{cyto3}$ fragment (residues 668-840). RACK1, MIBP, and SARA were each able to interact with $ST7_{cyto2}$. As expected, the negative control SV40 large T-antigen failed to interact with $ST7_{cyto2}$. We found that the $ST7_{cyto3}$ fragment alone activated reporter gene expression in AH109, thereby rendering it uninformative in these studies. Because our proteomic analysis of ST7 indicated that the juxtamembrane region of the cytoplasmic tail may constitute a functionally important domain, we further divided the N-terminal $ST7_{cyto2}$ fragment into three fragments, $ST7_{cyto4}$ (residues 498-625), $ST7_{cyto5}$ (residues 498-560), and $ST7_{cyto6}$ (residues 561-625). Again, the negative control SV40 large T-antigen protein did not interact with $ST7_{cyto4}$, $ST7_{cyto5}$, and $ST7_{cyto6}$. RACK1, MIBP, and SARA each interacted with $ST7_{cyto4}$ and $ST7_{cyto5}$. Neither MIBP nor SARA interacted with the $ST7_{cyto6}$, whereas RACK1 was able to interact with it. $ST7_{cyto6}$ lacks the highly conserved residues

Table IV

Interaction of prey proteins with truncated ST7 bait proteins

Full-length and truncated ST7 cytoplasmic domains (shown in Figure 5) were used as bait to identify the MIBP, SARA, and RACK1 binding sites in ST7. SV40 large T-antigen (SV40 T-Ag), which does not interact with ST7's cytoplasmic domain, served as the negative control. Plus (+) signs indicate that an ST7 bait protein interacted with the prey protein tested; minus (-) signs indicate that these proteins did not interact. NA, not applicable because the $ST7_{cyto3}$ bait protein self-activated the yeast reporter genes.

BAIT \ PREY	$MIBP_P$	$SARA_P$	$RACK1_P$	SV40 T-Ag
$ST7_{cyto1}$	+	+	+	-
$ST7_{cyto2}$	+	+	+	-
$ST7_{cyto3}$	NA	NA	NA	NA
$ST7_{cyto4}$	+	+	+	-
$ST7_{cyto5}$	+	+	+	-
$ST7_{cyto6}$	-	-	+	-

of the juxtamembrane region of ST7's cytoplasmic domain. These results demonstrate that the membrane-proximal 63 amino acids of ST7 contain the critical residues involved in its interaction with MIBP and SARA. Because RACK1 interacted with all of the bait proteins spanning residues 498-667, the critical residues for its interaction with ST7 must be contained in these truncated proteins.

Having mapped the MIBP binding domain to the membrane proximal region of ST7, co-immunoprecipitation studies using transiently co-transfected 293-T cells were carried out to determine whether MIPB interacts with additional ST7 proteins truncated within its juxtamembrane region. Specifically, we constructed expression plasmids encoding V5/His-tagged $ST7_{tr1}$, a truncated protein containing ST7's extracellular and transmembrane domains, but only the first ten residues of its 343 amino acid cytoplasmic domain (residues 1-507), and V5/His-tagged $ST7_{tr2}$ a truncated protein containing the ST7's extracellular and transmembrane domains, but only the first lysine of its cytoplasmic domain (residues 1-498). Sequencing verified that the truncated *ST7* cDNAs had been cloned in-frame with the epitope tags. 293-T cells were transiently co-transfected with plasmids encoding HA-tagged full-length MIBP and ST7-V5/His, $ST7tr_1$-V5/His, or $ST7_{tr2}$-V5/His. HA-MIBP was immunoprecipitated from lysates of the transiently co-transfected cells. The immunoprecipitation products were analyzed by Western blotting with a monoclonal V5 antibody to detect V5-tagged ST7 proteins and a monoclonal HA antibody to HA-MIBP. Both ST7-V5/His and the $ST7_{tr1}$-V5/His were strongly co-immunoprecipitated with HA-MIBP (Figure 6),

indicating an interaction between these protein pairs. Somewhat surprising was the finding, as shown in Figure 6, that the $ST7_{tr2}$-V5/His protein was also co-immunoprecipitated with HA-MIBP, although to a much lesser extent compared with ST7-V5/His and $ST7_{tr1}$-V5/His.

Figure 6. **The first ten amino acids of the ST7 cytoplasmic domain may suffice for MIBP binding**. Western blotting analysis of HA immunoprecipitation reactions from 293-T cells transiently transfected with ST7-V5/His and HA-MIBP, $ST7_{tr1}$-V5/His and HA-MIBP, or $ST7_{tr2}$-V5/His and HA-MIBP. The upper panel was probed with anti-V5 antibody to detect both the full-length and truncated V5-tagged ST7 proteins; the lower panel was probed with anti-HA antibody to detect HA-MIBP. . Lanes designated with a minus (-) sign contain negative control IP reactions, i.e., immunoprecipitation reactions without HA antibody; lanes designated with a plus (+) sign contain immunoprecipitation reactions with anti-HA; lanes designated as input contain 25 μg of the lysate used in the immunoprecipitation reactions; lanes designated as blank were not loaded.

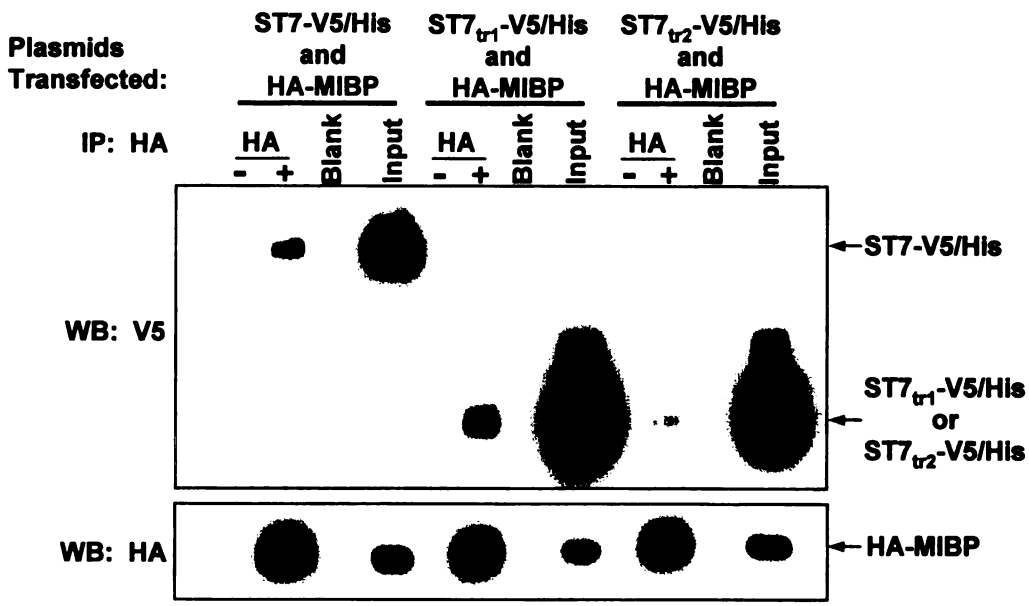

Plasmids Transfected:
ST7-V5/His and HA-MIBP
ST7tr1-V5/His and HA-MIBP
ST7tr2-V5/His and HA-MIBP
IP: HA
HA
-
+
Blank
Input
WB: V5
WB: HA
ST7-V5/His
ST7tr1-V5/His or ST7tr2-V5/His
HA-MIBP

Figure 6

DISCUSSION

Based on our analysis of ST7's protein sequence, we conclude that ST7 is a member of the low-density lipoprotein receptor (LDLR) superfamily. ST7 contains four critical structural motifs linking it to this superfamily: 1) an N-terminal signal sequence; 2) multiple LDLRA domains arranged in clusters in the extracellular region of the protein; 3) a single transmembrane domain; and 4) a cytoplasmic tail containing motifs believed to function in endocytosis and/or signaling. Moreover, the striking similarity between the structures of ST7, LRP3, and murine LRP9, shown in Figures 1 and 2B, indicates that these three proteins constitute an LDLR subfamily. Our findings agree with and strengthen those of Sugiyama et al. (2000), who first suggested that these proteins comprise a subfamily of the LDLR superfamily.

The extracellular region of every LDLR superfamily protein contains various numbers of LDLRA domain repeats arranged in clusters. These domains function in ligand binding (Yamamoto et al., 1984; reviewed by Hussain et al., 1999; Strickland et al., 2002). CUB domains are found in a diverse array of functionally unrelated extracellular proteins (reviewed by Bork and Beckmann, 1993). For example, the peripheral membrane protein cubilin, anchored in the external side of the plasma membrane through its N-terminus, contains 27 extracellular CUB domains that function in ligand binding (reviewed by Christensen and Birn, 2002). The LDLRA and CUB domains present in ST7 may very likely enable ST7 to bind to extracellular ligands. At this point, no ST7 ligands have been identified, but ongoing work in our laboratory is directed

toward this goal. Members of the LDLR superfamily also contain endocytic signals in their cytoplasmic domains. The presence of putative endocytic motifs in ST7's cytoplasmic tail (Figure 2B) suggest that, like other members of the LDLR superfamily, ST7 functions in endocytosis. In addition to putative internalization signals, we found (see Figure 2B and Table II) that ST7's cytoplasmic domain contains several putative signaling motifs as well as sites of potential phosphorylation. The presence of such motifs suggests that ST7 also plays a role in signal transduction. This is in agreement with recent studies that show that several other LDLR family proteins function in signal transduction (Trommsdorff et al., 1998, 1999; Gotthardt et al., 2000; Wehrli et al., 2000; Tamai et al., 2000; Pinson et al., 2000; Mao et al., 2001; Barnes et al., 2001; Boucher et al., 2002; Loukinova et al., 2002; Lutz et al., 2002).

The data presented in Figures 3 and 4 further support the hypothesis that the cytoplasmic domain of ST7, like other members of the LDLR superfamily, functions in signal transduction. Both RACK1 and SARA have been shown to be involved in signal transduction pathways. RACK1 was first identified as a shuttling protein for activated protein kinase C (PKC) (Ron et al., 1994; 1999). Because ST7's cytoplasmic tail contains six potential PKC phosphorylation sites (Table II), it may be that RACK1 shuttles activated PKC to ST7 to phosphorylate ST7. RACK1 consists of seven WD domains that fold to form a β propeller structure (Wall et al., 1995). Such a structure may allow RACK1 to bind several proteins simultaneously, thereby enabling it to function as a scaffold protein (Wall et al., 1995). Consistent with this structure, RACK1 has been shown to interact

with a wide variety of proteins in addition to PKC. These include the non-receptor tyrosine kinases Src and Fyn (Chang et al., 1998, 2001, 2002; Yaka et al., 2002), the cAMP-specific phosphodiesterase PDE4D5 (Yarwood et al., 1999), the Ras GTPase activating protein $p120^{GAP}$ (Koehler and Moran, 2001), the angiotensin receptor associated protein AGTRAP (Wang et al., 2002), and the signal transducer and activator of transcription 1 protein (Usacheva et al., 2001). RACK1 also associates with the cytoplasmic domains of cell surface receptors such as β1 integrins (Liliental and Chang, 1998), the insulin-like growth factor I receptor (IGF-IR) (Hermanto et al., 2002; Kiely et al., 2002), the protein tyrosine phosphatase PTPμ (Mourton et al., 2001), and the NR2B subunit of the NMDA receptor (Yaka et al., 2002). Because RACK1 interacts with a plethora of cellular proteins, it is believed to play a key role in regulating a variety of cellular signaling pathways (Chang et al., 2001).

The FYVE domain-containing protein SARA (also known as MADH-interacting protein, MADHIP) was first identified by Tsukazaki et al. (1998). They found that SARA serves as a scaffold protein to link unphosphorylated SMAD2/3 to active TGFβ receptor heterodimers. By localizing SMAD2/3 to the membrane, SARA facilitates phosphorylation of these SMADs by the type I receptor's kinase domain (Tsukazaki et al., 1998). Phosphorylated SMAD2/3 dissociate from SARA and associate with SMAD4 to translocate to the nucleus and regulate gene expression. More recently, it was determined that SARA localizes to early endosomes, suggesting that endocytosis plays a role in this SARA-mediated signaling pathway (Itoh et al., 2002; Penheiter et al., 2002; Hayes et al., 2002).

Although little is known about MIBP, it too appears to be involved in signal transduction. Li et al. (1999) first identified MIBP using a yeast two-hybrid assay to screen for proteins able to bind to the cytoplasmic tails of β1-integrins. They found that myocytes express a high level of MIBP prior to differentiation and a low level of MIBP after differentiation and that overexpression of MIBP in myocytes inhibits cell fusion and differentiation (Li et al., 1999). Therefore, MIBP likely plays an important role in signal transduction pathways regulating myocyte fusion and differentiation.

Our data showing that ST7's cytoplasmic domain binds to proteins related to signal transduction is consistent with the emerging role of LDLR superfamily proteins as signal transducers (reviewed in Strickland et al., 1995; Herz, 2001, 2002; van der Geer, 2002). For example, Trommsdorff et al. (1998, 1999) and D'Arcangelo et al. (1999) demonstrated that VLDLR and ApoER2 are co-receptors for the extracellular protein Reelin. Binding of Reelin to these receptors has two major consequences, the internalization of Reelin and the activation of a tyrosine kinase signaling cascade. This results in the phosphorylation of Dab1 protein bound to the cytoplasmic domains of these receptors. Alterations in this pathway cause dysfunction in brain development (Trommsdorff et al., 1999). Additionally, several groups demonstrated that LRP5 and LRP6 participate in the Wnt signaling pathway (Wehrli et al., 2000; Tamai et al., 2000; Pinson et al., 2000; Gong et al., 2001; Mao et al., 2001). Both proteins serve as co-receptors for extracellular Wnt glycoproteins. Mao et al. (2001) demonstrated that cytoplasmic domain of LRP5 binds to the

scaffolding protein axin to promote β-catenin stabilization and Wnt responsive gene expression. Mice lacking LRP6 die at birth as a result of severe developmental defects, including malformation of the skeleton, limbs, eyes, and urogenital tract (Pinson et al., 2000). Mutations in the human *LRP5* gene result in defects in bone density and eye development (Gong et al., 2001; Little et al., 2002; Boyden et al., 2002). Boucher et al. (2002) and Loukinova et al. (2002) showed that LRP1 serves as a co-receptor for PDGF BB and that the adaptor protein Shc binds to a phosphotyrosine residue in LRP1's cytoplasmic domain upon PDGF BB stimulation. Finally, yeast two-hybrid studies demonstrated that the cytoplasmic tails of LRP1, LRP2, LDLR, VLDLR, and ApoER2 interact with proteins known to be involved in MAP kinase signal transduction, synaptic transmission regulation, cytoskeletal organization, cellular adhesion, and endocytosis, adding further support to the idea that members of the LDLR superfamily participate not only in endocytosis but also in signaling (Gotthardt et al., 2000). The ST7-interacting proteins described in this paper can be classified into many of the same categories as those that interact with other LDLR superfamily members.

Our yeast two-hybrid studies also demonstrated that both SARA and MIBP bind to the juxtamembrane region of ST7's cytoplasmic domain (Figure 5 and Table IV). Because the bait construct containing ST7 residues 668-840 was able to self-activate the reporter genes, we cannot exclude the possibility that this region contains additional MIBP and SARA binding sites. However, the presence of MIBP and SARA binding sites in the juxtamembrane region of ST7 supports

our hypothesis that this region, which is highly conserved among the ST7 subfamily proteins, is important for ST7 function. Moreover, we determined that the first ten amino acids of ST7's cytoplasmic domain may suffice for MIBP binding (Figure 6). Based on signal intensity, we conclude that the MIBP binds equally well to ST7 and $ST7_{tr1}$. Somewhat surprising was the fact that a small amount of $ST7_{tr2}$ associated with MIBP. Because the cytoplasmic tail of this truncated protein contains only one residue, we did not expect it to interact with MIBP. It is always possible that the remaining lysine residue plays a critical role in the ST7-MIBP interaction, and by itself is able to interact with ST7, but does so much less efficiently. Another possible explanation is that residues of the transmembrane helix domain become exposed because of a conformational change when ST7 is active and participate in the ST7-MIBP interaction. The growth medium used in these studies, which included 10% fetal bovine serum, may contain ligands that bind to ST7's extracellular domain to activate the receptor and induce such conformational changes. Studies of integrin cytoplasmic domains suggest that ligand binding does induce conformational changes that affect the position of residues within and near the membrane (Armulik et al., 1999; Lu et al., 2001). Because the cells used in our co-immunoprecipitation experiments contain endogenous ST7 protein, it is possible that the latter protein was responsible for making it appear as if MIBP interacts with $ST7_{tr2}$. If dimerization of endogenous ST7 with $ST7_{tr2}$ were to occur, the observed interaction could have resulted from MIBP associating with the full-length cytoplasmic tail of endogenous ST7, instead of the truncated cytoplasmic

tail. Therefore, immunoprecipitation of MIBP would cause both endogenous and truncated ST7 to be co-precipitated. We prefer this possibility as the most likely explanation of our results with $ST7_{tr2}$. If dimerization explained that interaction, then it is possible that dimerization also explains the interaction between $ST7_{tr1}$ and MIBP. However, if it is responsible for both results, then we would have expected to see an equivalent amount of $ST7_{tr1}$ and $ST7_{tr2}$ pulled-down by MIBP. This was not the case (Figure 6).

ST7 contains at least one RACK1 binding site in the region spanning residues 498-667 (Table IV and Figure 5). If there is only one binding site for RACK1 in this region, it must fall between residues 498-625 because these residues enabled RACK1 to associate with ST7. Moreover, the observation that RACK1 interacts with both $ST7_{cyto5}$ and $ST7_{cyto6}$, two bait proteins that do not include any common residues, suggests that each of these proteins contain part of the RACK1 binding site. We cannot exclude the possibility that there is more than one RACK1 binding site in this region of ST7's cytoplasmic tail, and each bait protein contains one or more complete sites. Neither can our results rule out the possibility that residues 668-840 of ST7 contain one or more RACK1 binding sites.

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Appendix

Expression of ST7 Protein Inhibits the Tumor-Forming Ability of a Human Fibrosarcoma-derived Cell Line[1]

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[3] Manuscript in preparation, Battle, M.A., Maher, V.M., and McCormick, J.J. ST7 is a novel member of the LDLR superfamily with a cytoplasmic tail that interacts with intracellular proteins related to signal transduction pathways.

[4] The abbreviations used are: LDLR, low-density lipoprotein receptor; LRP, lipoprotein receptor-related protein; LDLRA, low-density lipoprotein receptor type A; CUB, Complement factor C1s/C1r, Urchin embryonic growth factor, Bone morphogenetic protein; RACK1, receptor for activated protein kinase C.

ABSTRACT

Previous studies using differential mRNA display to compare the infinite life span, non-tumorigenic human fibroblast cell strain MSU-1.1 to a carcinogen transformed, malignant MSU-1.1 derivative identified a novel gene, *ST7*. Compared to its parental strain, expression of ST7 was strongly down-regulated in the tumorigenic cells. A low level of ST7 expression was also observed in additional malignant human cell lines. Subsequent studies revealed that ST7 is a novel member of the low-density lipoprotein receptor (LDLR) superfamily, several proteins of which have been shown to participate in both endocytosis and signal transduction. To determine if ST7 acts as a tumor suppressor, the human fibrosarcoma-derived malignant cell line SHAC was transfected with plasmids encoding V5/His-tagged full-length ST7 or V5/His-tagged truncated ST7, which contains its extracellular and transmembrane domains, but only the first 10 of the 343 amino acids of its cytoplasmic domain. The tumorigenicity of two independent full-length ST7-V5/His-expressing transfectants, two independent transfectants expressing the V5/His-tagged truncated form of ST7, two independent vector-control transfectants, and two independent non-ST7-expressing control transfectants was assessed by subcutaneous injection of these cell strains into athymic mice. The results showed that overexpression of full-length ST7 inhibits the tumor-forming ability of SHAC cells. Tumors developed at a significantly lower frequency than that of the control cell lines/strains. In contrast, overexpression of the truncated form of ST7 failed to inhibit the tumor-forming ability of SHAC cells, supporting the hypothesis that

ST7 is a tumor suppressor and that its cytoplasmic domain plays a role in the protein's function as a suppressor.

INTRODUCTION

It is generally thought that tumors originate from a single cell that has acquired all of the properties necessary to become malignant, and replication of that cell gives rise to all of the cells of the tumor. Theories of carcinogenesis must provide an understanding of how the original tumor cell gained the necessary genetic and/or epigenetic changes to become malignant. At least five such changes are estimated to be required. To determine the number and nature of these changes, McCormick and his colleagues (reviewed by McCormick and Maher, 1996) established a model system in which normal human fibroblasts in culture can be transformed into malignant fibroblasts through the acquisition of a series of genetic changes, each providing a growth advantage that enables sequential clonal expansion. Using this model system, McCormick and his colleagues (Qing et al., 1997) carried out differential mRNA display to compare an infinite life span, non-tumorigenic human fibroblast cell strain, designated MSU-1.1, to a carcinogen-transformed, malignant derivative of MSU-1.1 cells. This comparison identified a novel putative tumor suppressor gene, *ST7*, which was predicted to encode a transmembrane protein (Qing et al., 1999). *ST7* expression in the tumorigenic cell line was significantly lower than that of the non-tumorigenic parental cell strain. Moreover, several other malignant human cell lines have a low level of ST7, suggesting that ST7 deficiency is a common characteristic of tumorigenic cells. Recent studies in this laboratory[3] revealed that ST7 is a member of a subfamily of the LDLR[4] superfamily of plasma membrane receptors. Its extracellular domain contains an N-terminal signal

sequence to target it to the membrane, five LDLRA domains, and two CUB domains. Its cytoplasmic tail, which is notably rich in serine, threonine, and proline, contains numerous putative phosphorylation sites and several motifs implicated in endocytosis and signal transduction.

Another member of the LDLR superfamily, *LRP1B*, has been identified as a candidate tumor suppressor gene. Liu et al. (2000a, 2000b) showed that the *LRP1B* gene is inactivated in ~45% of non-small cell lung cancer cell lines studied. They detected homozygous deletions within the *LRP1B* gene, and point mutations in the *LRP1B* alleles. Finally, some of the cell lines studied expressed abnormal truncated *LRP1B* transcripts. In contrast, none of the small cell lung cancer cell lines studied had any observable defects in the *LRP1B* sequence, and only one such line contained abnormal *LRP1B* transcripts. These findings strongly suggest that inactivation of *LRP1B* plays an important in the development of non-small cell lung cancer, but not in the development of small cell lung cancer. Most recently, Langbein et al. (2002) also documented alterations of the *LRP1B* gene in high grade urothelial cancer. In light of these data and of the earlier study of Qing et al. (1999) showing that ST7 expression is low in tumorigenic human fibroblasts, we tested the hypothesis that ST7 functions to suppress tumorigenicity. A tumorigenic cell line derived from a patient's fibrosarcoma, designated SHAC, was transfected with a plasmid encoding full-length ST7 or an ST7 protein with a severely truncated cytoplasmic domain, i.e., it contains ST7's extracellular and transmembrane domains, but only the first 10 of the 343 amino acids that comprise its cytoplasmic domain.

The tumorigenicity of independent transfectants expressing these proteins as well as of independent vector-control transfectants and non-ST7-expressing control transfectants was assessed by subcutaneous injection of these various cell strains into athymic mice. Tumors formed at a significantly lower frequency in athymic mice injected with SHAC cells expressing full-length ST7 than in athymic mice injected with control cell strains. Any tumors that developed did so with a greater latency. These data support the hypothesis that ST7 suppresses the tumor-forming ability of SHAC cells. Furthermore, expression of a truncated form of ST7 failed to inhibit the tumor-forming ability of SHAC cells, suggesting that the cytoplasmic domain of ST7 plays an important role in the protein's function as a tumor suppressor.

MATERIALS AND METHODS

Materials. The sources of specific materials used are: supplemented calf serum and fetal calf serum, Hyclone; restriction enzymes and T4 DNA ligase, New England Biolabs or Invitrogen; shrimp alkaline phosphatase, Promega; *Pfu* polymerase, Stratagene; oligonucleotides, the Michigan State University Macromolecular Structure, Sequencing, and Synthesis Facility; protease and phosphatase inhibitors, Sigma; SeeBlue® Plus 2 prestained protein molecular weight standards, Invitrogen; polyclonal ST7 antibody and monoclonal Na^+/K^+ ATPase antibody, Affinity BioReagents; monoclonal V5 antibody, Invitrogen; monoclonal actin antibody, Sigma; and horseradish peroxidase-conjugated secondary antibodies, Santa Cruz Biotechnology or Sigma.

Cells and cell culture conditions. Diploid human fibroblast cell lines SL89 and LG1 were derived from the foreskin of normal neonates. They were cultured in Eagle's minimal essential medium containing 0.2 mM L-aspartic acid, 0.2 mM L-serine, 1.0 mM sodium pyruvate, 10% supplemented calf serum, penicillin (100 units/ml), and streptomycin (100 μg/ml) (growth medium) in a 37°C humidified incubator with 5% CO_2 in air. SHAC cells, a cell line derived from a patient's fibrosarcoma, and all SHAC-derived cell strains were similarly cultured in growth medium, supplemented with 1 μg/ml hydrocortisone. The human embryonic kidney-derived cell line HEK 293-T was similarly cultured in high-glucose Dulbecco's modified Eagle's medium supplemented with 10% fetal calf serum and containing penicillin and streptomycin.

Stable transfection SHAC cells. Lipofectamine (Invitrogen) was used according to the manufacturer's instructions. Blasticidin (10 μg/ml) (Invitrogen) was used to select stably transfected independent clonal populations.

Preparation of cell lysates. Cells were washed in cold phosphate-buffered saline, collected in lysis buffer (50 mM Tris-HCl, pH 7.2, 150 mM NaCl, 50 mM NaF, 0.5% NP-40, 1 mM Na_3VO_4, 1 mM benzamidine, 1 mM phenylmethylsulfonyl fluoride, 25 μg/ml aprotinin, and 25 μg/ml leupeptin). Cells in lysis buffer were incubated on ice for 30 min. Lysates were clarified by centrifugation (25,000 x g) for 30 min at 4°C. The concentration of protein in each lysate was determined using the Coomassie protein assay reagent (Pierce) following the manufacturer's instructions, and aliquots of each lysate containing 50 μg of protein were denatured in 5X Laemelli sample buffer (0.125 M Tris-HCl, pH 6.8, 25% glycerol, 5% sodium dodecyl sulfate, 0.0125% bromophenol blue, and 25% β-mercaptoethanol as the reducing agent).

Preparation of plasma-membrane enriched fractions. Cells were washed in phosphate-buffered saline, collected in hypotonic lysis buffer (50 mM Tris-HCl, pH 7.4, 35 mM NaF, 5 mM $MgCl_2$, 1mM EGTA, 1 mM Na_3VO_4, 1 mM sodium pyrophosphate, 1 mM phenylmethylsulfonyl fluoride, 25 μg/ml aprotinin), and incubated on ice for 10 min. Cells were homogenized by 30 strokes in a dounce homogenizer. Homogenates were centrifuged (500 x g) for 5 min at 4°C. Supernatants were centrifuged (16,900 x g) for 1 h at 4°C. Pellets, which are enriched with plasma-membrane proteins, were resuspended in RIPA buffer (50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Triton X-100, 0.1% sodium dodecyl

sulfate, 0.5% sodium deoxycholate, 1mM EDTA, 1 mM Na_3VO_4, 2 mM phenylmethylsulfonyl fluoride, and 25 μg/ml aprotinin). To obtain cytosolic, soluble fractions, the supernatants were clarified by centrifugation (100,000 x g) for 2 h at 4°C. The concentration of protein in each fraction was determined using a bicinchoninic acid (BCA) protein assay reagent kit (Pierce) following the manufacturer's instructions. Aliquots of each plasma membrane-enriched fraction and cytosolic, soluble fraction containing 20 μg of protein were denatured in 5X Laemelli sample buffer.

Western blotting analysis. Denatured proteins were separated by sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred to Immobilon-P membrane (Millipore). The membranes were blocked with Tris-buffered saline containing 0.1% Tween-20 and 5% non-fat milk (blocking solution) for 2 h at room temperature and incubated with the appropriate primary antibody diluted in blocking solution for at least 2 h at room temperature. They were then incubated with an appropriate secondary antibody diluted in blocking solution for 1 h at room temperature. To detect proteins on the membrane, SuperSignal West Pico chemiluminescent substrate (Pierce) was used according to the manufacturer's instructions.

Tumorigenicity studies. The ability of cells to form tumors was tested in athymic mice (BALB/c). For each cell line/strain tested, two sites per athymic mouse (shoulder and flank or both flanks) were subcutaneously injected with 1.0 x 10^6 cells. Mice were examined weekly for tumor formation, and the tumor dimensions were measured using calipers. Tumor volume (in cm^3) was

estimated from the equation used to calculate the volume of a sphere. Tumor latency was defined in these experiments as the time (wk) required for a tumor to reach a volume of 0.5 cm^3. For statistical analysis, the cell strains were divided into three groups: group 1) ST7-V5/His expressing transfectants (ST7-1 and ST7-2); group 2) $ST7_{tr1}$-V5/His transfectants cell strains ($ST7_{tr1}$-1 and $ST7_{tr1}$-2); and group 3) non-ST7-expressing control and vector-control transfectants (NC-1, NC-2, VC-1, and VC-2). The average latency for each group of cell strains was computed. A t-test was used to compare the average latency observed for the full-length ST7-expressing group with that observed for the group expressing the truncated form of ST7 and with that observed for non-ST7-expressing control and vector-control group, i.e., group 1 compared with group2 and group 1 compared with group 3.

RESULTS

Construction of expression plasmids encoding V5/His-tagged full-length or a V5/His-tagged truncated form of ST7. To construct an expression plasmid encoding an ST7-V5/His fusion protein, a donor plasmid containing *ST7* cDNA was used as a template for PCR to generate an ST7 PCR product containing a mutated stop codon (TAG→TTG). This PCR product was digested with *EcoRI* and subcloned into the *EcoRI* digested, dephosphorylated expression vector pcDNA6-V5/HisA, in-frame with the vector's C-terminal V5 and His epitope tags. The resulting plasmid, which contains a gene encoding a selective marker (blasticidin), was designated pcDNA6-ST7-V5/His. To construct an expression plasmid encoding a V5/His-tagged truncated form of ST7 protein, the same donor plasmid was used as the template for PCR amplification of the region of ST7 encoding amino acids 1 through 507. The resulting plasmid, designated pcDNA-$ST7_{tr1}$-V5/His, encodes a protein containing the extracellular and transmembrane domains of ST7, but only the first 10 of the 343 residues of its cytoplasmic domain. Sequencing verified the fidelity of the PCR reactions.

Expression of full-length ST7 in a human fibrosarcoma-derived cell line, but not the truncated form of ST7, inhibits tumor forming activity. To test the hypothesis that *ST7* functions as a tumor suppressor, we transfected SHAC cells, a tumorigenic cell line derived from a patient's fibrosarcoma, with the ST7-V5/His expression plasmid, with the $ST7_{tr1}$-V5/His expression plasmid, or with an empty plasmid as a control. Transfectants were selected for resistance to blasticidin. Independent drug resistant clonal populations were isolated,

expanded, and screened for expression of the appropriate V5/His tagged protein using Western blotting analysis with a V5-specific monoclonal antibody. Figure 1 shows the level of expression of V5/His tagged full-length or truncated ST7 protein in such transfectants. The first two lanes contain protein isolated from cells transfected with the pcDNA6-ST7-V5/His plasmid, but that did not express the encoded V5/His-tagged ST7 protein (non-ST7-expressing control transfectants). We observed two V5-reactive bands in the lysates from the $ST7_{tr1}$-1 and $ST7_{tr1}$-2 cell strains. We interpret the lower band visible in lanes 5 and 6 to represent a product of proteolytic degradation.

These six SHAC-derived cell strains (shown in Figure 1), as well as two vector-transfected control cell strains, VC-1 and VC-2, i.e., SHAC-derived cell strains transfected with the empty pcDNA6-V5/HisA plasmid, and the parental non-transfected SHAC cell line were tested for their ability to form tumors in athymic mice. The cells were subcutaneously injected at two sites per mouse, and the latency of tumor formation was defined as time required for a tumor to reach 0.5 cm^3 in volume. The results are shown in Table 1. Because the mice were injected at two sites, the first tumor to arise sometimes grew faster than the other, most likely because of having a better blood supply. In those cases where one tumor reached 0.5 cm^3 in volume before the other, the mouse had to be sacrificed. Even though the second tumor had not reached 0.5 cm^3 in volume, examination by the pathologist was able to determine that the smaller tumor was malignant. Such tumors were excluded from the tumors analyzed for latency (Table 1, columns 2-4), but were included in the total number of tumors per sites

Figure 1. Presence or absence of V5/His-tagged proteins in SHAC transfectants. Protein (50 μg) was separated in a 10% SDS-polyacrylamide gel and transferred to PVDF membrane for Western blotting analysis with the anti-V5 monoclonal antibody. Lanes 1 and 2, the non-ST7-expressing control cell strains NC-1 and NC-2; Lanes 3 and 4, the ST7-V5/His-expressing cell strains ST7-1 and ST7-2, which showed a reduction in tumor-forming ability; Lanes 5 and 6, the $ST7_{tr1}$-V5/His expressing cell strains $ST7_{tr1}$-1 and $ST7_{tr1}$-2. The lower of the two bands present in lanes 5 and 6 is most likely a product of proteolytic degradation. Actin expression was measured as a loading control.

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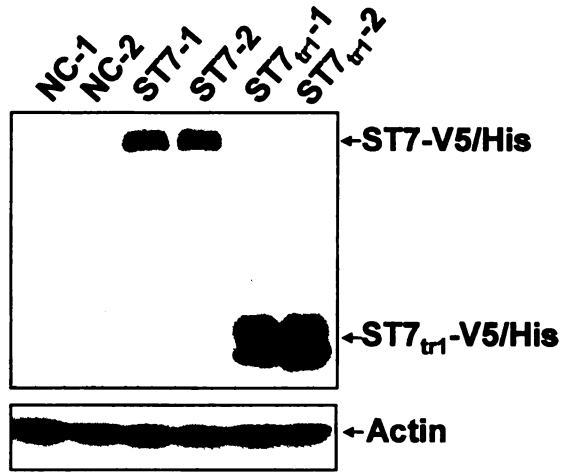

Figure 1

Table 1 *Expression of ST7 inhibits the tumor-forming ability of SHAC cells*

Cells tested[a]	Tumors (≥ 0.5 cm^3) per sites injected	Latency range (wk)[b]	Average latency	Total tumors per injected sites[c]
SHAC	7/8	4-6	4.8	8/8
VC-1	9/14	7-9	7.6	13/14
VC-2	4/6	4.5-8	5.8	6/6
NC-1	9/14	3-5.5	3.9	14/14
NC-2	12/14	4-7.5	5.8	14/14
$ST7_{tr1}$-1	16/16	3.5-5.5	4.7	16/16
$ST7_{tr1}$-2	15/16	4.5-7.5	5.7	16/16
ST7-1	6/14	10.5-16.5	13.6[d]	9/14
ST7-2	10/22	10-23	15.2[d]	10/22

[a] SHAC, parental non-transfected cells; VC-1&-2, vector-control transfectants; NC-1&-2, non-ST7-expressing control transfectants; $ST7_{tr1}$-1&-2, transfectants expressing a V5/His-tagged truncated form of ST7; ST7-1&-2, transfectants expressing V5/His-tagged full-length ST7.

[b] Time required for a tumor to reach 0.5 cm^3 in volume.

[c] See Results for explanation of this column.

[d] The difference in average latency between the ST7-expressing transfectants and the non-ST7-expressing control or vector-control transfectants is highly significant, $p < 0.0001$; the same is true of the difference between the ST7-expressing transfectants and the transfectants expressing the truncated form of ST7.

injected (Table 1, column 5). As expected, the parental SHAC cell line as well as the four non-ST7-expressing or vector control cell strains formed tumors at virtually all sites injected with a very short latency. The same was true for the cell strains expressing a truncated ST7 protein. In contrast, the ST7-1 and ST7-2 cell strains, which express full-length ST7-V5/His protein, formed tumors at only about half of the sites injected, and when tumors developed, the time to reach 0.5 cm^3 (latency) was significantly greater ($p < 0.0001$) than the time required for the non-ST7-expressing cells and those expressing a truncated ST7 protein to form such tumors.

The ST7-V5/His and ST7tr1-V5/His proteins are overexpressed in the SHAC cell strains generated. As shown in Figure 1, the level of expression of the truncated V5/His-tagged ST7 protein is higher than that of full-length V5/His-tagged ST7 protein. Even if the lower band present in the lysates of the transfectants expressing the truncated form of ST7 represents a degraded form of this protein, the level of expression of the intact form of V5/His-tagged truncated ST7 is higher than that of V5/His-tagged full-length ST7. To compare the level of exogenous full-length ST7-V5/His protein with that of endogenous ST7 protein, we first used the polyclonal antibody generated in 1997 by Qing et al. (1999). It proved no longer active. Therefore, we had antibody prepared in rabbits using another peptide from the cytoplasmic domain of ST7. This antibody, directed against residues 617-632 of ST7, is significantly more specific to ST7 than the anti-ST7 polyclonal antibody in 1997. To test the specificity of this antibody, protein from HEK 293-T cells transiently transfected with an ST7-

V5/His expression plasmid or a plasmid encoding a non-tagged ST7 protein, i.e., the donor plasmid referred to above or a derivative designated pcDNA6-ST7, was used for Western blotting analysis. A membrane containing three identical sets of samples was divided into three sections and probed with the new anti-ST7 polyclonal antibody, its corresponding pre-immune serum, or an anti-V5 monoclonal antibody. As expected, the pre-immune serum did not recognize any proteins; the polyclonal ST7 antibody recognized both the tagged and non-tagged proteins; and the anti-V5 monoclonal antibody recognized the V5/His tagged protein at the same position as that recognized by the new anti-ST7 antibody (data not shown). This new ST7 antibody was then used to compare the level of expression of full-length V5/His-tagged ST7 in the two isolated SHAC transfectants ST7-1 and ST7-2 with the level of expression of endogenous ST7 found in foreskin-derived human fibroblast cell lines, vector-control SHAC transfectants, or non ST7-expressing control SHAC transfectants. Parental SHAC cells were also included. The results, shown in Figure 2, indicate that the new ST7 antibody recognized the V5/His-tagged full-length ST7 in the SHAC transfectants ST7-1 and ST7-2 (Figure 2, lanes 8 and 9). This protein migrates at a slower rate than endogenous ST7 found in normal foreskin-derived cell lines (lanes 1 and 2). No such larger protein band was observed in the non-ST7-expressing control transfectants (Figure 2, lanes 6 and 7). However, in SHAC cells and its derivative transfectants, the new ST7 antibody also recognized a protein that migrated at the same position as that found in normal diploid human fibroblasts.

Figure 2. Expression of endogenous ST7 protein in normal human fibroblasts, SHAC cells, and SHAC-derived transfectants. Protein (50 μg) was separated in a 10% SDS-polyacrylamide gel, transferred to a PVDF membrane, and probed with a polyclonal anti-ST7 antibody. Lanes 1-2, foreskin-derived fibroblasts; Lane 4, SHAC cells; Lanes 5 and 6, empty-vector control transfected cell strains; Lanes 7 and 8, non-ST7-expressing control SHAC transfectants; Lanes 9 and 10, ST7-V5/His expressing SHAC transfectants. Actin expression was used as a loading control.

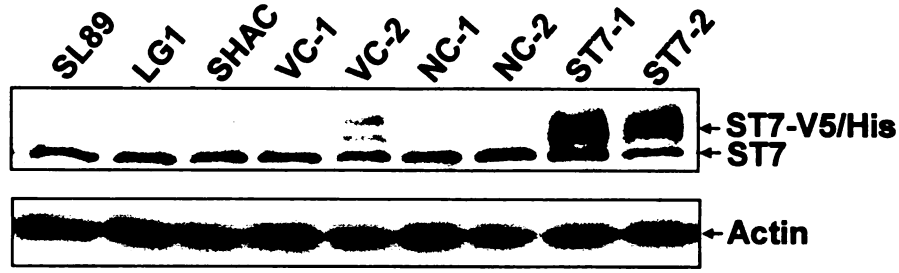

Figure 2

Plasma membrane localization of ST7-V5/His and ST7tr1-V5/His proteins. To determine whether exogenous ST7-V5/His and $ST7_{tr1}$-V5/His proteins localize to cellular membranes, as predicted by our analysis of the structure of ST7 protein[4], we isolated plasma membrane-enriched and soluble fractions from the ST7-1, $ST7_{tr1}$-2, and VC-2 SHAC transfectants and analyzed them by Western blotting using the anti-V5 antibody. Both ST7-V5/His and $ST7_{tr1}$-V5/His were detected in the appropriate plasma membrane-enriched fractions and were not in the matching soluble fractions, indicating that V5/His tagged full-length ST7 and V5/His tagged truncated ST7 localize to the plasma membrane (Figure 3). As a control, we also analyzed these fractions by blotting with an antibody against Na^+/K^+ ATPase, an integral membrane protein. As expected, this latter protein was detected in the plasma membrane-enriched fractions and was not in the soluble fractions.

Figure 3. The V5/His tagged ST7 proteins encoded by the transfected plasmids are localized in the membrane of the SHAC cell recipients. Plasma membrane-enriched (M) and soluble, cytosolic (S) fractions were isolated from a vector control transfected SHAC cell strain (lanes 1 and 2); from a transfectant overexpressing the truncated form of ST7 (lanes 3 and 4); and from a transfectant overexpressing full-length ST7 (lanes 5 and 6). A total of 20 μg of each fraction was separated in a 10% SDS-polyacrylamide gel, transferred to PVDF membrane, and probed with anti-V5 antibody (upper panel) and an antibody specific for a membrane protein, i.e., anti-Na^+/K^+ ATPase antibody (lower panel). The latter protein has multiple isoforms.

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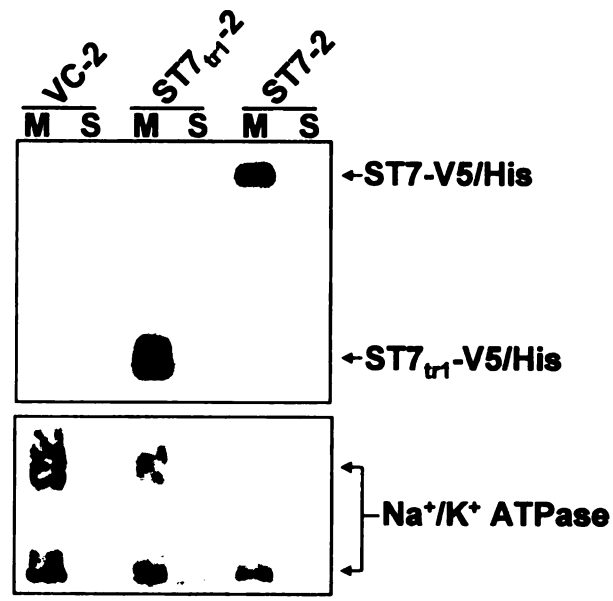

Figure 3

DISCUSSION

The results of the present study, as shown in Table 1, support the hypothesis that ST7 is a novel tumor suppressor. Overexpression of V5/His-tagged full-length protein inhibited the tumor-forming ability of SHAC cells, a cell line derived from a patient's fibrosarcoma. Moreover, overexpression of a severely truncated form of ST7, which contains its extracellular and transmembrane domains, but lacks the majority of its cytoplasmic domain, failed to inhibit the tumor-forming ability of SHAC cells. Therefore, we conclude that ST7 functions as a tumor suppressor in SHAC cells. Moreover, the data indicate that tumor suppression occurs as a result of ST7 protein function rather than merely protein overexpression, since the overexpressed truncated protein did not inhibit tumorigenicity. These results also strongly suggest that the cytoplasmic domain of ST7 plays a critical role in the protein's function as a tumor suppressor.

When we began our studies to test the hypothesis that ST7 functions as a tumor suppressor, we chose to use the human fibrosarcoma-derived cell line SHAC because research carried out in this laboratory in 1997 showed that SHAC cells fail to express endogenous ST7 protein (Qing et al., 1999). However, when we used a different polyclonal antibody to examine ST7 expression in both normal human diploid fibroblast cell lines and in SHAC and SHAC-derived cell strains, we detected endogenous ST7 protein in all of those cell lines/strains (Figure 2). Because there is no matched normal human cell line to use as the standard against which the ST7 expression level in SHAC cells can be compared, it is not possible to determine whether or not ST7 is down-regulated in

SHAC cells. However, the tumor data in Table 1 demonstrates that if the ST7 protein present in SHAC cells is functional, then its level is not sufficient to prevent these cells from rapidly forming tumors. Increasing the amount of full-length ST7 protein in these cells clearly inhibited their tumor-forming ability and greatly increased tumor latency. It is possible that the ST7 protein present in SHAC cells is non-functional. For example, point mutations in one or both of the alleles of the *ST7* gene could result in the synthesis of an inactive or mislocalized protein. Such mutations have been shown to inactivate LDLR, the prototype member of the LDLR superfamily (reviewed by Brown and Goldstein, 1986), and to cause familial hypercholesterolemia, an autosomal dominant disorder resulting in an increased concentration of plasma cholesterol and early onset heart attacks (Davis et al., 1986). At present, we cannot assess ST7 function in human fibroblasts. Moreover, ST7 belongs to an LDLR subfamily[3] that also contains two closely related proteins, LRP3 and LRP9. The functions of LRP3 and LRP9 have not yet been determined, but the striking similarity of these proteins with ST7 suggests that this subfamily of proteins has overlapping functions in human cells. Therefore, it is possible that SHAC cells are deficient in one or both of the other members of this subfamily and that overexpression of ST7 restores the function lost by LRP3 and/or LRP9 deficiency.

Both ST7-V5/His and $ST7_{tr1}$-V5/His proteins were found in plasma membrane-enriched cellular fractions and not in soluble, cytosolic cellular fractions, strongly suggesting that these proteins are localized in the plasma membrane (Figure 3). This agrees with Qing et al. (1999), who predicted that ST7 is a type I

transmembrane protein. Our related studies of ST7's cytoplasmic domain[3] provide at least one possible explanation for ST7's ability to suppress tumors. Using the yeast two-hybrid system, we isolated proteins that interact with ST7's cytoplasmic domain. One such ST7-interacting protein identified was RACK1, a scaffold protein that interacts with a plethora of cellular proteins and is believed to play a key role in regulating a variety of cellular signaling pathways (reviewed by Schechtman and Mochly-Rosen, 2001). Chang et al. (1998) showed that RACK1 binds to the nonreceptor tyrosine kinase Src and negatively regulates its kinase activity, thereby inhibiting Src-mediated proliferation of NIH3T3 fibroblasts. We also observed that ST7 overexpression inhibits the proliferation of SHAC cells (data not shown). It is possible that ST7, through its interaction with RACK1, plays a role in RACK1-mediated inhibition of Src-induced cellular proliferation. Binding of RACK1 to ST7's cytoplasmic tail would place RACK1 at the membrane in proximity to active Src, enabling RACK1 to bind to and inhibit Src. Inhibition of such a proliferative pathway by ST7 would provide cells with protection against abnormal cellular replication. Therefore, loss of ST7 function would disrupt this protective growth inhibitory pathway and provide tumor cells with a growth advantage. A better understanding of the mechanism through which ST7 functions to inhibit tumorigenesis may provide insights into the diagnosis and treatment of cancer.

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