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The *NF2* tumor suppressor gene product, merlin, mediates contact inhibition of growth through interactions with CD44

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ABSTRACT

The neurofibromatosis-2 (*NF2*) gene encodes merlin, an ezrin-radixin-moesin-(ERM)-related protein, that functions as a tumor suppressor. I found that merlin plays a critical role in the establishment and maintenance of contact inhibition of growth. At high cell density, merlin is activated and blocks proliferation with corresponding changes in cell cycle parameters. Merlin interfered with growth factor receptor or Ras-dependent signal transduction to MAP kinase and the step of interference was located downstream of Ras and Raf and upstream of MEK. Merlins growth inhibiting function depended on interaction with a specific domain of the cytoplasmic tail of CD44. In addition merlin activity and phosphorylation status depended on the extracellular ligands associated with the N-terminus of CD44. At high cell densities, in the presence of the extracellular ligand HA, merlin was dephosphorylated and bound directly to a basic amino acid motif in the cytoplasmic tail of CD44. Ezrin and moesin, which are also known to bind to the same basic amino acid motif in CD44 were absent within this growth inhibitory complex. Alternatively in logarithmically growing cells, merlin was inactive, phosphorylated and in a complex with ezrin and moesin. This growth permissive complex was also associated with the cytoplasmic tail of CD44. My data provides not only significant clues about how merlin functions as a tumor suppressor but revealed the existence of a novel molecular switch that, under the influence of ligands in the microenvironment, controls a cells decision to proliferate or growth arrest.

Das *NF2* Tumorsuppressor Genprodukt Merlin vermittelt durch Interaktion mit CD44 die Kontaktinhibition des Wachstums

ZUSAMMENFASSUNG

Das NF-2 Gen (Neurofibromatose-Typ 2) kodiert Merlin, ein Protein, das mit den ERM Proteinen (für Ezrin, Radixin, Moesin) verwandt ist. Merlin wirkt als Tumorsuppressor. In meiner Doktorarbeit entdeckte ich eine entscheidende Funktion von Merlin für die Hemmung des Wachstums von Zellen durch Kontaktinhibition. Wachsen Zellen in Gewebekultur bei hoher Zelldichte, dann wird Merlin aktiviert und hemmt die Zellproliferation durch Veränderung entsprechender Zellzyklusparameter. Für seine Funktion muß aktiviertes Merlin an das Transmembranprotein CD44 binden. Merlin interferiert mit signaltransduktionsabhängiger Aktivierung von MAP-Kinasen auf einer Stufe zwischen Ras/Raf und MEK. Die Aktivierung von Merlin und sein Phosphorylierungsstatus werden von Liganden, die extrazellulär an CD44 binden, gesteuert. Bei hoher Zelldichte wird Merlin unter dem Einfluß der Bindung von Hyaluronat dephosphoryliert und bindet direkt an ein basisches Aminosäuremotiv im zytoplasmatischen Teil des CD44 Proteins. Ezrin und Moesin, die ebenfalls an das gleiche Motiv in CD44 binden können, sind in dem Komplex, der zur Hemmung des Zellwachstums führt, nicht vorhanden. In logarithmisch wachsenden Zellen ist Merlin inaktiv, phosphoryliert und assoziiert mit Ezrin und Moesin. Dieser Komplex in proliferierenden Zellen ist ebenfalls mit dem zytoplasmatischen Teil von CD44 assoziiert. Diese Daten erklären nicht nur, wie Merlin als Tumorsuppressor wirkt, sondern beschreiben einen neuen molekularen Schalter, der unter dem Einfluß von extrazellulären Komponenten die Entscheidung der Zelle für Proliferation oder Stopp ihres Wachstums regelt.

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Abbreviations

AKAP	A-kinase anchoring protein
APC	adenomatous polyposis coli
APS	ammonium persulfate
BSA	bovine serum albumin
^{0}C	degrees celsius
CAMKII	calcium/calmodulin dependent protein kinase II
CD	cluster of differentiation
CD44s	CD44 standard
CD44v	CD44 variant
CFTR	cystic fibrosis transmembrane conductance regulator
CI	contactinhibin
cm	centimeter (10^{-2} meter)
DAG	diacylglycerol
DOX	doxycycline
DMEM	dulbecco´s modified eagles medium
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
DTT	dithiothreitol
E3KARP	Na^{+}/H^{+} exchanger type III kinase A regulatory protein
EBP50	ezrin binding phosphoprotein of 50 kDa
ECL	enhanced chemiluminescence
ECM	extracellular matrix
EDTA	ethylendiamine-N,N-tetracetate
EGF	epidermal growth factor
ENU	N-ethyl-N-nitrosourea
ERM	ezrin/radixin/moesin
ERMAD	ezrin/radixin/moesin association domain
FACS	fluorescence-activated cell sorting
FAK	focal adhesion kinase
FCS	foetal calf serum
FERM domain	4.1, ezrin, radixin, moesin-like domain
FGF	fibroblast growth factor

FITC	fluorescein isothiocyanate
g	gram
G418	geneticin
GAP	GTPase-activating protein
GDI	guanine-nucleotide dissociation inhibitor
GDP	guanosine diphosphate
GEF	guanine-nucleotide exchange factor
GEM	glycolipid-enriched microdomain
GFP	green fluorescent protein
GST	glutathione S-transferase
GTP	guanosine triphosphate
HA	hyaluronate
HEV	high endothelial venule
HGF/SF	hepatocyte growth factor/scatter factor
hr	hour
HRP	horseradish peroxidase
HS	heparan sulphate
ICAM	intercellular adhesion molecule
Ig	immunoglobulin
IL	interleukin
kDa	kilodalton
LFA-1	lymphocyte function associated antigen type I
LPA	lysophosphatidic acid
m	milli
M	molar
mAb	monoclonal antibody
MDCK	Madin Darby canine kidney
merlin	moesin, ezrin, radixin-like protein
mg	milligram (10^{-3} gram)
min	minute
ml	milliltre (10^{-3} l)
MMP	matix metalloproteinases
NF2	neurofibromatosis type II
NHE-RF	Na^+/H^+ exchanger regulatory factor

NHE3	Na^+/H^+ exchanger type III
NRSB	non reducing sample buffer
OD	optical density
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PDGF	platelet-derived growth factor
PI	phosphoinositide
PI3K	phosphatidylinositol 3-kinase
PI4P5K	phosphatidyl 4-phosphate 5-kinase
PIP_2	phosphatidylinositol 4,5-bisphosphate
PKA	cAMP-dependent protein kinase A
PKC	protein kinase C
PKN	protein kinase N
PLC	phosphatidylinositide specific phospholipase C
PMA	phorbol 12-myristate 13-acetate
PMSF	phenylethylsulphonyl fluoride
PTP	protein tyrosine phosphatase
RHAMM	receptor for hyaluronate-mediated motility
ROCK	Rho-associated kinase
SDS	sodium dodecyl sulphate
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
sec	second
SMC	smooth muscle cells
TEMED	N,N,N´,N´-tetramethylethylenediamine
TGFß	transforming growth factor-beta
TNF	tumor necrosis factor
TRIS	tris-(hydoxymethyl)-aminomethane
UDP	uridine diphosphate
UV	ultra violet

INTRODUCTION

Growth control and contact inhibition

Regulation of cell proliferation is a key event in the establishment and maintenance of a multicellular organism. The coupling between physical adhesion and developmental signalling provides a mechanism to tightly regulate tissue morphogenesis, a coordination that is essential to achieve the intricate patterns of cells in tissue. In the context of organized tissue, maintaining structural integrity, cells fill the space allotted to them and then stop proliferating when touching each other or dense extracellular matrix (ECM) (Holley, 1975). This process, referred to as “contact inhibition” of growth, was first described more than 25 years ago (Hakomori and Siddiqui, 1974; Rubin and Everhart, 1973; Weber and Rubin, 1971). The mechanisms, however, by which a cell senses when it should stop growing during contact are still poorly understood (Fagotto and Gumbiner, 1996). It is plausible that cells have a number of cell surface receptors that signal growth arrest, and inhibit the proliferation program of gene expression. Alternatively, as cells proliferate, such sensors should be in a mode that is permissive for growth. Moreover, these growth promoting or growth inhibiting modes must be dynamic since an appropriate switch from quiescence to proliferation can be initiated in response to physiological injury and inappropriately during tumorigenesis. Identifying these putative contact sensing complexes and understanding their function would not only further understanding of the malignant process but also aid in developing important targets for therapeutic intervention.

Previously the phenomenon of contact inhibition of growth was thought only to be a consequence of cell-cell contact. Earlier experiments demonstrated that plasma membrane preparations when added to sparse growing cultures, mimicked contact inhibition (Nakamura *et al*., 1983; Natraj and Datta, 1978; Peterson and Lerch, 1983; Whittenberger and Glaser, 1977; Wieser and Oesch, 1986). Moreoover cell-cell contacts via specific plasma membrane glycoproteins whose glycan moieties interact with specific receptors were later thought to be the main growth regulatory principle (Wieser *et al*., 1990). Cell-cell interactions have demonstrated the importance of E-cadherin, a homophilic cell-cell adhesion molecule, in contact inhibition of normal epithelial cells (Bracke *et al*., 1997; Kandikonda *et al*., 1996; Levenberg *et al*., 1999; Spiryda and Colman, 1998, St. Croix, 1998 #405; St. Croix *et al*., 1998). In many carcinomas, cadherins are lost or downregulated, resulting in a reduced level of intracellular adhesion (Kandikonda *et al*.,

1996). Little is known about the molecular pathways by which homophilic cell-cell interactions inhibit cell growth but loss of intracellular adhesion obviously endows tumor cells a relative growth advantage over normal contact-inhibited cells.

Models of cell-cell interactions have expanded to other mechanisms including for example expression of soluble proteins and cellular interactions with ECM. The former is a more novel mechanism proposing that at high cell densities, there is an increase in a soluble inhibitor “contactinhibin (CI) that binds to its membrane bound receptor CI receptor inhibiting growth (Gradl *et al*., 1995; Wieser *et al*., 1995). The precise mechanism, however is still unknown. The ECM is made up of different combinations of collagens, elastin, hyaluronate, proteoglycans and various glycoproteins such as fibronectin. More evidence is accumulating that these ECM components have not only structural functions but interact with cells directly and much of the control of cell adhesion, migration and differentiation appears to be mediated by these interactions. Moreover, cell adhesion to the ECM appears to play a critical role in growth control. Although much has been learned about the mechanisms by which cell-ECM interactions promote growth (Guan and Chen, 1996; Newby and George, 1996; Simmons *et al*., 1997) evidence for ECM in growth inhibition is limited. One of these examples is ECM binding via integrins, promoting the growth and survival of cells (Bates *et al*., 1995; Boudreau and Jones, 1999; Guan and Chen, 1996; LaFlamme and Auer, 1996). Levels of individual integrins can increase, decrease, or remain unchanged in tumor cells, suggesting that growth promotion by integrins is only part of the story. Indeed, some types of integrins suppress cell growth inhibiting cell cycle progression at late G1 phase (Hazlehurst *et al*., 2000). Whether a cell proliferates or is growth inhibited may depend on the type of integrin expressed suggesting that integrins are “bimodal” in function. Alternatively structurally distinct forms of specific integrin ligands can influence growth. For example, polymerized collagen fibres, specifically regulating integrin signalling, arrested the smooth muscle cells (SMCs) in G1 phase of the cell cycle while monomer collagen supported SMC proliferation (Koyama *et al*., 1996). Taken together, it is plausible that not only differences in integrin expression is decisive in growth control but the structural quality of one particular ligand is important. In addition, how the intracellular signalling pathways downstream of these putative bimodal complexes are organized remains to be investigated. More than likely subtle functional differences in the proteins proximal to the membrane are an immediate target of these extracellular signals.

Other cell surface adhesion molecules that bind to the ECM include the heterogenous family of CD44 proteins. The ECM component addressed by these transmembrane proteins is hyaluronate (HA) an abundant component of the ECM (Aruffo *et al*., 1990). Proliferative functions to CD44 have been suggested, since it is detected more abundently in proliferative tissue. Most notably, members of the CD44 family have been implicated in a number of aspects in tumor progression and metastasis (Foekens *et al*., 1999; Günthert *et al*., 1991; Herrlich *et al*., 1993; Ponta and Herrlich, 1998; Sherman *et al*., 1996). Interestingly tumor formation in vivo was due to the ability of CD44 to interact with its ligand HA (Bartolazzi *et al*., 1994). In addition the transgenic disruption of CD44 expression in keratinocytes caused skin abnormalities, including a build up of HA in the superficial dermis leading to a decreased proliferation ability of the keratinocytes (Kaya *et al*., 1997). However, as with integrins, there are other studies that demonstrate that enhanced binding of HA to CD44 reduced tumorigenicity (Takahashi *et al*., 1995; Tanabe *et al*., 1995). Overall it is apparent that cells binding to HA can be both growth promoting or growth inhibiting, depending on the cell line and probably the type of HA used in the studies. Despite the overwhelming correlation of CD44 involvement in disease progression, fundamental questions on the function of the CD44 proteins and its ability to influence inhibition or proliferation of cells remains unanswered.

In my PhD thesis I have detected a candidate complex that fulfills the predicted requirements for a bimodal contact sensor. I found that tumor suppressor protein merlin plays a critical role in the establishment and maintenance of contact inhibition of growth. At high cell denstiy, merlin is activated and blocks proliferation. Its growth inhibitory function is associated with dephosphorylation and depends on interaction with a specific domain of the cytoplasmic tail of CD44. In logarithmically growing low density cell cultures, however, CD44 assembles complexes of ezrin-radixin-moesin (ERM) proteins associated with phosphorylated merlin. The extracellular CD44 ligand HA could induce rapid merlin dephosphorylation and arrest of proliferation in these low density cultures, thus mimicking contact inhibition of growth as observed at high cell density. My data provide not only significant clues about how merlin functions as a tumor suppressor but revealed the existence of a novel molecular switch that, under the influence of ligands in the microenviroment, controls a cells decision to proliferate or growth arrest. In the following chapters I will introduce the players of this bimodal contact sensor complex.

Introduction to hyaluronate (HA)

The largest component of the ECM is a hydrated polysaccharide consisting of glycosaminoglycans such as HA. HA is a linear polymer made up of repeats of the disaccharide unit D-glucuronic acid and N-acetyl-D-glucosamine and usually has a molecular weight of several million Da. HA is synthesised by many cell types including mesothelial cells (Honda *et al*., 1991) and fibroblasts (Teder *et al*., 1995). HA synthase (HAS) (Prehm, 1989) acts at the cytoplasmic face of the plasma membrane and utilises both sugar substrates UDP-N-acetylglucosamine and UDP-glucuronic acid and Mg^{2+} to synthesise HA. Large HA polymers are simultaneously synthesised and protruded into the extracellular space, thereby avoiding the presence of the high molecular weight molecule within organelles of the cell. This is thought to be especially important due to its high viscosity (Weigel *et al*., 1997). Due to the flexibility of the polysaccharides chain, the polymer is arranged into a helical structure and forms an expanded or random coil in solution (Laurent *et al*., 1996). In addition, the highly negative charge brought about by so many disaccharide units attracts positively charged cations which facilitates the incorporation of water into the structure via osmosis, causing it to be highly hydrated. This high water content provides HA with a high volume to mass ratio enabling it to occupy a large amount of space at low concentrations and to function as a compression resistant gel.

Functions of HA

Due to the large molecular weight, HA functions as a structural and space filling component of the ECM. It is also an abundant component of cartilage where it is non-covalently linked to proteoglycans such as aggrecan and stabilised in aggregates by binding to link protein (Knudson and Knudson, 1993). The high viscosity of HA also enables it to control the diffusion rates of molecules within the matrix and may also act as a filter to exclude large signalling molecules, cytotoxic lymphocytes and viruses from cells with HA containing pericellular matrices. HA is also thought to have a more specialised role as a lubricant in the synovial fluid of the joint and in the vitreous fluid of the eye. In addition to these passive structural functions, HA is thought to play a role in the morphogenesis and differentiation of tissues, for example in the neural crest during embryonic development. HA has also been shown to increase the healing of ear wounds in rats, possibly by increasing epithelial migration (Laurent and Fraser, 1992) and it is often found to be deposited in areas where migration is about to occur. It has been suggested that HA may

open migration paths within tissues and thereby promote locomotion and invasion. In accordance with this idea, HA is often enriched in tumors, which are known to both produce HA and stimulate surrounding cells to produce it (Knudson *et al*., 1984). The HA content of a tumor can also play an important role in cell division as it is produced maximally by dividing cells and in particular may facilitate detachment of cells from the matrix. HA synthesis can be stimulated by several growth factors such as epidermal growth factor (EGF), platelet-derived growth factor (PDGF), transforming growth factor-beta (TGFβ) and cytokines such as TNF and IL-1 (Laurent and Fraser, 1992). The principle receptor, by which cells can bind to HA are the CD44 proteins, which are a large group of transmembrane glycoproteins widely expressed in many cell types and tissues of the body.

The protein structure of CD44

The CD44 proteins having a high degree of cross species conservation are a distinct family that are structurally unrelated to other families of cell surface molecules such as the integrins, cadherins, selectins, syndecans and immunoglobulins (Ig). The human cDNA sequence encoding the 85 kilodalton (kDa) standard form of CD44 (CD44s) was cloned independently by two groups from lymphoid cell lines (Goldstein *et al*., 1989; Stamenkovic *et al*., 1989). The sequence published by Stamenkovic and colleagues encoded a protein with a 19 amino acid signal peptide, cleavage of which yielded a putative type-I transmembrane protein of 341 amino acids with a predicted molecular weight of 37 kDa. The mature protein consisted of a 248 amino acid extracellular domain, a 21 amino acid hydrophobic (putative transmembrane) domain and a 72 amino acid cytoplasmic domain. The disparity between the predicted and the observed molecular weights is more than likely due to extensive addition of both N- and O-linked sugars and differential folding of the protein.

(Stamenkovic *et al*., 1991) et al identified multiple isoforms of CD44. For example, a cDNA from the HT29 human colon carcinoma cell line was identified which expressed a 160 kDa isoform. The cDNA sequence revealed that it was identical to CD44s, except for deletion of three amino acids (Ala221-Arg223) in the extracellular domain, a concurrent insertion at this position of a 135 amino acid sequence and a Glu/Gly substitution at residue 371. This epithelial isoform (CD44E) was also identified in human neonatal foreskin keratinocytes. QH56 squamous carcinoma cells (Brown *et al*., 1991) and from a human myelomonocytic

cell line (KG1a) (Dougherty *et al*., 1991). Many other CD44 isoforms containing different amino acid sequences inserted at the same position have subsequently been identified.

Gene structure of CD44

The multiple CD44 isoforms are encoded by a single gene which spans 50-60kb (Screaton *et al*., 1992) and is located on the short arm of chromosome 11 in the human (Goodfellow *et al*., 1982) and chromosome 2 in the mouse (Colombatti *et al*., 1982). Both the mouse and human CD44 genes are composed of 20 exons and the various isoforms, such as CD44E, are brought about by alternative splicing of these exons (Screaton *et al*., 1992). The extracellular domain of CD44s is encoded by exon 1-5 and 15-18 the transmembrane domain is encoded by exon 18, and exon 19 and 20 encode the cytoplasmic domain. The variant isoform contain this core structure with the additional inclusion between 5 and 15 of one or more of ten variant exons, exon 5a-14 which are commonly denoted variant exons 1-10 (v1-v10). As both the standard and variant isoforms usually contain exons 1-5 and 15-18 these regions are sometimes referred to as the 5' and 3' constant regions respectively. At least 20 different CD44 transcripts have been identified, for example the previously described CD44E (CD44v8-v10) (Stamenkovic *et al*., 1991) and CD44v3-v10 a 230 kDa isoform which is expressed in kerotinocytes (Hofmann *et al*., 1991). In general exons v3-v10 are the main variant exons used in CD44 splice isoforms reviewed by (Lesley *et al*., 1993) and CD44 isforms containing v1 are probably not expressed in human tissues as the human v1 exon encodes a stop codon (Screaton *et al*., 1993). Individual cells are able to express one or a combination of CD44 isoforms (Brown *et al*., 1991; He *et al*., 1992). There are only a few known exceptions to the pattern of splicing detailed above. One such exception, is CD44v4-v7, the pMeta-1 isoform, which lacks exon 15 in the 3' constant region This isoform was identified due to its ability to confer metastatic potential when expressed in a rat carcinoma cell line (Günthert *et al*., 1991).

CD44 extracellular domain is heavily modified by glycosylation

In addition to the heterogeniety of CD44 created by alternative splicing, further diversity is achieved by the extensive post-translational addition of sugar moeties. The CD44s isoform contains consensus sites for three types of sugar modification. 1) six consensus sites for N-linked glycosylation of the sequence Asn-X-Ser/Thr (where X is any amino acid

except proline) five of which are located in the link protein homolgy domain 2) a region containing a high proportion of clustered serine and threonine residues in combination with acidic residues and prolines in the membrane proximal domain which is indicative of O-linked glycosylation and 3) four Ser-Gly consensus sites for glycosaminoglycan (GAG) addition.

Although multiple GAG attachment sites are present, the only one utilized in the constant regions appears to be in exon 5 which is modified predominantly by chrondrotin sulphate (CS) (Goodfellow *et al*., 1982; Greenfield *et al*., 1999). CD44s usually contains some GAG modification, the amount of which can be dependent on the cell type. Multiple sites for N- and O-linked glycosylation and GAG attachment are present in the variant exons, for expample CD44E (CD44v8-v10) can be additionally modified by both N- and O- linked glycosylation (Brown *et al*., 1991). The v3 exon also contains a Ser-Gly-Ser-Gly GAG attachment site which enables isoforms containing this exon to be modified by heparan sulphate (HS) as well a CS (Bennett *et al*., 1995). The same CD44 isoform can also be differentially modified depending on the cell type in which it is expressed (Stamenkovic *et al*., 1991).

Post-translational modification of the CD44 cytoplasmic and transmembrane domains

The sequence of the CD44 transmembrane and cytoplasmic domains is highly conserved between species. CD44 is subject to constitutive phosphorylation on serine residues in a number of cell lines (Camp *et al*., 1991; Carter and Wayner, 1988; Isacke *et al*., 1986; Kalomiris and Bourguignon, 1988). Of the 7 serine residues in the human CD44 cytoplasmic domain, 4 (Ser291, Ser316, Ser323 and Ser325) are completely conserved between species and another Ser337 is subject only to a conservative amino acid change to a threonine residue in the rat sequence. Mutation of these residues to threonines either singly or in combination, has however, demonstrated that Ser325 is the major site of phosphorylation and the kinase acting at this site is serine specific (Peck and Isacke, 1998). Phosphorylation at Ser325 is known to be important for the ability of CD44 to mediate cell migration on HA (Peck and Isacke, 1996). There is also evidence that Ser291 can be phosphorylated by protein kinase C (PKC). This residue is situated proximal to a number of basic residues which form a putative PKC consensus sequence (Pearson and

Kemp, 1991) and can be phosphorylated by PKC in vitro (Kalomiris and Bourguignon, 1989).

The CD44 transmembrane domain is subject to post-translational modification by palmitoylation (Bourguignon *et al*., 1991; Guo *et al*., 1994). Such lipid modification of cell surface receptors may regulate protein:protein interactions and protein:lipid interactions and has been suggested to mediate CD44 signal transduction (Guo *et al*., 1994) and enhance CD44 association with the cytoskeleton (Bourguignon *et al*., 1991). The transmembrane domain can also facilitate receptor dimerisation via cysteine interactions which are mediated through Cys286 in the human CD44 sequence and have been reported to influence HA binding and signalling (Liu and Sy, 1997).

CD44 is a major cell surface receptor for HA

The N-terminal 100 amino acids of CD44 extracellular domain has a shared sequence homology of approximately 30% with both the chicken and rat cartilage link proteins and the rat proteoglycan core protein (Deak *et al*., 1986; Doege *et al*., 1986; Neame *et al*., 1987). Typically link protein contains a proteoglycan tandem repeat motif or link module consisting of three disulphide bonded loops. CD44 contains a single region which has homology to two of the link domains. This homologous region of CD44 contains 6 cysteine residues, of which 5 are conserved in all CD44 species. These residues are thought to enable CD44 to form a disulphide bonded loop structure with the sequence between the middle cysteine residues, Cys77-Cys97, forming the apex of the loop. Link module consensus sequences are found not only in link protein but also in a number of other proteins including the proteoglycan core proteins versican, aggregan, neurocan and brevican, the tumor necrosis factor-stimulated gene 6 (TSG-6) (Lee *et al*., 1992), and the lymph-specific HA receptor LYVE-1 (Banerji *et al*., 1999). CD44 binds to HA via the link domain and these characteristics of a HA binding pocket are similar to those of the carbohydrate binding interactions described for E-selectin (Kohda *et al*., 1996).

It is now well established that CD44 is a major cell surface receptor for HA. The residues within CD44 extracellular domain which mediate HA binding have now been elucidated. Primarily the N-terminal homologous link protein domain is sufficient for HA binding (He *et al*., 1992). However further in vitro mutational analysis indicated that the membrane proximal domain can also contribute to binding (Peach *et al*., 1993). Overall residues, in the link protein homology domain (Glu21-Ser45 and especially Arg41) and residues in a

cluster of basic amino acids situated in the membrane proximal domain (Thr144-Asp167) were found to be important for binding. A HA binding motif has been proposed, comprising of a B(X_7)B motif where B represents a basic amino acid (Arg or Lys) and X_7 a stretch of any seven amino acids including one basic residue and no acidic residues (Yang *et al*., 1994). CD44 therefore appears to mediate interactions with HA using a combination of the disulphide bonded link homolgy and regions of positively charged amino acids (Yang *et al*., 1994).

Regulation of HA binding

Expression of CD44 by a cell does not always confer HA binding ability. Three HA binding states of CD44 have been described as follows reviewed by (Lesley *et al*., 1992) 1) constitutive binding: the presence of CD44 on the cell surface correlates directly to HA binding ability, 2) inducible binding: although the cells express CD44 they cannot bind HA constitutively but can be induced to do so with various anti-CD44 mAbs or by reagents to cause activation of cells such as PMA, 3) non-binding: in some cells the presence of high levels of CD44 can not enable HA binding and binding cannot be induced with mAbs or stimulatory agents. The regulation of HA binding is complex and can occur by a number of different mechanisms, for example, inclusion of variant exons, glycosylation, CS addition, shedding of the extracellular domain, regulation by the cytoplasmic domain and regulation by the transmembrane domain.

Inclusion of variant exons

CD44 splice isoforms are constitutively expressed in a highly tissue specific manner. Transient increases in expression of CD44 isoforms have also been documented in a number of instances such as in tumor cells and on activation of a number of cell types such as lymphocytes (Arch *et al*., 1992). It is clear that although the variant isoforms all contain the HA binding sites and mediate HA binding via these sites they often show differential HA binding abilities. For example isoforms containing the v3 exon do not bind HA (Bartolazzi *et al*., 1995), the CD44E isoform (CD44v8-v10) is also unable to bind or binds poorly to HA (Bennett *et al*., 1995; Stamenkovic *et al*., 1991) and the CD44v4-v7 isoform has been reported to bind HA with a higher affinity than CD44s (Sleeman *et al*., 1996; Sleeman *et al*., 1996). Many of the isoforms contain additional N- and O-glycosylation sites which can modify HA binding (Bennett *et al*., 1995). Additionally, as

CD44 antibodies raised against epitopes outside the HA binding sites can affect HA binding by affecting the protein conformation, it is possible that inclusion of variant exons may also change function in this way.

Glycosylation

Glycosylation of CD44 is known to regulate HA binding. Inhibition of N-linked glycosylation has been shown to inhibit (Bartolazzi *et al*., 1996) or enhance HA binding (Esford *et al*., 1998; Lesley *et al*., 1995). Inhibition of O-linked glycosylation can either enhance (Bennett *et al*., 1995) or have no effect on HA binding (Lesley *et al*., 1995). Decreases in glycosylation converts inducible binding forms to constitutive binding forms or non binding forms to inducible binding forms (Esford *et al*., 1998; Lesley *et al*., 1995).

CS addition

Effects on HA binding have also been observed with differences in CS addition. Inhibition of CS synthesis or removal of CS using chondroitinase ABC has been shown to convert non HA binding forms of CD44 to inducible binding forms (Lesley *et al*., 1995). Esford and colleagues have also demonstrated using a cell line deficient in CS synthesis that CS is required for HA binding (Esford *et al*., 1998) Addition of CS also confers the ability to bind alternative ligands to HA.

Shedding of the extracellular domain

One mechanism by which CD44 can be down regulated is via receptor shedding. This occurs via proteolytic cleavage of the extracellular domain close to the membrane. (Bazil and Strominger, 1994; Campanero *et al*., 1991; Katoh *et al*., 1994). Soluble CD44 is known to be upregulated in some disease situations. For example in certain immune diseases and during tumor growth (Katoh *et al*., 1994) and CD44 is shed by PMA-differentiated macrophages on phagocytosis of certain mineral particles (Trabelsi *et al*., 1998) or on stimulation of neutrophils with TNF-α or PMA (Campanero *et al*., 1991) or from glioma cells by TPA or ionomycin (Okamoto *et al*., 1999). The protease(s) responsible for cleavage of CD44 have not been identified, although some studies have implicated a metalloproteinase as cleavage could be inhibited with specific inhibitors of these enzymes (Okamoto *et al*., 1999). It is possible that changes in the conformation of the extracellular domain are required for activation of these proteases as isoforms

containing the v3 exon are inhibited in their ability to shed (Bartolazzi *et al*., 1995). In addition, a mutation which induced increased N-linked glycosylation at Asn255 in the membrane proximal domain lead to an increase in spontaneous shedding (Bartolazzi *et al*., 1996). Shed CD44 is believed to function as a competitor to cell surface CD44 HA binding (Bartolazzi *et al*., 1995; Katoh *et al*., 1994).

Regulation by the cytoplasmic domain

The cytoplasmic domain also regulates HA binding as CD44 receptors lacking the cytoplasmic domain (tailless CD44) bind soluble HA poorly in AKR1 cells (Lesley *et al*., 1992) and Jurkat T cells (Liao *et al*., 1993). The addition of the IRAWB14 mAb, which induces HA binding in cell types such as T lymphoma cells still induced the binding of tailless CD44 (Lesley *et al*., 1992). The effect of the IRAWB14 antibody was subsequently found to be dependent on its ability to form multivalent complexes as monovalent Fab fragments could not mediate the induced binding (Lesley *et al*., 1993). Cells expressing tailless CD44 can however bind to HA substrata, although not as well as the full length receptor (Lesley *et al*., 1992). The mechanism by which the CD44 cytoplasmic domain mediates efficient HA binding is not fully understood. Lokeshwar and colleagues demonstrated that the ankyrin binding domain sequence was required for this effect (Lokeshwar *et al*., 1994), but in a similar study deletion of this region was found to have no effect on HA binding (Perschl *et al*., 1995). The cytoplasmic domain is not thought to be required for suppression of HA binding in inducible HA binding cell lines as removal of the cytoplasmic domain or replacement of the CD44 cytoplasmic domain with other sequences does not induce constitutive binding (Lesley *et al*., 2000).

Regulation by the transmembrane domain

The transmembrane domain can facilitate receptor dimerisation via cysteine interactions mediated by Cys286 in the human CD44 sequence (Liu and Sy, 1996; Liu and Sy, 1997). Mutation of this cysteine residue prevented soluble fluorescinated-HA binding in CD44 transfected Jurkat cells in response to activating CD44 mAb or PMA, suggesting that dimerisation via the transmembrane domain is required for inducible HA binding (Liu and Sy, 1996). Receptor clustering can be induced by antibody or PMA but is insufficient to induce binding until covalent dimerisation occurs, as a CD44 receptor in which Cys286 is mutated to an alanine residue can still be clustered by these reagents but not induced to

bind HA. Additionally, the requirement for the cytoplasmic domain could also be overcome by substitution of the CD44 transmembrane domain with the transmembrane domain of CD3ζ chain (Perschl *et al*., 1995). Suggesting that the cytoplasmic domain may in some way be required to change the distribution or conformation of the receptor, possibly by receptor clustering, and that the same effect can be mediated by binding of the mAb IRAWB14, or by immobilised HA. Indeed, induction of clustering via the cytoplasmic domain using cytoplasmic domain chimeric molecules increased the constitutive HA binding, especially at low levels of CD44 expression, similar to that observed using the CD3ζ chain transmembrane chimeras (Lesley *et al*., 2000). Cytoplasmic domain mediated clustering may then enable dimerisation between transmembrane domain cysteines which is required for induced binding. As the CD3ζ chimeras strongly dimerise constitutively, without clustering, this may explain why the cytoplasmic domain can be removed from these chimeras without affecting HA binding. Clustering via the transmembrane domain or cytoplasmic domain is not able, however, to confer constitutive binding in an inducible HA binding cell line (Lesley *et al*., 2000).

Other ligands of the CD44 extracellular domain

In addition to HA, CD44 can also bind other molecules via interactions with its extracellular domain. Many of these interactions, however, are less well characterised than for HA. Other GAGs such as chrondriotin-4 sulphate and chrondriotin-6 sulphate (CS) are able to compete with CD44s HA interactions (Aruffo *et al*., 1990). This binding to non-HA GAGs was dependent on the presence of both exons v6 and v7 and was mediated by the HA binding site in the N-terminal constant region of CD44 as mutation of the critical residue Arg41 but not a double mutation in the membrane proximal HA binding site (mutation of residues Lys 162 and Arg 166) ablated binding to soluble CS (Sleeman *et al*., 1997). CD44 also binds to mucosal adressin (Picker *et al*., 1989), collagen type I (Faassen *et al*., 1992), fibronectin (Jalkanen and Jalkanen, 1992), MIP-1β (Tanaka *et al*., 1993), the CS form of invariant chain (Naujokas *et al*., 1993), serglycin, a heavily CS modified glycoprotein secreted by a cytotoxic T cell line (Toyama-Sorimachi *et al*., 1995), and osteopontin (Weber *et al*., 1996). Some of these interactions are thought to be mediated by binding to sugar moieties on CD44, especially CS, and therefore isoform or cell type specific. Although CD44 can bind to collagen it cannot mediate adhesion to collagen,

however CS modification of CD44 is required for melanoma cell invasion into collagen gels (Faasen *et al*., 1992).

Several CD44 splice variants are able to bind heparin binding growth factors. This binding is dependent on the inclusion of the CD44 v3 exon which is modified by HS and cannot be mediated by isoforms modified with CS. Growth factors such as basic fibroblast growth factor (FGF), and heparin binding-epidermal growth factor (HB-EGF) (Jackson *et al*., 1995), hepatocyte growth factor/scatter factor (HGF/SF) (van der Voort *et al*., 1999), FGF-4 and FGF-8 (Sherman *et al*., 1998) bind CD44. A role for v3 exon containing isoforms of CD44 in presentation of FGF-8 during limb development has been proposed in which presentation but not binding of FGF-8 could be blocked by antibodies specific for epitopes in both v3 and v6 exon encoded sequences (Sherman *et al*., 1998). Additionally, these isoforms are expressed on activated macrophages where they can bind and present growth factors. Expression of v3 exon containing CD44 isoforms were found to be upregulated in tissue sections from rheumatoid arthritis patients suggesting they may be involved in regulating the inflammatory response (Jones *et al*., 2000).

Signal transduction via CD44

The role of many transmembrane receptors is to transduce extracellular signals from the outside to the inside of the cell. These signals typically involve cell-cell adhesion, adhesion to ECM or binding of growth factors to transmembrane receptors which transmit signals via phosphorylation events in their cytoplasmic domain. A good example of CD44 involvement in signal transduction is in lymphocytes. Although the physiological ligand is not known CD44 antibodies can trigger a signal transducing pathway similar to one activated by T-cell receptor/CD3-complex in T-cells. Moreover the transmembrane domain of CD44 may mediate CD44 signalling events via membrane association with signalling molecules. In particular the tyrosine kinases p56lck (Lck) (Taher *et al*., 1996) and Fyn can be co-immunoprecipitated with CD44 from T lymphocytes. Lck is anchored to the cytoplasmic face of the plasma membrane by lipid modifications (palmitoylation and myristoylation) and is known to interact with other transmembrane receptors such as CD4. CD44 has been demonstrated to partition to the same membrane fraction as Lck and Fyn (Ilangumaran *et al*., 1998), and stimulation with various CD44 mAbs causes upregulation of tyrosine

phosphorylation of these molecules and phosphorylation of the Lck substrate ZAP-70 in human peripheral B lymphocytes (Ilangumaran *et al*., 1998; Taher *et al*., 1996).

In addition CD44 can modulate integrin function. For example binding of some anti-CD44 mAbs to T-cells causes increased integrin mediated cell:cell and cell:substrate adhesion. Addition of anti-CD44 mAbs to T-lymphocytes caused LFA-1 integrin ($\alpha_1\beta_2$) mediated homotypic (Koopman *et al*., 1990) and heterotypic (Bruynzeel *et al*., 1993) cell:cell adhesion. Similar effects on LFA-1 integrin activation have also been observed in colorectal cancer cells where HA fragments or crosslinking of CD44 using an anti-CD44 mAb caused increased activation and upregulation of LFA-1 and also upregulation of cMet, the HGF/SF receptor (Fujisaki *et al*., 1999). Similarly, CD44 has been implicated in the induction of VLA-4 integrin ($\alpha_4\beta_1$) mediated from adhesion of activated T-cells to endothelial cells (Brocke *et al*., 1999; Siegelman *et al*., 2000). Although the mechanism by which CD44 induced integrin activation is mediated is unknown, several reports have demonstrated that it is inhibited by cytochalasin B which prevents formation of microfilaments.

Recently, HA binding by CD44 has been demonstrated to induce Rac activation (Oliferenko *et al*., 2000). Addition of HA to EpH4 mammary epithelial cells induced the formation of lamellipodia which was inhibited by an anti-CD44 mAb or micro-injection of a dominant-negative mutant of Rac. Furthermore, the mechanism via Rac activation is mediated has been proposed to be via recruitment of Tiam1, a Rac specific guanine exchange factor (GEF) (Bourguignon *et al*., 2000). A direct association between CD44 cytoplasmic domain and Tiam1 has been demonstrated and Tiam1 from cells stimulated with HA had an increased ability to activate Rac in vitro. Although phosphorylation of the CD44 cytoplasmic domain on serine residues is likely to facilitate CD44 signal transduction, at the present time the physiological stimuli and downstream components regulating these have not been determined.

The CD44 cytoplasmic domain can interact with the cytoskeleton

Some cell adhesion molecules which interact with ECM components can often associate with the cytoskeleton via their cytoplasmic domains. Early biochemical studies of CD44 suggested that it could form associations with cytoskeleton. After detergent extraction of cells, CD44 was observed to be co-distributed with cytoskeletal components (Carter and

Wayner, 1988). Furthermore, the amount of NP-40 insoluble material was reduced in thioglycollate-elicited macrophages compared to resident macrophages and the dephosphorylated form of CD44 was preferentially associated with the NP-40 insoluble fraction (Camp *et al*., 1991). As no actin binding sites have been identified within the CD44 cytoplasmic domain the association of CD44 with the cytoskeleton is unlikely to be direct. Rather, it is likely that CD44 associates indirectly with the cytoskeleton via interaction(s) with cytoskeletally associated proteins. One such protein is ankyrin and an ankyrin binding site within the CD44 cytoplasmic domain has been mapped (Lokeshwar *et al*., 1994). In addtion Tsukita suggested that CD44 could associate with an alternative family of cytoskeletal components, the ezrin, radixin, moesin (ERM) family of proteins (Tsukita *et al*., 1994). These proteins were proposed to act as adaptor molecules linking actin to transmembrane receptors and are therefore likely candidates to perform this role.

Introduction to merlin

Identification and cloning of merlin

Neurofibromatosis 2 (NF2) is an inherited disorder which, despite its name, is not characterised by the formation of neurofibromas, like the more frequent neurofibromatosis 1 or von Recklinghausen disease (Gutmann *et al*., 1997). Instead, NF2 involves a pedisposition to the formation of schwannomas and meningiomas, tumors that endanger the patient not by progression to malignancy but rather by compression of nervous system structures. The disorder affects 1 in 40000 persons, displaying autosomal transmission and causes severe morbidity, shortening the average lifespan to less than 40 years. The characteristic tumor found in NF2 patients is the vestibular schwannoma, a benign, slow growing tumor on the vestibular branch of the acoustic nerve that can cause loss of hearing and balance. Vestibular schwannomas may also occur as single isolated tumors in the general population, but in NF2 patients they usually occur bilaterally. This feature, along with the occurrence in the same patients of multiple other tumors such as spinal schwannomas, meningiomas and occasional ependyomas first suggested that NF2 might result from loss of a tumor suppressor gene. Two independent groups using both loss of heterozygosity in tumors and a recombination analysis in NF2 families to focus on the disease locus, resulted in the identification of the *NF2* gene located on chromosome 22 (Rouleau *et al*., 1993; Rouleau *et al*., 1987; Trofatter *et al*., 1993). Transmission of a

mutant gene through the germline of NF2 patients predisposes to tumor formation, which is initiated by somatic mutation of the remaining wild-type copy of the gene (Rouleau *et al*., 1990; Rouleau *et al*., 1987). Mutational analysis of both germline and somatic alterations in the *NF2* gene has supported the tumor suppressor model, revealing a wide variety of inactivating mutations, the vast majority of which are predicted to truncate the protein. Merlin inactivation appears to be involved in most if not all sporadic vestibular schwannomas and in about half of sporadic meningiomas. Inactivating mutations of the *NF2* gene are also frequent in malignant mesothelioma (Bianchi *et al*., 1995; Deguen *et al*., 1998; Sekido *et al*., 1995).

The *NF2* gene encodes a protein which has particular high similarity with three proteins, moesin, ezrin and radixin (ERMs) members of the protein 4.1 superfamily. These proteins including protein 4.1 are implicated as linkers between integral membrane proteins and the actin cytoskeleton. Consequently, it was named merlin for moesin, ezrin, radixin, like protein (Trofatter *et al*., 1993). Merlin shares 46% sequence identity to ezrin and is most homologous in its N-terminal domain. Since the identification of the ERM proteins and merlin, a number of other proteins have been identified which contain regions which are homologous to the N-terminal of band 4.1. The high degree of homology in this region and the role of this domain in mediating interactions with components of the plasma membrane prompted the characterisation of this domain as the 4.1 protein, ezrin, radixin, moesin (FERM) domain (Chishti *et al*., 1998). Merlin is encoded by 17 exons with alternative splicing of the penultimate exon producing two major isoforms. Isoform 1, encoded by exons 1-15 and exon 17 is a 595 amino acid protein. Merlin isoform 1 possesses a FERM domain that precedes a long α-helical stretch which is interrupted by a proline-rich region and is followed by a charged carboxy-terminus. Isoform 2 differs from isoform 1 only at the carboxy-terminus. Insertion of exon 16 into the mRNA provides a new stop codon, resulting in merlin isoform 2, a 590 amino acid protein that is identical to isoform 1 over the first 579 residues (Bianchi *et al*., 1994).

Merlin and growth suppression

The tumor suppressor action of merlin has been well documented. Both overexpression studies and reduction of merlin expression using antisense and knockout mouse technologies have been used. In the RT4-D6P2T and JS1 rat schwannoma cell lines as well as fibroblast cell lines overexpression of wild type merlin inhibits growth (Bianchi *et al*.,

1994; Lutchman and Rouleau, 1995; Sherman *et al*., 1997; Tikoo *et al*., 1994). Further merlin isoform 1 (lacking exon 16) and not isoform 2 is growth inhibiting and both N-terminal and C-terminal domains of wild type merlin are required. (Sherman *et al*., 1997; Tikoo *et al*., 1994). Reduction of merlin expression by antisense technique in a schwannoma cell line (STS26T) resulted in small increases in cell proliferation (Huynh and Pulst, 1996). In Drosophila, somatic mosaic analysis showed that groups of cells lacking merlin function over proliferate relative to neighboring cells (LaJeunesse *et al*., 1998). In addition Schwann cells from human schwannomas proliferated in the absence of added growth factors while normal human Schwann cells did not (Pelton *et al*., 1998; Rosenbaum *et al*., 1998). In every case when merlin levels are diminished, increased cell proliferation is apparent. Targeted homozygous disruption of the murine *Nf2* gene causes embryonic lethality prior to gastrulation (McClatchey *et al*., 1997). Heterozygous *Nf2* +/- mice were found to be cancer prone (McClatchey *et al*., 1998). In the mice, the form of cancer was found to be more severe than in human NF2 patients as the mice were prone to a variety of malignant tumors such as osteosarcomas, but did not develop schwannomas or other tumor types seen in NF2 patients. This observation that other malignant tumors developed identified *Nf2* as an important tumor suppressor gene in many other cell types. The fact that *Nf2* hemizygous mice did not develop schwannomas might be explained by insufficient rate of second allele inactivation in the Schwann cell compartment. The conditional homozygous *Nf2* knockout mice with Cre-mediated excision of *Nf2* exon 2 in Schwann cells showed however, characteristics of neurofibromatosis type-2, including the development of schwannomas (Giovannini *et al*., 2000) establishing a model of the human disease.

Clues about merlins function, lessons learned from related ERM proteins

Merlins structural similarity to the ERM proteins has provided a wealth of information concerning the potential behaviour and activities of this protein. I will begin this section with information concerning ERMs, since these proteins, in some instances, have been better characterised. The following section will then be concerned with comparing merlins activity to the ERMs.

Membrane binding of ERM proteins

ERM proteins are found in a variety of areas of membrane remodelling, such as membrane ruffles, microvilli, filopodia and the cleavage furrow (Tsukita and Yonemura, 1999). The anchorage of the ERM family to the plasma membrane occurs in different ways. Indirect binding of ERM to different proteins with transmembrane domains, such as the cystic fibrosis transmembrane conductase regulator (Short *et al*., 1998) or the Na+/H+ antiporter (Murthy *et al*., 1998), involving an adaptor protein such as EBP 50 (Reczek *et al*., 1997). ERM can also be linked directly to the cytoplasmic tail of transmembrane proteins including CD44, CD43, siaolophorin, intercellular adhesion molecules 1 and 2 (ICAM1 and ICAM2), and syndecan-2 (Heiska *et al*., 1998; Legg and Isacke, 1998; Serrador *et al*., 1998; Yonemura *et al*., 1998). The first reported candidate for a membrane associated binding partner for ERM proteins was CD44 (Legg and Isacke, 1998; Tsukita *et al*., 1994). With purified fusion proteins the interaction was nailed down to the N-terminal half of the ERM protein containing the FERM domain (Hirao *et al*., 1996). In the transmembrane proteins CD44, CD43 and ICAMs the sequence interacting with ERMs is a basic amino acid motif located in the membrane proximal regions of their cytoplasmic domains (Legg and Isacke, 1998; Yonemura *et al*., 1998).

ERM proteins bind to actin

Ezrin fusion proteins bind to purified filamentous actin (F-actin) (Turunen *et al*., 1994). A C-terminal actin binding site in the last 34 residues of ezrin (amino acids 553-586) accounts for this binding. In addition, to this domain, a second actin binding site has been suggested in the N-terminal half of the ERM proteins (Roy *et al*., 1997). However, the physiological relevance of this actin binding site is not clear. The C-terminal actin binding region contains a sequence of basic amino acids which is homologous to regions found in other actin binding proteins, for example the CapZ β subunit. It seems to facilitate interactions with an acidic amino acids in actin. Further the actin binding region contains two threonine residues (Thr567 and Thr576 in ezrin) and one conserved tyrosine (Tyr565) of which Thr567 and Tyr565 lie within the basic motif. Phosphorylation of the threonines, appears to enhance actin binding by changing the conformation of the protein (Huang *et al*., 1999; Simons *et al*., 1998).

ERM proteins and self association

Regulation of ERM linker function is thought to occur through conformational changes (Bretscher *et al*., 1997). The ERM proteins possess two conserved domains, the N-terminal domain is responsible for the membrane targeting, whereas the C-terminal domain contains the F-actin binding site (Turunen *et al*., 1994). These two domains interact strongly with eachother, and have been termed N- and C-ERMADs, standing for ERM association domains (Andreoli *et al*., 1994; Gary and Bretscher, 1995; Gary and Bretscher, 1993). In ezrin, N-ERMAD has been mapped to the first 296 amino acids and C-ERMAD to the last 107 amino acids. As a consequence of the intramolecular N/C-ERMAD interactions, most ERM proteins are in a cytosolic dormant form, in which binding sites for membrane components and F-actin are masked. Intermolecular N/C-ERMAD interactions also form ERM oligomers. In purified placental microvilli, ezrin dimers, trimers, tetramers, and higher order oligmers were identified, suggesting head-to-tail assembly. These oligomers are proposed to be associated with the cytoskeleton and to be involved in microvillar morphogenesis (Berryman *et al*., 1995). Oligomerization is not specific to ezrin, since mixed oligomers containing different ERM members were observed (Gary and Bretscher, 1993). To engage ERMADs in intermolecular interactions, cytosolic dormant monomers are thought to be subjected to a gross conformational change and this does not occur spontaneously, rather ERMADs require an activation step (Bretscher *et al*., 1995).

Phosphorylation of ERM proteins

Phosphorylation has been proposed to regulate ERM activation, since phosphorylation of ERM proteins correlates with their cytoskeletal association (Simons *et al*., 1998). Ezrin is phosphorylated on tyrosine residues upon growth factor stimulation (Crepaldi *et al*., 1997; Gould *et al*., 1989). In response to EGF, ezrin phosphorylation on tyrosine 145 and 353 is concomitant with an increase in dimer formation, suggesting a casual relationship between phosphorylation and oligomerization (Berryman *et al*., 1995; Krieg and Hunter, 1992). However mutations of these tyrosines into phenylalanines does not alter ezrin localization in microvilli, and production of this mutated ezrin does not affect cell morphology (Crepaldi *et al*., 1997). Rather than controlling its cytoskeletal association, tyrosine phosphorylation of ezrin appears to transduce signals. For example, phosphorylation of tyrosine 353 was found to signal cell survival during epithelial differentiation (Gautreau *et al*., 1999). Another phosphorylation site that is a better candidate to activate ERM cytoskeletal linkage is a

phosphothreonine residue. This phosphothreonine residue, originally identified in moesin (Nakamura *et al*., 1995) is localized in a conserved C-terminal region of ERM proteins (T567 in ezrin, T564 in radixin, and T558 in moesin). Using phosphospecific antibodies, this phosphorylated residue was detected in the ERMs from a variety of cells and tissues, and phosphorylated ERM proteins were shown to be present in actin-rich membrane structures (Hishiya *et al*., 1999; Matsui *et al*., 1998; Nakamura *et al*., 1996; Oshiro *et al*., 1998). Two kinases, protein kinase C-theta (PKC-θ) and Rho-kinase, and two phosphataes, myosin phosphatase and type 2C protein phosphatase (PP2C), were found in different systems to regulate the phosphorylation status of this conserved threonine (Fukata *et al*., 1998; Hishiya *et al*., 1999; Matsui *et al*., 1998). The primary consequence of this phosphorylation event is to impair N/C-ERMAD interaction. For example, in an overlay assay, phosphorylation of T564 in radixin C-terminal domain impairs its association with the N-terminal (Matsui *et al*., 1998). Similarly, the T558D mutation of moesin, which mimics the phosphorylated state, was shown to affect the N/C-EMRAD. The phosphorylation of an isolated C-terminal fragment of ERM proteins does not affect its affinity with F-actin interaction (Huang *et al*., 1999). However, in full length ERM proteins, phosphorylation of this conserved threonine is required to bind to F-actin (Huang *et al*., 1999; Matsui *et al*., 1998). These results suggest that phosphorylation of this residue activates ERM cytoskeletal association by unmasking the cryptic F-actin binding site. Futhermore, phosphorylation of this conserved threonine regulates membrane-specific transition from oligomers to monomers, which are the active plasma membrane-actin cytoskeleton linkers (Gautreau *et al*., 2000).

ERM proteins are involved in GTPase mediated cytoskeletal reorganisation

ERM have attracted a great deal of interest because their functions have been shown to be regulated by the small GTPases. The small GTPase Rho, Rac and Cdc42 are key regulators of cytoskeletal organisation (Hall, 1998), and are known to control actin polymerisation and enable the formation of specific actin structures. For example, in fibroblast stimulation of Rho causes the formation of stress fibres and focal adhesions, Rac the formation of lamellipodia, membrane ruffles and focal contacts (Ridley and Hall, 1992) and Cdc42 the formation of filopodia and focal contacts (Nobes and Hall, 1995). These proteins act as switches, controlling this process by virtue of their ability to bind and

hydrolyse guanosine triphosphate (GTP) to guanosine diphosphate (GDP). In the GTP bound form the proteins are active and exert their control on the cytoskeleton via interactions with a number of effector proteins. In the inactive GDP bound form however, these interactions can not occur. In turn their activity is known to be regulated both positively by guanine-nucleotide exchange factors (GEFs) and negatively by guanine-nucleotide dissociation inhibitors (GDIs) and GTPase-activating proteins (GAPs).

Stimulation of GTPases by a number of different techniques such as by addition of GTPγS, a non-hydrolysable form of GTP, lysophosphaditidic acid (LPA) or by micro-injection of constitutively active GTPase mutants causes the activation of ERM proteins. Upon activation of Rho a rapid, transient increase in phosphorylation of ERM proteins at their C-terminal threonine residue is observed (Matsui *et al*., 1998; Oshiro *et al*., 1998; Shaw *et al*., 1998). This causes unfolding and activation of ERM proteins with concurrent redistribution of the proteins from the cytoplasm to sites of the plasma membrane such as microvilli (Hayashi *et al*., 1999; Matsui *et al*., 1998; Oshiro *et al*., 1998; Shaw *et al*., 1998). In addition phosphorylation and microvillus formation could be reconstituted by overexpression of phosphatidyl 4-phosphate 5-kinase type-Iα (PI4P5K) but not by a kinase inactive mutant of PI4P5K (Lys138/Ala138). This kinase is a known effector of Rho (Chong *et al*., 1994) and catalyses the production of PIP_2 from PIP. As PIP_2 itself can bind to an activated ERM protein, this presents the possibility that PIP_2 binding exposes the threonine phosphorylation site, enabling another kinase to phosphorylate it.

It has been suggested that ERM proteins are themselves involved in the activation of GTPases, for example Rho, by interacting with GTPase regulatory proteins (Takahashi *et al*., 1997). Micro-injection of the N-terminal domain of radixin caused the formation of stress fibres but not filopodia or lamellipodia (Takahashi *et al*., 1997). There may be an interaction between the N-terminal domain of ezrin, radixin and moesin with RhoGDI and they can suppress the ability of RhoGDI to inhibit GDP-GTP exchange (Takahashi *et al*., 1997). Furthermore, as well as binding this inhibitory regulator, it was demonstrated that radixin can bind Dbl, a GEF which can activate Rho (Takahashi *et al*., 1998). The association of ERM members with these positive and negative regulators may serve to recruit inactive Rho bound to GDI and promote its activation at the membrane. However, this cannot be the whole story as only the truncated N-terminal domain of ERM proteins

were able to bind these regulators in vitro, suggesting that prior activation of the ERM protein is required. As ERM protein activation is thought to be downstream of Rho it remains to be determined how this may come about and how the system is negatively regulated.

Merlin functions different to ERMs

Studies of merlin deficient systems suggested that merlin disrupts some aspect of intracellular signalling that leads to a signal to proliferate. The fact that none of the ERM proteins has been found to be at the root of a tumor suppressor syndrome like NF2 and that the ERMs are expressed in the absence of merlin in NF2 tumors suggests that the critical signalling pathway affected by merlin may not be identical to those involved with the ERMs. However, the similarity between merlin and the ERMs suggests that some aspects of merlin activity may be closely related to ERM proteins activity.

Alternative C-terminal

In cultured cells, merlin is expressed as a protein that migrates on SDS-PAGE with an apparent size of 70 kDa, close to that predicted by its sequence (Gonzalez-Agosti *et al*., 1996). This is in contrast to the ERMs which all migrate with an apparent molecular mass greater than would be predicted form their actual size. The anomalous migration has been attributed to the structure of the C-terminal half of the ERMs, suggesting that merlin differs from its relatives in properties of this region. This is in agreement with the lack of homology in the C-terminus between merlin and the ERMs. The most apparent diffference in the C-terminal of merlin is the lack of the conserved C-terminal actin binding site. Merlins interaction with actin is proposed to be mediated by a region in the N-terminal half of the protein (Huang *et al*., 1998; Xu *et al*., 1998), however, this binding is still a matter of debate. The N-terminal half of merlin shows the greatest similarity to the ERMs and, by analogy, would be expected to direct the tumor suppressor protein to targets in the membrane. Indeed, like the ERMs, merlin localizes in various cell types to areas of membrane remodelling, particularly membrane ruffles (Gonzalez-Agosti *et al*., 1996). In addition, when merlin is overexpressed, it inhabits additional actin-rich cortical structures such as filopodia and microvilli, co-localizing with ERMs (Shaw *et al*., 1998; Xu *et al*., 1998).

Merlin and self association

Merlins activity seems to be regulated by self association similar to that of ERMs, but the details of activation and the precise residues responsible are not well characterised. Interestingly, the two merlin isoforms appear to differ significantly in their regulation. Isoform 1 behaves like the ERMs, with an N-terminal to C-terminal intramolecular interaction that supports a closed conformation and that must be disrupted to produce the open state (Xu and Gutmann, 1998). Isoform 2, on the other hand, does not display this intramolecular interaction, leaving the protein always in the open state in which it is presumably available for interaction with putative protein partners (Sherman *et al*., 1997).

Merlins phosphorylation state

Merlin is known to be a phophoprotein and two proteins differing slightly in their molecular weight are observed by immunoblotting with anti-merlin mAbs. The bands reflect phoshorylated and non-phosphorylated forms of the proteins (Shaw *et al*., 1998). Merlin level and phosphorylation status is regulated by both cell:cell contact formation and by growth factors. In confluent or serum starved cultures of NIH-3T3 cells, merlin protein levels are increased and an increased abundance of the non-phosphorylated form is observed. This regulation appears to be specific to cells in G0/G1 phase arrest as reagents causing arrest at other stages of the cell cycle do not have this effect. Addition of serum rapidly down regulates merlin expression with the non-phosphorylated form being preferentially downregulated. Phosphoaminoacid analysis demonstrated that this phosphorylation was present solely on serine and threonine residues. Increasing amounts of the non-phosphorylated form of merlin were also observed on adhesion of cells in suspension when replated onto tissue culture dishes but not in the presence of cytochalasin D, an actin cytoskeleton disrupting agent which enable the cells to adhere but not spread.

Merlin interacting partners

To date only a few of the proteins that are likely to interact with merlin have been identified. As would be expected from observed intermolecular interactions between ERMs, merlin is capable of interacting with the ERMs themselves (Gronholm *et al*., 1999; Meng *et al*., 2000). As merlin and the ERMs possess similar FERM domains, it is likely that merlin is capable of binding to the ERM membrane partners such as CD44, or to other membrane

proteins that display a cytoplasmic domain with clustered positively charged residues. However, such binding has not yet been directly demonstrated (Legg and Isacke, 1998; Yonemura *et al*., 1998). Interestingly, studies of the Drosophila homolog of merlin suggest that its growth suppressing properties reside with the N-terminal half of the protein. The N-terminal domain of human merlin has been reported to interact with microtubules (Xu and Gutmann, 1998) and several as yet unidentified proteins that do not interact with the ERMs (Takeshima *et al*., 1994). In the C-terminal region, merlin isoform 2 has been shown to bind to beta II-spectrin, which could act as a connector to cytoskeletal actin in the absence of direct C-terminal merlin-actin binding (Scoles *et al*., 1998). The weaker interaction of isoform 1 with beta II-spectrin might be due to masking of the binding site by merlin self-association. Merlins capacity to interact directly with participants in the signal transduction pathways that mediate cytoskeletal reorganization has yet to be tested.

Merlin, like ERMs, interacts with a protein called ezrin binding phosphoprotein of 50 kDa (EBP50) (Murthy *et al*., 1998). EBP50 is a 358 amino acid protein containing two PDZ domains followed by a 120 amino acid C-terminal tail. The protein is the human homologue of the rabbit Na^+/H^+ exchanger regulatory factor (NHE-RF) (Weinman *et al*., 1995). NHE-RF enable the cAMP-dependent protein kinase A (PKA) dependent regulation of renal Na^+/H^+ ion exchanger (Yun *et al*., 1997). The C-terminal 30 residues of EBP50/hNHE-RF interact with ezrin (Reczek and Bretscher, 1998) enabling the N-terminal PDZ domains to interact with the NHE3 (Yun *et al*., 1997). These interactions are likely to be important in polarised epithelial cells where ERM members are co-localised with these proteins in apical microvilli where they may prevent endocytosis and down regulation of membrane transporters. Thus, NHERF and probably NHERF-2 may link merlin to a number of different ion channels and receptors that provide new possibilites for effects on intracellular signalling.

Aim

The principle aim of this thesis is to study the function of the tumor suppressor protein merlin. Merlin belongs to a family of proteins including ERMs that act as linkers between integral membrane proteins and actin cytoskeleton. ERM proteins are known to bind via their N- terminus to a number of transmembrane proteins, including CD44. Since merlin is most homologous in that region my overall goal is to test whether merlin can also bind to CD44 and whether this binding is important for its growth inhibiting function. For that purpose I propose to make a conditional wild type and mutant merlin expression system in a transformed cell line. The first goal would be to test merlins effect on several growth assays including in vitro colony formation and cell counts in tissue culture, in addition tumor outgrowth in vivo will be tested. If merlin demonstrated to be growth inhibiting my next goal would be to test whether merlin can bind to CD44 and whether this binding in necessary for its function. Since ERMs are modified by extracellular signals I want to test whether merlin is similarly modified. Further if merlins function depends on binding to CD44 my future goal would be to test whether extracellular CD44 ligands can influence merlin activity.

MATERIAL AND METHODS

Materials

Chemicals. All general chemicals were purchased from *Carl Roth GmbH & Co.*, Karlsruhe, *Merck*, Darmstadt or *Sigma Chemie GmbH*, Deisenhofen and were of the highest quality. Radiochemicals were obtained from *Amersham*..

General Methods

A number of protocols and recipies for commonly used buffers used in this project were taken from the laboratory manual of (Maniatis *et al*., 1989) and Current Protocols in Molecular Biology (Ausubel *et al*., 1987) unless otherwise stated.

Phenol/Chloroform extraction of nucleic acid

The removal of unwanted protein contaminants from nucleic acids. An equal volume of Tris-buffered phenol, chloroform and isoamylalcohol (2-propanol) at a ratio of 25:24:1 was added and the mixture vortexed. The two phases were separated by centrifugation at 10000xg for 10 min. The upper aqueous nucleic acid containing phase was transferred to a new reaction tube and subjected to a further round of extraction with chloroform/isoamylalcohol (24:1).

Ethanol (or 2-propanol) precipitation of nucleic acids

In order to recover nucleic acids from solution, the salt concectration was brought to 200 mM with 3 M Na-acetate (pH 4.8-5.0), and 2.5 volumes of ethanol or 1 volume of 2-propanol were added. After 30 min to overnight incubation at -20^{o}C or 15 min at -80^{o}C (only ethanol precipitation). The precipitate was pelleted by centrifugation at 10000xg for 15-20 min. The pellet was washed with 80% ethanol to remove the salt and was then dried.

Determination of nucleic acid concentration

The concentration of nucleic acids was determined by measuring their optical density (OD) at 260 and 280 nm. An OD_{260} = 1 is equivaent to 50 μg/ml double stranded DNA or 40 μg/ml RNA or 20 μg/ml single stranded oligonucleotide. The OD_{280} is used as an indication of the purity of the nucleic acid; it should be approximately 50% of the OD_{260}

Restriction endonuclease digestion of DNA

Usually 2-3 units of a restriction enzyme for each μg DNA were used. DNA was digested at a concentration of 1 μg/10 μl in a buffer recommended by the supplier. The reaction was carried out for 2 hours to overnight at 37^{o}C (unless otherwise recommended by the supplier) and was stopped by a phenol/chloroform extraction. The DNA was precipitated with ethanol. The quality of the digest was controlled by gel electrophoresis.

DNA Ligation

All ligation reactions were performed in a total of 20 μl and incubated overnihgt at 14^{0}C, followed by heat inactivation of the ligase at 70^{0}C for 5 minutes before storing at -20^{0}C.

Sub-cloning

The cloned fragment of DNA was released from the vector using appropriate restriction endonucleases, purified by agarose gel electrophoresis and subsequently cloned into the new vector using compatible sites or through blunt end ligation

Size separation of nucleic acid by agarose gel electrophoresis

The required amount of agarose (SeaKem, Biozym Diagnostik, Hameln, final concentration between 0.8 and 2%) was dissolved in 50 ml electrophoresis buffer (TBE: 90 mM Tris-base, 90 mM boric acid, 2.5 mM EDTA, pH 8.3). Ethidium bromide was added at a concentration of 0.3 μg/ml. The molten gel was pured into a horizontal (13.5x8 cm) chamber. Combs with the appropriate number and size of the teeth were used to make the loading slots. The gel (when set) was with 200 ml electrophoresis buffer and run at 35-45 mA (50-100 V) at room temperature for the required time. Samples were loaded onto the gel in loading buffer (10 mM EDTA, 10% glycerol, 0.1% SDS, 0.02% bromophenol blue). DNA was visualised by transillumination with 302 nm ultraviolet radiation.

Isolation/purification of DNA from agarose gels

A number of methods were employed to isolate DNA from agarose gels once electrophoresis was complete.

Electrophoretic isolation of DNA

Once the DNA has migrated the desired distance, a slit was cut into the gel with a scalpel just below the chosen DNA band. A strip of DE81 DEAE-cellulose membrane (Schleicher & Schuell, Dassel) was inserted into the slit ad electrophoresis continued until the DNA fragment had run into the membrane. At this point the membrane was removed, rinsed briefly with distilled H_2O and the DNA eluted by incubation in 400 µl 1.5 M NaCl, 10 mM Tris-HCL pH 7.5 and 1 mM EDTA for 30 minutes at 65^0C with shaking. After two extractions with phenol/chloroform, the DNA was precipitated with ethanol, air dried and dissolved in an appropriate volume of water.

Direct isolation from agarose gels

The DNA band of choice was cut out from the gel, under long wave UV light with the aid of a scalpel. DNA containing gel strip was placed inside 1.5 ml reaction tube and crushed in an equal volume of T/E buffer. An equal volume of phenol was added and vortexed, the reaction tube was then immersed into liquid nitrogen for 1-2 min and centriguged at high speed for 5-10 min. The upper aqueaous phase was transferred to a new reaction tube and precipitated with ethanol.

DNAeasy kit (Biozyme) DNA isolation from agarose gels

As above with the exception that the gel strip containing the DNA was added to 3x it´s weight to volume of "salt buffer" (all reagents provided in the kit). The gel piece was melted in the buffer by incubation at 65^0C before the binding resin was added. After two subsequent washing steps the resin with bound DNA was air dried and the DNA eluted by addition of bi-dest H_2O.

Preparation of competant bacteria (E.Coli)

Chemical competent E.Coli (calcium chloride method)

A single colony of E.coli DH5α was taken to inoculate 5 ml of LB medium (10g/lt tryptone, 5 g/lt yeast extract, 5 g/lt NaCl) and allowed to grow overnight at 37^0C with shaking (220 rpm). 4 ml was removed and added directly to 400 ml of LB medium. The bacteria were grown to an OD_{590} of 0.375 before incubating the bacteria on ice for 10 min. The

bacteria were sedimented by centrifugation without brake at 3600x g for 7 min at 4^0C and the pellet carefully resuspended in 20 ml of ice cold 0.1 M $CaCl_2$ and allowed to stand on ice for a further 10-15 min. The cells were centrifuged again under the same conditions and resuspended in a further 20 ml of ice cold $CaCl_2$. This process was repeated once more and the final pellet resuspended in 2 ml of ice cold $CaCl_2$ with 10% glycerol. After a short period on ice, the bacteria were dispensed in 100 µl aliquots in pre-chilled reaction tubes and snap-frozen in liquid nitrogen before storing at -80^0C.

Electrocompetent E.Coli

As above, a single colony of E.Coli DH5α was taken to inoculate 1 ml of YENB (7.5g/lt Bacto yeast extract, 8.0g/lt Bacto Nutrient broth) medium and the culture grown overnight at 37^0C with shaking. This is very important as it eliminates all the steps needed to remove the salts. Salts are known to reduce the efficiency of electrotransformation and cause arcing in the electroporation cuvette. 500 ml of fresh YENB medium was inoculated with the 1 ml overnight culture. Grown at 37^0C with shaking, cells were harvested between an OD_{600} of 0.5 to 0.9. To harvest cells, a flask was chilled on ice and spun at 4000x g for 10 min at 4^0C. Medium was discarded and the pellet was washed in 100 ml of cold water twice and centrifuged as in previously described. Supernatant was discarded and cells resuspended in 10 ml of cold 10% glycerol and centrifuged and supernatant discarded. Cells were resuspended in a final volume of 2 ml of cold 10% glycerol. The cell number in the suspension should be 1.5-3x10^{10} cells/ml. These competent cells can be used fresh or be frozen for future use. To freeze competent cells, cells were aliquoted into reaction tubes (40 µl/tube and placed on dry ice until frozen. Stored at -80^0C. Thawed on ice before use. These competent cells are good for 1-2 years.

Transformation of E.Coli

Chemically

Depending on the application, 5 ng of super coiled plasmid or 1 µl of a ligation mix (usually a 1/10 of the ligation) was added to 200 µl of competent cells and left on ic for a period of 30 min. Following this, the cells were heat-shocked at 42^0C for 90 seconds before rapidly

returning the tube to ice for a few min. After addition of 1 ml SOC medium (2.0% tryptone, 0.5% yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgSO_4$ and 20 mM glucose) the bacteria were transferred to a shaker and incubated for 45 min at 37^0C. The cells were then pelleted lighly by a short centrigugation (3 min at 1000x g) and 1 ml of the supernatent was removed before resuspending the cells in the remaining 200 µl. A volume of 50-200 µl was plated out on LB-agar aplates supplemented with the correct antibiotic and the plates incubated 18-24 hours at 37^0C.

Electroporation

To one 40 µl aliquot of competent cells 1-5 µl of DNA or 1/10 of a ligation (Salt can be removed from ligation mix by phenol/chloroform extraction and then ethanol precipitated). Mixed well and placed on ice for 1 min. Mixture was transferred to a cold 0.2-cm electroporation cuvette (Bio-Rad) and electroporated in a Bio-Rad Gene Pulser ^{R} according to the manufacturers instructions (1.8 Kv). The cuvette was removed from the chamber and immediately 1 ml of YENB medium was added to the cuvette. Cells were resuspended, transferred to a polypropylene tube (17 x 100 mm) and incubated with or without shaking at 37^0C for 1-3 hours. Cells were plated on selective medium plates.

Mini-prep plasmid preparation from E.Coli

Standard Method

Individual colonies were picked from a LB agar plate and used to inoculate 3 ml of LB medium, containing appropriate selective antibiotic. The inoculated bacteria were then incubated with shaking (220 rpm) overnight at 37^0C until a stationary phase had been reached upon which 1.5 ml was removed and the bacteria pelleted by centrifugation at 4000 x g for 5 min. The pellet was resuspended in 100 µl of solution I (50 mM Glucose, 25 mM Tris-HCL pH 8.0, 10mM EDTA and 300 µg/ml RNase A) and left at room temperature for 5 min before addition of 200 µl of solution II (0.2 M NaOH and 1.0% SDS). Once the resulting mixture appeared clear, it was neutralized wih 150 µl of solution III (3 M Na-Acetate pH 5.2) and mixed by gentle inversion of the reaction tube. Following 15 min incubation on ice, the precipitated protein and chromosomal DNA was pelleted by centrifugation at 10000 x g for 10 min before the aqueous supernatent was removed.

Extraction of the supernatent with phenol/chloroform was followed by precipitation of the plasmid DNA with ethanol and the resulting DNA pellet resuspended in 50 µl of bi-dest H_2O.

Wizard Mini-prep kit (Promega)

Resuspension, lysis and neutralising of the bacerial pellet was carried out according to and using the manufacturer´s buffers provided. The plasmid DNA in the retained supernatent was isolated using the supplier´s DNA-binding resin and suction manifold. The resulting plasmid DNA was then eluted in 50 µl of bi-dest H_2O. This method was primarily used for the production of sequencing-grade plasmid DNA.

Large scale plasmid preparation from E.Coli

Usually, a volume of 200-250 ml of LB or 2TY (16g/lt tryptone, 5 g/lt yeast extract and 5 g/lt NaCl) medium supplemented with the relevant antibiotic was inoculated with a single bacterial colony and incubated with shaking (220 rpm) at 37^0C overnight intil the bacteria had reached a stationary growth phase. The bacteria were pelleted by centrifugation in a fixed angle rotor at 4000 x g for 10 min and the pellet resuspended in 10 ml of solution I (10 mM EDTA, 50mM Tris-HCL pH 8.0 and 400 µg/ml of RNase A). Following 5-10 min incubation at room temperature, the cells were lysed by addition of 10 ml of solution II (200 mM NaOH and 1% SDS). Once the solution had taken an opaque appearance, the mixture was neutralized with 10 ml of solution III (3 M potassium acetate pH 4.8) the entire contents gently inverted to aid mixing of the solutions. After an additional 10-20 min on ice, the cell wall fragments and the bacterial chromosomal DNA were sedimented by centrifugation at 13000 x g for 20 minutes at 4^0C in a fixed angle rotor. The retained supernatent was then added directly to a pre-equilibrated Quiagen-tip 500 column (Qiagen Inc.) and the plasmid DNA was recovered according to and using the manufacturer´s supplied buffers. The purified DNA was precipitated using 0.8-1.0 vols of isopropanol, washed twice in 70% ethanol before resuspending the DNA to a final concentration of 1-3 mg/ml in bi-dest H_2O and stored at -20^0C.

Sequencing of double-stranded template DNA

α- ^{33}P-ddNTP method

The Thermo Sequenase radiolabelled terminator cycle sequencing kit (Amersham) was used for the sequencing of DNA templates. The kit combines two revolutionary innovations for sequencing DNA. First, the label is incorporated into the DNA reaction products by the use of four α^{33}P-ddNTP terminators and secondly, the use of an engineered Thermo Sequenase DNA polymerase which allows for efficient incorporation in cycling sequencing protocols. Sequencing of desired templates was carried out according to the manufacturer´s guidelines. Approximately, 0.5 μg of plasmid DNA was taken together with 1-2.5 pmol of an appropriate primer (usually M13 Forward or Reverse universal primers) with 8 U of the Thermo Sequenase polymerase in a total volume of 20 μl. From this mixture 4.5 μl was aliquoted into each termination vial (`G´, `A´, `T´, `C´) and cycled in a Perkin Elmar 9600 PCR machine using the following parameters: 95^{0}C, 30 sec, 55^{0}C, 30 sec and 72^{0}C, 1 min for a total of 35 cycles. Upon completion, 3 μl was loaded in each lane and resolved over a 6% polyacylamide 6 M urea TBE gel. Once the the run had reached the desired length, the gel was removed, dried on Whatmann 3MM paper at 80^{0}C for 2 hours on a vacuum gel dryer before autoradiography. Films were developed after 18-36 hours exposure.

Stable and transient transfection of cells

DOTAP liposomal transfection protocol

Cells used for transfection were split and reseeded 24 hours before transfection commenced. Usually, stable transfections were performed in 6 well petri dishes containing 3-4x10^5 cells, giving a confluency of 60-80%. Transfection was performed using the liposomal transfection reagent DOTAP (Boehringer Mannheim) exactly as described in the protocol provided, using 2.5 μg of maxi-prep plasmid DNA per transfection. The cells were reseeded 24-36 hours post-transfection and placed under appropriate antibiotic selection to generate clones. After a period of 1-2 weeks, visible clones were picked and placed in 24 well petri dishes and propagated further under selection until sufficient cell number had been reached, whereupon, the clones were expanded further in 10 cm dishes. For co

transient transfections, a ratio of 1:5 reporter plasmid to test plasmid was used in each transfection (0,5 μg : 2.5 μg). No selection was used and 24-36 hours post-transfection cells were lysed and the reporter plasmid was monitored with the appropriate method to be described later.

ANALYTICAL METHODS

Separation of proteins by polyacrylamide gel electrophoresis (PAGE)

Unless otherwise indicated for most applications a polyacrylamide separating gel of 10-12% was made and a 5% stacking gel. Reagents for the stacking gel were 8.3 ml of acrylamide/bis-acrylamide (30:0.8, Carl Roth GmbH & Co, Karlsruhe) 6.25 of 1.5 M Tris-HCl pH 8.8, 0.125 ml of 20% SDS, and 10.05 ml of H_20 (for a 12% separating gel, the amount of acrylamide/bis-acrylamide added was 10 ml and the H_20 reduced accordingly to give the same end volume). To the mixture was added 250 μl of 10% ammonium persulphate (APS) and the reaction initiated with 20 μl of TEMED. The gel mix is poured between two glass plates with spacers between and allowed to polymerize. Upon completion, a stacking gel is poured on top. This was made up of 1.7 ml of acrlamide/bis-acrylamide, 2.5 ml of 0.5 M Tris.HCl pH 6.8, 50 ml of 20% SDS, 5.65 ml of H_20, 100 μl of 10% APS and 7.5 μl of TEMED. The gel was then run in 1x laemmli-running buffer (25 mM Tris-HCl pH 8.3, 0.2 M glycine and 0.1% SDS) until the desired distance had been reached.

Preparation of protein probes

Depending of the application, medium was removed and 2x laemmli was added (160 mM Tris.HCl pH 6.8, 4% SDS, 16% glycerol, and 0.005% bromophenol blue). The slurry scrapped and transferred to a 1.5 ml reaction tube and the genomic DNA sonified (Branson cell disruptor B15, output 6) to reduce viscosity. Before loading the samples, for reducing conditions 100 mM DTT was added and proteins denatured by heating for 3 min at 95^0C.

EXPERIMENTAL PROCEDURES

Growth Factors and Reagents

Recombinant human platelet-derived growth factor BB (PDGF) (Biomol, Hamburg); doxycycline (Sigma, Deisenhofen); hyaluronate (HA) (Healon; high molecular weight; Pharmacia & Upjohn, Erlangen); hyaluronidase type VI-S from bovine testes (Haase) (Sigma); glutathione agarose (Santa Cruz, CA); Nonidet P-40 (NP40; Boehringer Mannheim).

Antibodies

Rabbit polyclonal antibodies used to detect merlin: A-19, N-terminal epitope; C-18, C-terminal epitope were from Santa Cruz. Antibodies against the cytoplasmic tail of CD44 were produced according to standard methods and will be described elsewhere. Sources of other antibodies: to the retinoblastoma protein (Rb; Santa Cruz: C-15); phosphorylated Erk (New England Biolabs, Schwalbach), against Erk (K23), ezrin (C-19) and actin (I-19) from Santa Cruz; to hemagglutinin tags (12CA5; Boehringer Mannheim); to CD44 (IM7, Pharmingen, SanDiego; KM81, ATCC; Hermes-3, gift of Sirpa Jalkanen, Turku). The CD44-specific antibody 5G8 has been described (Sleeman *et al*., 1996).

Plasmid constructs

pUHD17-1 encoding the reverse tetracycline-dependent transactivator (rtTA), pUHC13-3 rtTA-responsive luciferase reporter, and pUHD10-3 rtTA-responsive cloning vector (Gossen and Bujard, 1992; Gossen *et al*., 1995) were kindly provided by Hermann Bujard (Heidelberg). EcoR1 fragments encoding either the complete merlin cDNA (NF2.17) or merlin mutant cDNA (NF2.L64P), (Gutmann *et al*., 1999) were subcloned into pUHD10-3.

CMV promoter driven expression constructs encoding the truncated extracellular domain of CD44 (Aruffo *et al*., 1990), a mutant of the hyaluronate binding motif (Bartolazzi *et al*., 1994) and the plasmid encoding the cytoplasmic tail of CD44 inserted into pEBG-3x (Legg and Isacke, 1998) were prepared as described as was the mutant CD44 tail construct defective in ezrin binding (alanine substitutions for arginines at positions 293 and 294 and for lysines at positions 298, 299 and 300). The plasmids used for transfection of RP-MC cells express CD44s or CD44s mutated in the ezrin binding domain (see above) in the

pcDNA3 vector (Invitrogen, DeShelp). Expression constructs encoding activated oncogenes were as follows: Ras (leu61) (Medema *et al*., 1991), RafBXB (Bruder *et al*., 1992) obtained from Martin Schwartz, Scripps; MEK-1 DD subcloned into pcDNA3.1 (Mansour *et al*., 1994) obtained from Axel Knebel, Dundee. The hemagglutinin-tagged Erk-2 plasmid was a gift of Axel Ullrich (Martinsried).

Production of soluble CD44

Expression constructs were transfected into COS-1 cells and soluble protein purified and quantitated as described (Bartolazzi *et al*., 1994).

Cell cultures

The RT4-D6-P2T schwannoma cell line, NIH3T3 mouse fibroblasts and COS-7 cells were purchased from the European Collection of Animal Cell Cultures (Salisbury). RP-MC cells were kindly provided by Dr. I. Stamenkovic (Boston). All cells were grown in Dulbecco´s modified Eagle´s medium (DMEM; Gibco-BRL, Karlsruhe) supplemented with 10% fetal bovine serum (Gibco-BRL), 100U/ml penicillin G and 100 μg/ml streptomycin and maintained in a humidified atmosphere with 5% CO_2 at 37 ^{o}C. Primary rat Schwann cells were prepared and grown as described (Kim *et al*., 1997).

Generation of a dox-inducible merlin in the RT4-D6-P2T rat schwannoma cell line

All transfections made using DOTAP (Boehringer Mannheim) according to the manufacturers´s instructions. One day prior to transfection, cells were trypsinized counted and 1.5x10^{5} cells added to 6 well plates. The pUHD17-1 (rtTA) plasmid (2.5 μg) was co transfected with pBabe plasmid (0.5 μg) (puromycin resistance) after 7 hours medium was changed and the cells were left overnight to recover. Transfected cells were then selected with puromycin 1 μg/ml.

The pUHD13-3 luciferase reporter plasmid was used to screen for transgene activity inorder to obtain founder clones with low background and high dox dependent transcriptional induction. Clones from rtTA cells were transiently transfected with

pUHD13-3, (0.5 μg) in the presence or absence of 1 μg/ml dox. After 24 hours cells were harvested and assayed for luciferase activity.

In selected founder cell lines, either the pUHD10-3(NF2.17), pUHD10-3(L64P), pUHD10-3(NF217 C term) or pUHD10-3(NF217 N term) was co transfected with pcDNA3 plasmid (G418 resistance; invitrogen). Transfected cells were selected with G418 500 μg/ml. To screen for dox-inducible NF2-17 protein (merlin), $2.5x10^4$ cells from individual clones were added, in duplicate, to 24 well plates, overnight dox was added or excluded. Cells were then harvested in 2x Laemmli sample buffer and cell extracts were separated on a 10% SDS-polyacrylamide gel. Proteins were transferred to membrane. Immunoblotted with merlin specific antibodies. Clones containing merlin halves were selected on the basis of equal overexpression using endogenous merlin as a standard control.

Generation of stable wild type ezrin, N- terminal ezrin and N- terminal merlin clones in dox-inducible cells

Stably expressing wild type ezrin, N- terminal ezrin and N- terminal clones were generated in the dox-inducible merlin RT4-D6-P2T rat schwannoma cell line. All transfections were made using DOTAP as previously described. Either the pcDNA3-ezrin, pcDNA3-ezrin N term or pcDNA3 NF217 Nterm (2.5 μg) plasmid was co transfected with pCEP4 plasmid (0.5 μg) (Hygromycin resistance). Transfected cells were selected with hygromycin 150 μg/ml. Clones were selected for high stably expressing protein by western blot analysis using either an ezrin antibody for wild type protein, 12CA5 antibody to detect N terminal half of ezrin or a N terminal Merlin specific antibody. Cells were harvested in 2x Laemmli sample buffer and cell extracts were separated on a 10% SDS-polyacrylamide gel. Proteins were transferred to membrane. Immunoblotted with GSTCD44 specific antibody (CTI).

Generation of stable soluble GSTCD44 wild type and mutant cytoplasmic tail clones in dox-inducible cells

Stably expressing soluble GSTCD44 wild type and mutant cytoplasmic tail clones were generated in the dox inducible merlin RT4-D6-P2T rat schwannoma cell line. All transfections were made using DOTAP as previously described. The EbgGSTCD44 (2.5 μg) plasmid was co transfected with pCEP4 plasmid (0.5 μg) (Hygromycin resistance).

Transfected cells were selected with hygromycin 150 μg/ml. Clones were selected for high stably expressing GSTCD44 protein by western blot analysis using GSTCD44 tail specific antibody (CTI) using endogenous CD44 as a standard control. Cells were harvested in 2x Laemmli sample buffer and cell extracts were separated on a 10% SDS-polyacrylamide gel. Proteins were transferred to membrane. Immunoblotted with GSTCD44 specific antibody.

Measurement of tumor growth in vivo

Dox (100 μg/ml) was added or excluded to the drinking water of nude mice, one week prior to and continued through out the experiment. Cells were detached from plates with 5 mM EDTA in PBS, and resuspended in PBS free of calcium and magnesium. $5x10^5$ cells in a single cell suspension were injected subcutaneously into the right flank of nude mice (bred and maintained at the Institute für Genetik Animal Facility, Karlsruhe, Germany). Tumor volumes were determined every 4 days via caliper measurements. Each data point represents the mean + - the standard error of the mean (s.e.m) of tumor volumes from 4 animals.

Determination of cellular growth rate in vitro

Soft agar assay, cells were detached from plates with 0.25% trypsin, resuspended in complete media and counted. $1x10^4$ cells were resuspended in 3.2 ml of medium (DMEM) and 400 μl of FCS. Resuspension divided into two, and dox was added to one. To each mixure 200 μl of heated soft agar (stock 3.3%) was added and mixed. Rapidly added into 24 plates at 500 μl volume and cooled for 2 minutes at 4^0C. Colony formation was counted after 7 days.

For growth rates in cultrue dishes, cells were detached from plates with 0.25% trypsin, resuspended in complete media and counted. Cells were seeded at a low density, therefore $5x10^3$ cells were added to each 24 well plate in triplicate. 1 μg/ml dox was added or excluded. Each day for 5 days cells were counted. Treatment of logarithmic cells with either HA (Healon), sCD44 or sCD44mut, $2.5x10^3$ cells were added to 24 well plate in triplicate. Following day either 100 μg/ml of HA, 10 ng/ml of sCD44 or sCD44mut was added to culture medium in the presence or absence of dox. Each day for 3 days

cells were counted. Treatment of confluent cells with either sCD44 or sCD44mut, $2x10^4$ cells were added to 24 well plate in triplicate. The following day 10 ng/ml of sCD44 or sCD44mut was added to culture medium in the presence or absence of dox. Each day for 3 days cells were counted.

Proliferation measured by DNA synthesis

DNA synthesis was evaluated by ^3H thymidine incorporation assays. $2.5x10^4$ cells were plated in triplicate onto 24 well plates. After overnight induction with dox, confluent cells were labelled with 2uCi/ml ^3H thymidine (Amersham) for 3 hours. Cells were washed twice with phosphate buffered saline (PBS: 145 mM Nacl, 5 mM KCl, 10 mM sodium phosphate pH 7.4), solubilized in 200 μl of 0.2 M NaOH. Counts were then determined in a scintillation counter. For BrdU incorporation assays, cells were plated (see below) into 8-well chamber slides (Lab-Tek) and BrdU incorporated for 60 min. Staining for BrdU was done as recommended by the manufacturer (Oncogene Research Products).

Cell cycle distribution

Flow cytometry analysis was performed using a FACScan (Becton Dickinson). Confluent cells from 10 cm plate ($2x10^6$) treated or untreated overnight with dox. Cells were trypsinized, washed in medium rinsed in PBS, and fixed for 10 min with cold 4% paraformaldehyde/1% methanol. Cells washed with PBS and then resuspended in PBS 0.5% tween for 10 min. Cells were pelleted and resuspended in a large volume of PBS/5% FCS/0.5% tween containing 2 μg/ml of Hoechst dye and then incubated on ice for 30 min. Total cells were analyzed for cell cycle profile.

Analysis of Rb protein, dox-inducible cells were plated in duplicate, (at confluency), into 24 wells ($5x10^4$) treated or untreated overnight with dox. Cells were harvested in 2x Laemmli sample buffer and cell extracts were separated on a 10% SDS-polyacrylamide gel. Proteins were transferred to membrane. Immunoblotted with a Rb specific antibody.

Analysis of $p21^{Cip/Waf1}$ and $p27^{Kip1}$ cell cycle inhibitor proteins. Dox-inducuble cells were plated, in duplicate, at a low density, into 6 wells ($1.5x10^4$) treated or untreated overnight with dox. For NIH3T3 cells were plated at low density into 6 wells ($1.5x10^4$).

Following day either 100 μg/ml of HA or 10 ng/ml of sCD44 was added for 30 minutes. Cells were harvested in 2x Laemmli sample buffer and cell extracts were separated on a 10% SDS-polyacrylamide gel. Proteins were transferred to membrane. Immunoblotted with either $p21^{Cip/Waf1}$ and $p27^{Kip1}$ specific antibody, for loading control an actin antibody was used.

Meausurement of Erk activity

For endogenous Erk activity. Cells were plated in duplicate, logarithmic cells, $1.5x10^4$ cells were added to 6 well plates, confluent cells $2.5x10^4$ cells were added to 24 well plates. Following day cells were serum starved for 24 hours and for the last 8 hours one sample was treated with dox. Alternatively $2.5x10^4$ cells, in duplicate, were mixed with 0.8% methylcellulose in medium without serum and placed in 24 well plates (24 well plates were coated with 1% agar) the last 8 hours one sample was treated with dox. Cells were stimulated for 5 minutes with 5 ng/ml of PDGF. Cells were harvested in 2x Laemmli sample buffer and cell extracts were separated on a 10% SDS-polyacrylamide gel. Proteins were transferred to membrane. Immunoblotted with a phopho Erk specific antibody stripped and re blotted with Erk antibody for loading control.

For assays of transfected HA-tagged-Erk activity in confluent cells. $2x10^5$ cells in 6 well plates were co transfected with 0.5 μg of HA-tagged-Erk and 2.5 μg of constitutively activated mutants, ras, rafBxB and MEK1. Cells recovered from transfection overnight and were then serum starved for 24 hours. For the last 8 hours dox was added or excluded. Cells were washed once in ice-cold PBS before lysing the cells on ice with 1 ml of lysis buffer RIPA (10 mM Tris pH7.4, 150 mM NaCl, 1 mM EDTA, 1% Tx-100, 0.1% SDS, 0.5% DOC, 10 mM NaF) containing protease inhibitors, PMSF 1 mM, Aprotinin 10 μg/ml, Leupeptin 10 μg/ml and 1mM Na vanadate. Lysed cells were scraped together and transferred to a reaction tube and incubated on ice for 15 minutes. After DNA was sheared through a 26-guage needle, the lysate was cleared by centrifugation for 5 min 10000x g at 4^0C. Supernatent was transferred to new reaction tube and 5 μg/ml of antibody (12CA5) and 30 μl of protein A/G agarose was added (Oncogene science) and rotated for 3 hours at 4^0C. Immuncomplexes were recovered by centrifugation and washed 4x with cold lysis buffer. 50 μl of 2x Laemmli sample buffer was added. Samples

were separated on a 10% SDS polyacrylamide gel. Proteins were transferred to membrane. Immunoblotted with a phopho Erk specific antibody stripped and re blotted with Erk antibody for loading control.as described above.

For the measurement of endogenous Erk activity in logarithmic cells after pre treatment with either HA or soluble CD44. $1.5x10^4$ cells were added to 6 well plates, following day cells were serum starved for 24 hours and for the last 8 hours one sample was treated with dox. Cells were then pre treated with either 100 μg/ml of HA or 10 ng/ml of sCD44 for 5 minutes then stimulated for 5 minutes with 5 ng/ml of PDGF. Cells were harvested in 2x Laemmli sample buffer and cell extracts were separated on a 10% SDS-polyacrylamide gel. Proteins were transferred to membrane. Immunoblotted with a phopho Erk specific antibody stripped and re blotted with Erk antibody for loading control.

Examination of modulated Merlin protein

Endogenous merlin or exogenous dox-induced merlin cells were either plated at a low density, ($2.5x10^4$) in 6 wells or at high density, ($2.5x10^4$) in 24 well. The following day cells were harvested in 2x Laemmli sample buffer and cell extracts were separated on a 8% SDS-polyacrylamide high resolution gel. Proteins were transferred to membrane. Immunoblotted with a merlin specific antibody. Treatment of logarithmic cells with either HA (Healon) or sCD44, $2.5x10^4$ cells were added to 6 well plates dox was added overnight. Following day either 100 μg/ml of HA, 10 ng/ml of sCD44 was added to culture for 5 minutes. Cells were harvested in 2x Laemmli sample buffer and cell extracts were separated on a 8% SDS-polyacrylamide high resolution gel. Proteins were transferred to membrane. Immunoblotted with a merlin specific antibody

NIH3T3 were plated in 6 wells $2.5x10^4$. The following day, KM201 was added at 30 μg/ml for 5 min. 30 μg/ml of HA was then added for 5 minutes. Cells were harvested in 2x Laemmli sample buffer and cell extracts were separated on a 8% SDS-polyacrylamide high resolution gel. Proteins were transferred to membrane. Immunoblotted with a merlin specific antibody.

HAase treatment of confluent cells. $2.5x10^4$ cells were plated in 24 wells in duplicate. The following day cells were serum starved for 24 hours and the last 8 hours doxycycline was

added or excluded. HAase pretreatment 5u/μl for 2 hours was used, cells were then stimulated for 5 minutes with 5 ng/ml of PDGF. Cells harvested in 2x Laemmli sample buffer and cell extracts were separated on a 10% SDS-polyacrylamide gel. Proteins were transferred to membrane. Immunoblotted with a phopho Erk specific antibody stripped and re blotted with Erk antibody for loading control.

Immunoprecipitation

For the immunoprecipitation of exogenous merlin, cells were grown to confluency in 10 cm dishes ($2x10^6$), washed once in ice-cold PBS before lysing the cells on ice with 1 ml of lysis buffer (50 mM Tris pH7.4, 150 mM NaCl, 3 mM $MgCl_2$, 0.5% NP-40) containing protease inhibitors, PMSF 1 mM, Aprotinin 10 μg/ml, Leupeptin 10 μg/ml. Lysed cells were scraped together and transferred to a reaction tube and incubated on ice for 15 minutes. After DNA was sheared through a 26-guage needle, the lysate was cleared by centrifugation for 5 min 10000x g at 4^0C. Supernatent was transferred to new reaction tube and 5 μg/ml of antibody was added and rotated overnight at 4^0C. 30 μl of protein A agarose was added (Oncogene science) and rotated for another 3 hours at 4^0C. Immuncomplexes were recovered by centrifugation and washed 4x with cold lysis buffer. 50 μl of 2x Laemmli sample buffer was added. Samples were separated on 10% SDS polyacrylamide gel and immunoblotted with a merlin specific antibody. For CIP (calf intestinal phosphatase) (NEBiolabs) treatment, immunoprecipitates were divided into equal fractions and 1 unit of CIP was added to one fraction and incubated at 30^0C for 1 hour (as recommended by supplier). Cells were harvested in 2x Laemmli sample buffer and cell extracts were separated on a 8% SDS-polyacrylamide high resolution gel.

Coimmunoprecipitation of exogenous merlin with endogenous CD44, at confluency, as described above with the following exceptions: Lysis buffer 20 mM Tris pH7.4, 50 mM NaCl, 3 mM $MgCl_2$, 0.5% NP40. Lysate was pre cleared for 2 hours with protein A/G agarose (Oncogene Science) before addition of 5 μg/ml of the CD44 antibody 5G8 and rotated overnight at 4^0C. 30 μl of protein A/G agarose was added for 3 hours at 4^0C.

Coimmunoprecipitation of exogenous merlin with soluble GSTCD44 or GSTCD44mutant cytoplasmic tail as described above with following exceptions: Lysis buffer 20 mM Tris

pH7.4, 50 mM NaCl, 3 mM $MgCl_2$, 0.5% NP40. 30 µl of glutathione agarose (Santa Cruz) was added and rotated overnight 4^0C. Immunocomplexes were recovered by centrifugation and washed 4x with cold lysis bufer.

Immunoblotting

After gel electrophoresis proteins were transferred to Immobilon membranes (Millipore Corporation). The blots were incubated in blocking buffer, 10% skimmed milk, 0.1% Tween in 10 mM Tris pH 7.6, 100 mM NaCl (TBS) for 1 hour at room temperature. Incubation with primary antibodies were in blocking buffer (for phopho specific antibodies 5% BSA, 0.1% tween in TBS) for 1 hour room temperature (for phopho specific antibodies overnight 4^0C). Washed 3x for 10 min in TBS 0.1% tween. Incubation with secondary antibodies were in blocking buffer for 1 hour room temperature. Washed 3x for 10 min in TBS 0.1% tween. Blots were developed using enhanced chemiluminescence (Amersham International plc) and visualized with autoradiography film (Fuji Photo Film Co. Ltd).

RESULTS

Merlin inhibits rat schwannoma growth in vitro and in vivo

Introduction

To study the functions and biochemical activities of merlin, I looked for a cell system in which merlin expression was either lost or aberrantly regulated and which was amenable to biochemical and molecular manipulation. The ideal source would be human schwannoma cells from NF2 patients, (Hung *et al*., 1999; Pelton *et al*., 1998; Rosenbaum *et al*., 1998). However these cells cannot be maintained in long-term cultures and are difficult to use in biochemical and molecular studies. I therefore chose to examine the possibility of using an ENU-induced rat schwannoma cell line, RT4-D6P2T. The RT4-D6P2T cell line was generated from immature cells of the rat Schwann cell lineage exposed transplacentally to N-ethyl-N-nitrosourea (ENU). Such Schwann cells are transformed into immortalized malignant schwannomas with mutations in a number of genes including an activating mutation of the *neu* protooncogene (Bargmann and Weinberg, 1988; Nikitin *et al*., 1991; Weiner *et al*., 1989). At confluency, these transformed cells, including the RT4-D6P2T cell line, are not contact inhibited. Instead, as with other transformed cell types, these cells continue to proliferate and form extensive foci.

To determine whether RT4-D6P2T cells were an appropriate cell system to study merlin function, I compared merlin expression in RT4-D6P2T cultures and in normal rat Schwann cells under different culture conditions. In a variety of cell lines, merlin expression is significantly increased in cells that are growth-arrested due to confluency or serum deprivation (Shaw *et al*., 1998). This holds also for normal Schwann cells. I grew passage-matched primary cultures of normal rat Schwann cells at low and high cell density, assayed for equal amounts of total cellular protein and then tested merlin levels by Western blotting. Merlin was expressed at 2-3 fold higher levels in confluent cultures as compared to logarithmic cultures, determined by scanning densitometry (Fig 1). In contrast, merlin expression was not increased in RT4-D6P2T cells grown at high cell density in the same culture conditions as the rat Schwann cells (Fig 1). These rat schwannoma cells have therefore lost their ability to regulate cell density-dependent merlin expression. I therefore considered the rat schwannoma cells a suitable experimental model

to study merlin action, since the overexpression of merlin might re-establish the increased merlin in confluency and therefore counteract transformation.

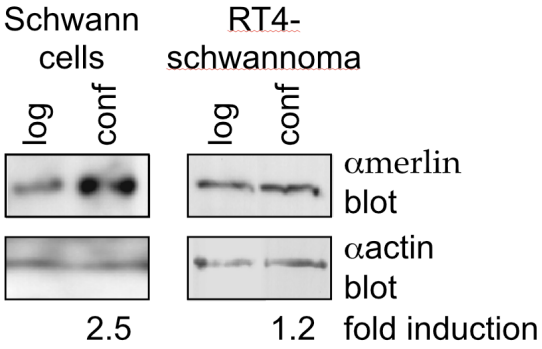

Figure 1. **At confluency merlin expression is increased in primary rat Schwann cells but not in the RT4-D6P2T schwannoma cell line.** Primary rat Schwann cells at early passage (2-3) were plated on poly-l-lysine coated 100 mm dishes in DMEM + 10% FCS at high and low cell densities. The RT4-D6P2T schwannoma cells were plated in the same manner but on uncoated plates. After 24 hrs, Schwann cells and the density-matched RT4-D6P2T cells were lysed and subjected to a 10% SDS-PAGE mini gel. Merlin expression was assayed by Western blotting using the anti-merlin antibody C18 (αmerlin) showing a merlin band at 70kDa. Twenty-five micrograms of total protein was loaded into each lane. Protein loading was confirmed by Ponceau S staining and verified using an anti-actin antibody (αactin) showing an actin band at 43kDa.

Establishment of a cell system with conditional merlin activity

It was previously found that stable overexpression of merlin in RT4-D6P2T cells and in another rat schwannoma cell line, JS1, inhibited cell growth both in vitro and in vivo (Sherman *et al*., 1997). However, the potent growth-inhibitory activity of merlin resulted in loss of merlin expression after several cell doublings, making a mechanistic biochemical analysis of merlin's function difficult. To overcome these problems, I generated clones of RT4-D6P2T cells that can be induced to express merlin at various levels (Gossen *et al*., 1995). More than 25 clones were selected and 4 clones were used in the experiments. Figure 2 shows an example of some selected clones that, in the non-induced state, expressed merlin at approximately the level of the endogenous gene and in which merlin expression could be induced either 6-fold (clone 6_7) or 28-fold (clone 5_4) above background following treatment with doxycycline (dox). As negative controls, I also generated clones of cells bearing only empty vectors (not shown) and clones that inducibly expressed an NF2 patient-derived inactive merlin mutant, L64P, at 4.7-fold levels above background (Fig. 2).

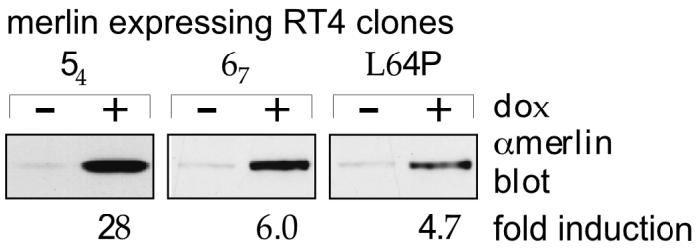

Figure 2. **Dox-inducible merlin expression clones**. RT4-D6P2T cells were stably cotransfected with a dox-inducible merlin vector and the r-tet regulator. Two different wild type clones (5_4, and 6_7) and one mutant clone (L64P) were examined for merlin expression. Cells were plated at a low cell density (70% confluency) and harvested at 8 hours after addition of dox. Lysates were subjected to 10% SDS-PAGE (minigel) and Western blotting with the anti-merlin antibody C-18 showing a merlin band at 70kDa.

Inducible expression of merlin inhibits rat schwannoma cell growth

The dox-inducible clones revealed what was expected from the data of constitutive stable expression clones. Elevation of merlin by dox treatment reduced cell growth in vivo and in vitro. In vivo, injection of $1x10^5$ cells subcutaneously into isogenic mice in the absence of dox led to tumors of 200 mm^3 within about 14 days and of more than 2000 mm^3 within 28 days. In the presence of dox in the drinking water, growth of the merlin-expressors in the animals was inhibited by approximately 55% (Fig. 3).

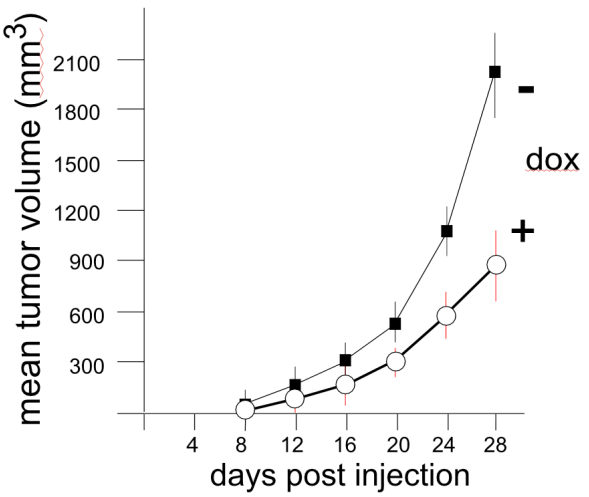

Figure 3. **Merlin inhibits tumor growth in vivo**. Subcutaneous tumor growth after injection of clone 5_4 cells into nude mice (see material and methods). Where indicated, dox was added to the drinking water. The experiments were also done with clone 6_7 with similar results.

In vitro, the soft agar colony formation assay represents a good correlate of tumorigenic transformation. Induction of both low (clone 6_7) and high (clone 5_4) levels of merlin resulted in 40 to 50% reductions in colony formation compared to uninduced cells (Fig 4). The growth of dox-treated vector control cells and cells induced to express the L64P mutant were unchanged. Thus, increases in merlin expression to levels, similar the endogenous ones found in confluent normal Schwann cells, inhibit the growth of these transformed

cells. The finding that a mutant merlin protein failed to inhibit cell growth demonstrates the specificity of merlin's activity in this system.

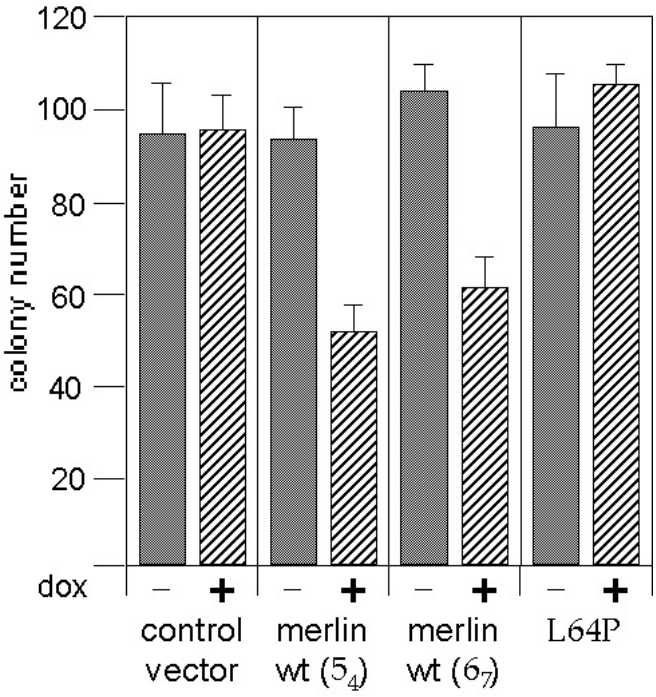

Figure 4. **Wild type merlin reduces agar colony formation**. Dox-inducible clones expressing either wildtype merlin (clones 5_4 and 6_7), or the mutant L64P, or vector control cells were placed in soft agar (see material and methods). Average number of colonies per well are plotted and standard errors are indicated.

Previous findings indicated that the growth-inhibitory effect of merlin depended on the presence of both it´s N- and C-terminal domains, either of the contigous protein or even if present as separate halves in the same cell (Sherman *et al*., 1997). I explored, whether this cooperation between N- and C-terminal half molecules is also required in the dox-inducible system. I introduced into the schwannoma cell system dox-inducible constructs that encoded either merlin N-terminal or the C-terminal halves. The dox-induced overexpression of either transfectant caused no reduction of soft agar colony formation (Fig. 5A). The expression levels of either merlin N-terminal or C-terminal, was monitored by Western blots, showing expression levels relative to the endogenous merlin band (Fig. 5A left and middle panel). Interestingly co-expression of both constructs as two separate peptides behaved like the full merlin protein and reduced colony formation (Fig. 5A). Double expressor clones were selected on the basis of equal expression of both N- and C-terminal domains, (Fig. 5A right panel, Western blots). This either indicates that the two domains exert separate functions or that the association of the N- and C-terminal domains

in the cell is important for merlin function. Coimmunoprecipitation from extracts of double expressors, both in the stable transfectants or in transiently transfected COS cells (Fig. 5B) indeed confirmed that a re-association of the half molecules can exist.

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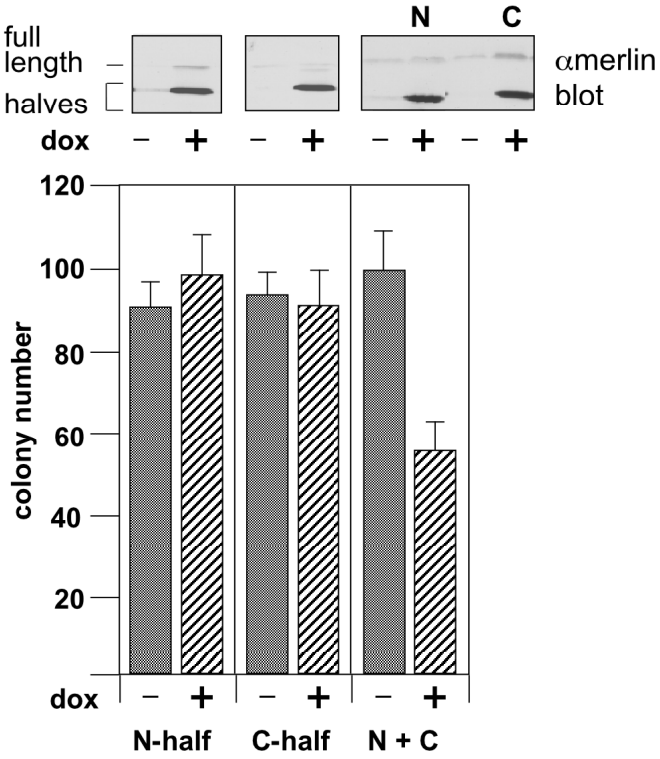

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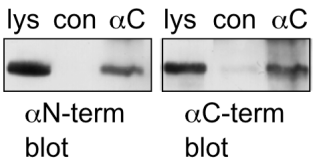

Figure 5. **N- and C- terminal halves of merlin are required for growth inhibition. Separate N- and C-terminal half peptides of merlin reassociate in vivo.** (A) Dox-inducible clones expressing either, merlin N- or C- half molecules or clones doubly expressing N- and C- terminal halves of merlin were placed in soft agar. The clones were selected for equal expression. Expression after addition of dox was detected by Western blotting using the antibodies directed against either the C-terminus (C-18; panels 2 and 3) or N-terminus (A-19; panels 1 and 3)) of merlin. In panel 3 the left two lanes represent the Western blot with A-19, the right two lanes that of C-18. Average colony number per well are plotted. The experiments were also done with three other independent dox-inducible clones with similar results. (B) N-terminal halves of merlin coprecipitate with the C-terminus. Cos-1 cells were transiently transfected with pcDNA3-N-terminal and pcDNA3-C17-terminal merlin constructs. Proteins were immunoprecipitated from the lysates with a C-terminal merlin-specific antibody (C-18). Immunocomplexes subject to Western blotting left panel with a N-terminal merlin specific antibody (A-19) the right panel demonstrates immunoprecipitation of merlin C-terminal fragments the blot was reprobed with the C-terminal merlin-specific antibody C-18. Whole cell lysate (lys), control IP (con) the same cell lysates treated with A-sepharose beads alone.

In summary, the induced overexpression of merlin supplemented the endogenous complement of merlin. Activity was also demonstrated even when the N- and C-terminal domains were expressed as separate peptides. Merlins overexpression demonstrates its tumor suppresser activity. Merlin inhibits tumor growth in vivo and in vitro and the effects of merlin depended on its wild type sequence. This conditional experimental system mimicks human pathology and is therefore suitable to further study the mechanism of growth inhibition of merlin.

Merlin activity is modulated by cell density.

The finding that merlin expression is highest in Schwann cells grown in confluent cultures suggests that merlin's growth-inhibitory activity may be linked to culture density. In both the nude mouse and colony formation assays described above, cells would have been in close contact with one another, similar to conditions in confluent monolayer cultures. To determine whether increased merlin expression also influences the growth of cells in culture dishes in a density-dependent manner, inducible merlin cells were plated at low cell density in the presence and absence of dox and assayed for growth over several days. Upon merlin induction, both low and high expressing schwannoma clones were growth-inhibited but only at high cell density, which was reached after three days under these experimental conditions (Fig. 6. clones 5_4 and 6_7). During log phase growth, however, neither low nor high level induction of merlin expression influenced cell growth. Dox treatment of vector control or cells inducibly expressing mutant (L64P) merlin had no effect on cell growth at either high or low cell density (Fig. 6).

This reduction of cell counts in confluent cultures was due to the inhibition of growth rate. There was no morphological sign of a merlin-dependent increase in apoptosis in these cells. Moreover after a period of 48 hours, the presence of elevated merlin in confluent cultures did not cause an increase of cells carrying sub-G1 amounts of DNA. Rather, cell cycle profiles indicated that more cells were in G1, 83 % with dox-induced merlin (Fig 7A right panel) instead of 70 % without dox (Fig 7A left panel).

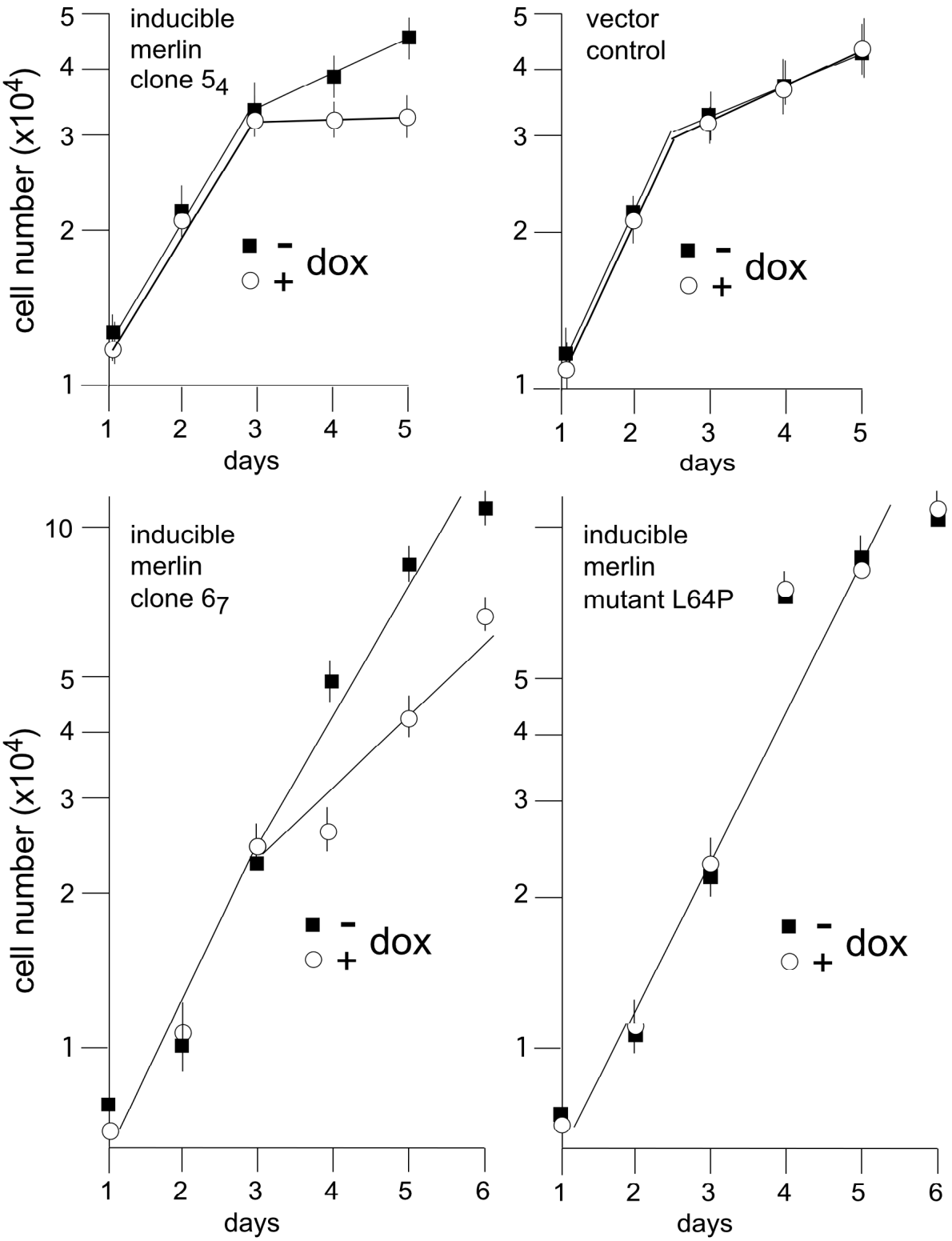

Figure 6. **Merlin is activated at high cell density**. Wild-type merlin reduces growth in culture dishes but only at high cell density. RT4-D6P2T clones 5_4, 6_7 and L64P and vector control cells were seeded at low density (triplicates in 24-well dishes) and cultivated in the presence or absence of dox. Cells per well were counted at 24-hour intervals and the numbers plotted starting 24 hours after seeding. Standard errors are indicated.

In agreement with the cell cycle profiles dox-induced merlin expressors incorporated 30% less ^{3}H-thymidine at high cell density, in comparison to the empty vector control, following a 3 hour pulse, as measured by scintillation counting (Fig. 7B). In non-transformed cells the consequence of high cell density or of contact inhibition, includes an increase in the cyclin-dependent kinase inhibitors p21 and p27, a decrease in cyclin D1, and accumulation of hypophosphorylated Rb (St. Croix *et al.*, 1998). In agreement, I found in confluent cultures of dox-induced merlin expressing cells an increase of hypophosphorylated Rb, as compared to non-induced cells (Fig. 7C left panel). These data are consistent with the notion that merlin interferes with signals that influence cell cycle progression. Further I compared logarithmic and confluent dox-inducible cultures for the expression of p27 and p21. There was an increase in the level of p27 (Fig 7C right panel) and p21 (not shown) in the confluent as compared to the logarithmically growing cultures. However the amount of p27 in both non-induced and induced states were the same suggesting that p27 induction alone is not sufficient to arrest these transformed cells.

Taken together, increasing the abundance of merlin in cells is not in itself sufficient to suppress growth. Rather, merlin appears to function in two different states, growth-suppressive at high cell density and inactive or growth permissive at low cell density. The finding that increased merlin expression can inhibit the growth of malignant rat schwannoma cells upon reaching confluency suggests that merlin plays a role in restoring signalling of contact inhibition of growth in these transformed cells. The following sections deal with these questions: Which step of signal transduction is effected by merlin? What determines merlin activity? How is this activity related to cell-cell contact?

Active merlin inhibits Map kinase signalling

Loss of contact inhibition is a feature of transformed cells, and is likely linked to the activation of oncogene-driven signalling cascades. Consistent with my findings, a previous report indicated that merlin overexpression reversed anchorage-independent growth in soft agar of v-Ha-Ras-transformed NIH3T3 cells (Tikoo *et al.*, 1994) suggesting that merlin might influence the Ras-MAP kinase signalling cascade.

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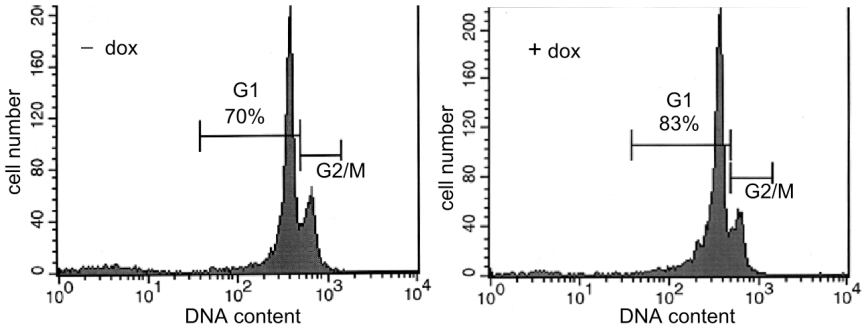

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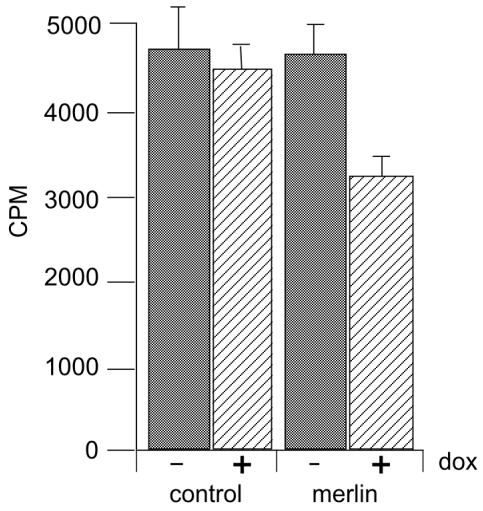

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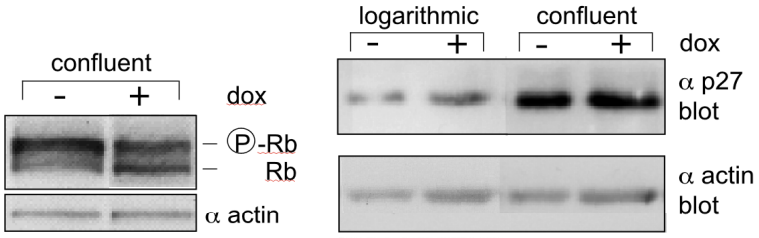

Figure 7. Merlin reduces cell proliferation at high density. (A) DNA profiles of schwannoma cells with or without dox at high cell density. Cells were fixed 24 hours following dox treatment, incubated with propidium iodide and analysed on a fluorescent activated cell sorter. (B) ^{3}H thymidine incorporation into schwannoma cells with or without exogenous merlin expression. Either vector control or dox-inducible merlin cells were seeded in triplicate at a high density, into 24 well plates. Cells treated with dox overnight where indicated. Counts were determined in a liquid scintillation counter and were plotted as mean CPM and standard errors calculated. (C left) Immunodetection of Rb protein from clone 5_4 at day 3 of culture (high density) in the presence or absence of dox demonstrating that at high cell density in the presence of dox, Rb is hypophosphorylated (C right) Equal number of dox-inducible cells (clone 5_4) from logarithmic or confluent cultures were treated overnight with dox. Western blotting with a anti p27 antibody and anti actin antibody to contol for loading.

The RT4-D6P2T cell line expresses a constitutively active mutant form of ErbB2 (Pfeiffer and Wechsler, 1972; Schubert *et al*., 1974; Tomozawa and Sueoka, 1978), a receptor tyrosine kinase that functions, in part, through the Ras-MAP kinase pathway (Janes *et al*., 1994). To examine the possibility that merlin restored contact inhibition in the inducible rat schwannoma system through interference with Ras-MAP kinase signalling, I examined merlin´s influence on the immediate activation of components of this signalling pathway in response to stimulation by a growth factor. Despite the fact that the schwannoma cells are constantly stimulated by constitutively active ErbB2, they still reacted to the addition of serum or PDGF by strongly elevating Erk phosphorylation (Fig. 8A). As expected from the stimulus chosen, JNK and p38 activities were also induced but only weakly. Pretreatment with dox of merlin expressors for 8 hours, prevented or reduced the PDGF dependent activation of Erk, but only if the cells were in a confluent condition or grown in methocel (Fig. 8A left and right panel). Logarithmically growing cells showed no reduction in the PDGF dependent activation of Erk (Fig 8A bottom panel).

Both Erk activation and colony formation in methocel served as endpoints to characterize the step of interference with signalling by merlin. I transiently cotransfected dominant active mutant constructs of Ras, Raf or MEK together with an expression contruct encoding a tagged version of Erk (haemagglutinin-tagged [HA-tagged]) to be able to examine only transfected cells in transient transfection assay. I then examined the influence of merlin expression on Erk phosphorylation. Merlin should only be able to inhibit Erk phosphorylation if acting downstream of the constitutive signalling component. I immunoprecipitated the HA-tagged Erk and measured the effect of activated dox-induced merlin at high cell density. Both Ras and Raf induced Erk activation was prevented by merlin (Fig. 8B Western blots), however merlin could not prevent Erk phosphorylation by constitutive active MEK. A similar result was obtained in soft agar colony assay. Dox-inducible cells were stably transfected with the same constitutive active mutants of signalling components. Pools of transfectants were placed in methocel and treated or not treated with dox. Merlin reduced colony formation in the control and in the transfectants with constitutively active Ras or Raf (Fig. 8B). Merlin however failed to inhibit colony formation supported by constitutively active MEK (Fig. 8B). I conclude from these data that merlin acts to promote contact inhibition at least in part by interfering with the Ras-MAP kinase signalling downstream of Raf, but prior to activation of MEK.

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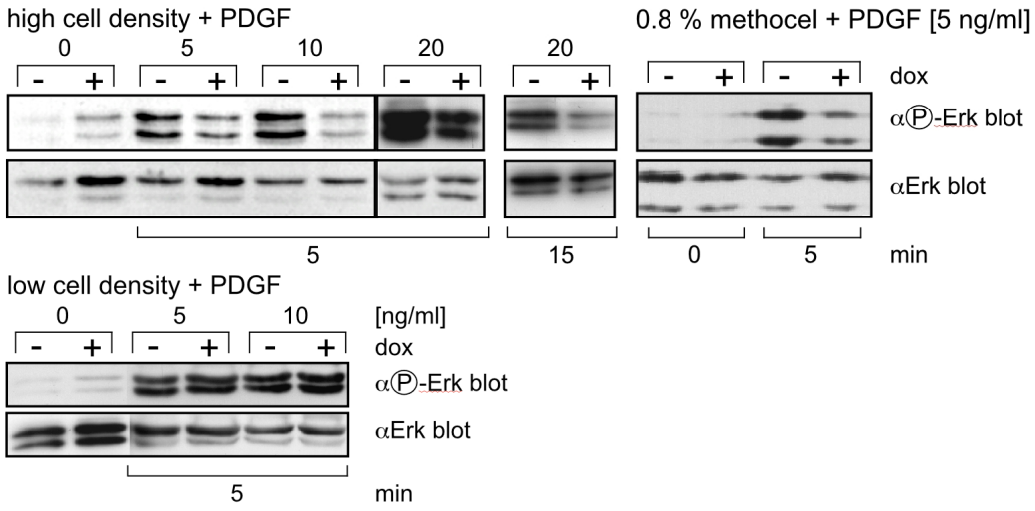

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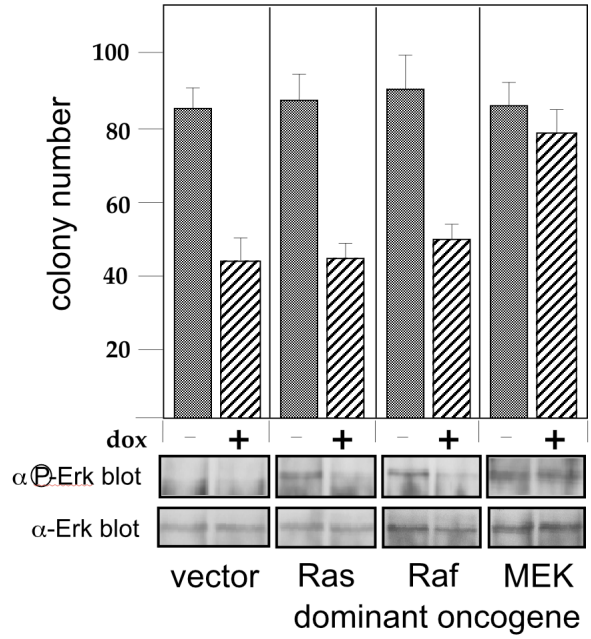

Figure 8. **Merlin interferes with signal transduction**. **Merlin´s interference with signal transduction is below Ras and Raf but prior to MEK**. (A) PDGF-dependent Erk-phosphorylation. Dox-inducible clone 5_4 cells were plated into culture dishes at a low or high density, or in methylcellulose (methocel), and serum starved for 24 hours. Dox was added, where indicated, for a period of 8 hours prior to treatment with PDGF (5ng/ml). Cells were harvested 5 min later and the lysates subjected to SDS-page and Western blotting for phorphorylated Erk and total Erk. (B Western blot) Interference between Raf and MEK with oncogene.dependent Erk phosphorylation. Dox-inducible clone 5_4 was cotransfected in a 5:1 ratio with constructs encoding the constitutively active mutants of Ras, Raf or MEK, and with a HA-tagged version of Erk. Cells were plated at a high cell density serum-starved for 24 hours and treated with dox for 8 hours prior to lysis and Western blotting. Similar results was obtained with clones 2_3, 6_3 and 6_7. (B) Interference between Raf and MEK with oncogene-dependent agar colony formation. Dox-inducible clone 5_4 was cotransfected with expression constructs encoding constitutively active mutants Ras, Raf or MEK with a hygromycin vector. Clones were selected with hygromycin (100μg/ml) and then grown in soft agar. Average number of colonies per well are plotted and standard errors are indicated.

Inhibition of the Map kinase signalling induces cell cycle arrest in the rat schwannomas

Hypophosphorylated Rb accummulates at high cell density (Fig 7C) in the dox-induced merlin expressor cells, suggesting that merlin influence signals to the cell cycle machinery. In the previous section I demonstrated that merlin promotes contact inhibition by interfering with the Ras-MAP kinase signalling pathway. With the following experiment I wanted to test whether inhibition of the MAP kinase pathway could explain the cell cycle arrest. Using a potent specific MEK inhibitor on logarithmically growing cells I could show that within two hours there was an increase of hypophosphorylated Rb and an accumulation of the cell inhibitor p27 (Fig. 9). The fact that the inhibition of the Ras-MAP kinase pathway, using the MEK inhibitor, in these schwannoma cells was sufficient to induce cell cycle arrest would therefore account for merlin action.

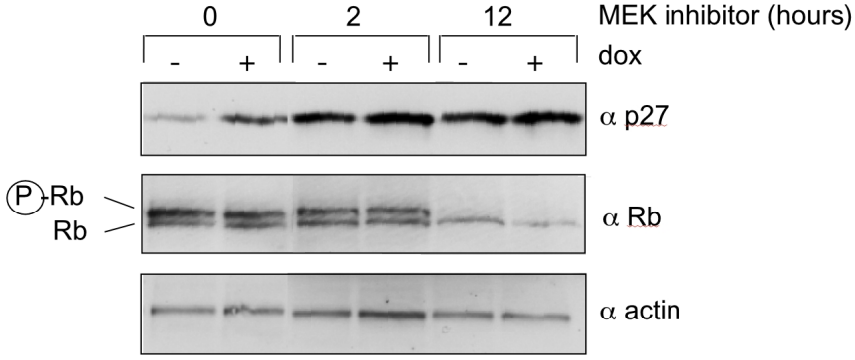

Figure 9. **MEK inhibition leads to the accumalation of p27 and to an increase of hypophosphorylated Rb**. Dox-inducible cells were plated onto 6 well plates at a low density. Where indicated dox was added. The following day a MEK inhibitor (U0126 Promega) was added for 2 or 12 hours. 0 indicated no addition of the MEK inhibitor. Lysates were resolved on a SDS-PAGE gel and immunoblotted with either an anti p27 antibody, an anti Rb antibody and for a loading control an anti actin antibody was used.

Merlin is hypophosphorylated at high cell density

The observation that merlin can be activated in cell culture during confluency suggested that a posttranslational mechanism existed for merlin activation. Using NIH3T3 cells, Shaw and co-workers (1998) reported that merlin could be phosphorylated and that merlin´s levels of phosphorylation decreased at high cell density or when the cells lost substratum adhesion. I therefore examined the phosphorylation status of merlin in the dox-induced system under different growth conditions. By SDS gel electrophoresis and Western blotting two merlin bands could be resolved (Fig. 10A left panel). A slower migrating band predominated in the logarithmically growing cultures while a second faster-

migrating band appeared only upon confluence. This latter seems to be a hypo-phosphorylated form since the total of merlin was converted to the faster-migrating band by calf intestinal phosphatase digestion (Fig. 10B). Both the endogenous (Fig. 10A, left panel) and the dox-induced population of merlin molecules (Fig. 10A, right panel) occurred in these two forms. Notably, the L64P mutant remained phosphorylated in both logarithmically growing and confluent cultures (Fig. 10C). Since the hypo-phosphorylated form of merlin is only detected at confluency, I conclude that it is this form that has growth-suppressive properties.

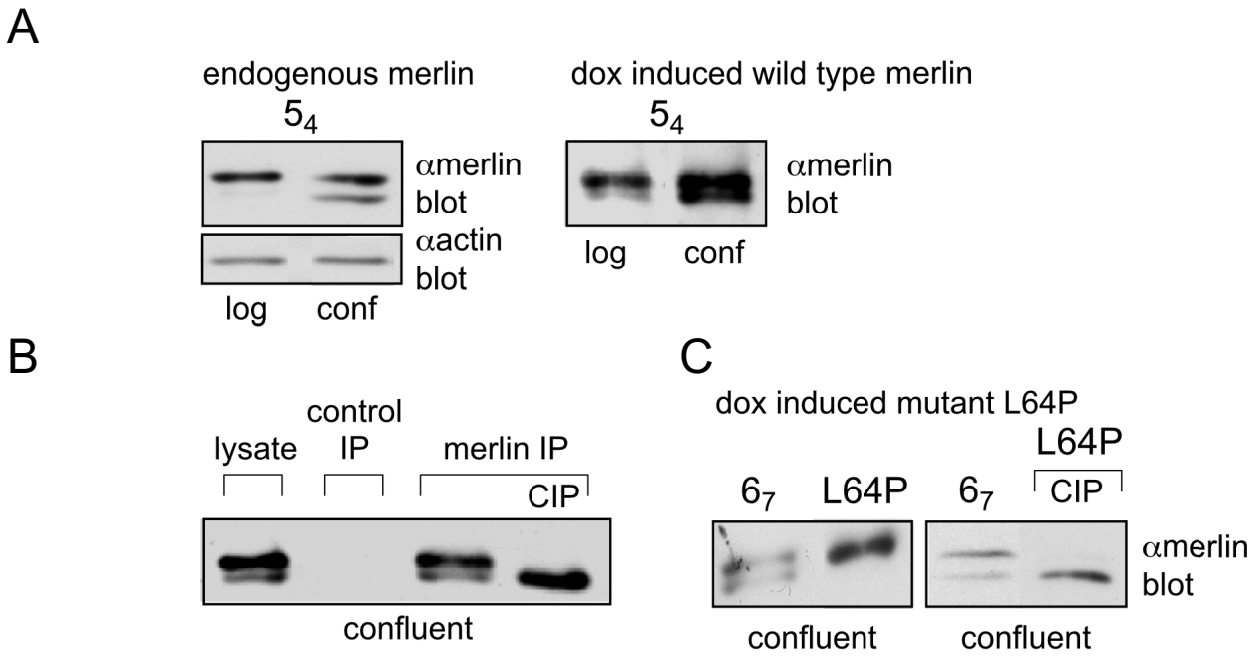

Figure 10. **A hypophosphorylated form of the endogenous merlin can be resolved in confluent cultures** (A) left blot. Clone 5_4 cells were cultivated without dox ("endogenous merlin"). Equal cell numbers from either logarithmic or confluent growth conditions were lysed and the lysates subjected to a 8% SDS-PAGE (higher resolution gel) and Western blotting (upper panel) using the anti-merlin antibody (C18). The loading was controlled by Western blotting using an anti-actin antibody (lower panel). **A hypophosphorylated form of doxycycline-induced merlin can be resolved in confluent cultures** (A right blot). Clone 5_4 cells were cultivated in the presence of doxycycline. Equal numbers of either logarithmically growing or confluent cells were lysed and treated as in A (upper panel, exposure about 30 times shorter than in A). (B) Shows a Western blot of merlin immunoprecipitates derived from lysates of confluent 5_4 cells that had been treated with calf intestinal phosphatase (CIP) or non-treated. The slower migrating band disappears upon digestion with CIP. "Control IP" defines a precipitation with sepharose A beads alone. **L64P mutant merlin remains phosphorylated even at cell confluency** (C). Cells of clone 6_7 or clone L64P were cultivated with dox. Confluent cells were lysed, subjected to a 8% SDS-PAGE (higher resolution gel) and Western blotted with C18 as in A. The slower migrating band of L64P represents the phosphorylated form in that it was converted to a faster migrating band upon digestion with CIP. Apparent molecular weights: 70 kDa for the phosphorylated merlin form, 69 kDa for the hypophosphorylated form. Marker proteins were run with each gel and the sizes confirmed in all experiments.

Hypophosphorylated merlin, but not ezrin or moesin, binds the cytoplasmic tail of CD44 at high cell density

Exrin, radixin and moesin (ERM) proteins are known to interact, via their N-terminal ends, with a number of transmembrane proteins, including CD44 reviewed by (Sherman *et al*., 1994; Tsukita and Yonemura, 1997). The striking similarity of the N-terminal half of merlin with the N-termini of ERM, suggested that merlin may also bind to CD44. To test this assumption, CD44 co-precipitation experiments were performed using the dox-inducible cell clones. With the CD44-specific antibody 5G8 (Sleeman *et al*., 1996) merlin was co-precipitated, when the cells were grown at high cell density (Fig. 11 left panel). More interestingly, exclusively the hypophosphorylated form of merlin was coprecipitated with CD44. Thus either only the activated form of merlin could bind to CD44 or the dephosphorylation was an immediate consequence of that binding. Under identical culture conditions, the L64P merlin mutant protein, which is not hypophosphorylated, did not co-immunoprecipitate with CD44 (Fig. 11 right panel) verifying that this association is not simply due to protein overexpression. No ezrin or moesin could be detected in these protein complexes although the lysates contain considerable amounts of these proteins (Fig. 11).

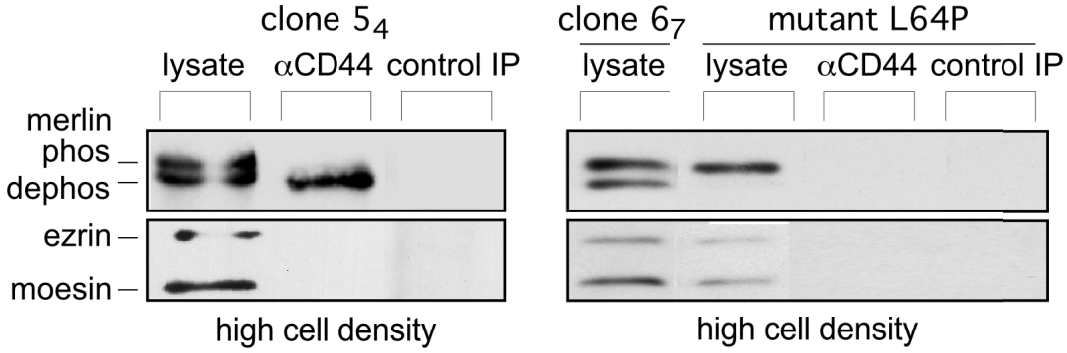

Figure 11. **Dox-induced hypophosphorylated merlin, but not ezrin and moesin, coprecipitates with CD44.** For coimmunoprecipitation experiments, cells were seeded at high cell density and treated with doxycycline for 8 hours. Cell lysates from clone 5_4 (left panel) or L64P (right panel) were incubated overnight with the CD44 antibody 5G8 and then treated with protein A sepharose. Whole cell lysates and immunoprecipitates were then subjected to 8% SDS-PAGE (higher resolution gel). Lysate from clone 6_7 at high cell density was included to control for the resolution of the L64P mutant merlin. Western blotting was performed using either an anti-merlin antibody or an anti-ezrin antibody which also recognizes moesin, showing ezrin at a molecular weight of 80 kDa and moesin at a molecular weight of 76 kDa. “Control IP” defines precipitates with protein A sepharose alone.

Merlin function depends on the interaction with the transmembrane protein CD44

To explore whether merlin required the association with CD44 for its function, the following experiments were performed. I generated stable clones in the dox-inducible cells overexpressing a soluble cytoplasmic peptide of CD44 fused to GST. The assumption was that if the location at the cell membrane via endogenous CD44 was required for merlin function, the soluble CD44 tail-GST fusion protein would sequester merlin from its site of activation and block merlin activity. The abundance of the CD44 cytoplasmic tail-GST fusion protein was monitored via western blots, showing at least 20 fold overexpression as compared to the endogenous CD44 (Fig. 12A right panel). The influence of overexpression of soluble CD44 cytoplasmic tails was tested in a soft agar colony assay. The CD44 cytoplasmic tail-GST fusion protein abolished growth inhibition by merlin while expression of GST alone had no effect on merlin function (Fig. 12A).

Ezrin binds in vitro to two clusters of basic amino acids in a membrane-proximal nine amino-acid motif of the CD44 cytoplasmic tail (Legg and Isacke, 1998). To determine whether merlin interacted with CD44 through the same domain as ERM proteins, I generated a CD44 tail-GST fusion protein with mutations in the ERM protein-binding domain and stably expressed it in the inducible merlin cell lines (as described above). Overexpression of this mutant protein could not abrogate merlin-mediated growth inhibition in soft agar (Fig. 12A). Furthermore, in pull down experiments from cell lysate using glutathione agarose, merlin bound to wild type, but not the mutant CD44 tails verifying the specificity of this effect (Fig. 12B).

These data indicate that the attachment of hypophosphorylated merlin to the membrane-anchored CD44 tail, or to other transmembrane proteins with an ERM-binding amino acid motif, is an essential determinant of merlin's function as a negative growth regulator at high cell density conditions.

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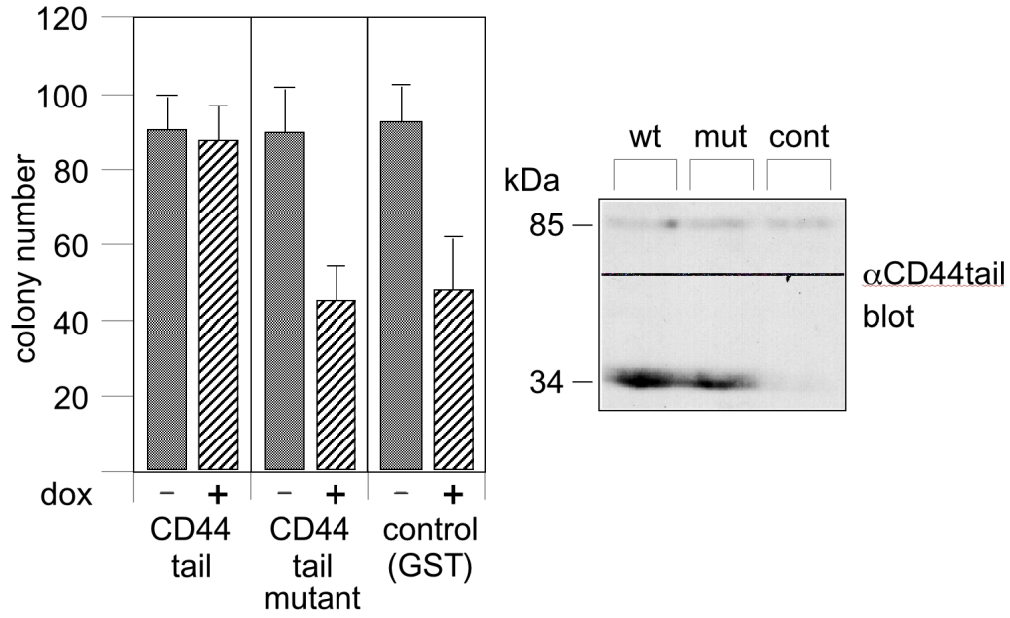

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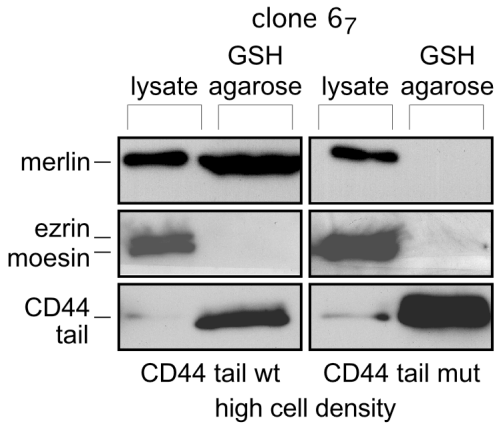

Figure 12. **Sequestering of merlin by binding to overexpressed soluble CD44 cytoplasmic tails abolishes merlin function** (A). The dox-inducible merlin expressing clone 5_4 was stably transfected with expression constructs encoding either a wild type or mutated CD44 cytoplasmic tail fused to GST. Control cells were transfected with expression constructs encoding GST alone. The mutant form was chosen for its inability to bind ezrin (see text and material and methods). Subclones with high expression levels (see Western blot with an anti-CD44 tail-specific antibody (αCD44tail)) were selected, placed in soft agar and their colony forming ability (see material and methods) was determined in the presence or absence of dox.

Overexpressed wild type, but not the mutant cytoplasmic tail of CD44 pulls down merlin at high cell density (B). Cells of clone 6_7 transfected with either wild type or mutant CD44 tail constructs, were plated at high cell density. 8 hours after dox addition, the cells were lysed and the CD44 tails enriched by GSH agarose. Lysates were subjected to 10% SDS-PAGE (minigel). Western blotting was performed using either anti-merlin (C-18), anti-ezrin or anti-CD44 tail antibodies.

At low cell density, ezrin, moesin and the phosphorylated form of merlin are associated with CD44

The finding that hypophosphorylated merlin, but not ERM proteins, bound CD44 through the ERM binding motif in high cell density cultures (Fig 11) suggested that ERM proteins and merlin may associate with CD44 under different cellular conditions. I therefore repeated the CD44-merlin binding experiments in low density cultures of the dox-inducible merlin cells. In contrast to high cell density cultures, ezrin and moesin were co-immunoprecipitated with CD44 in low cell density cultures (Fig. 13A). Interestingly, merlin was also found in the complex, but only in its phosphorylated form. This result was confirmed in pull-down experiments using the CD44 cytoplasmic tail-GST fusion proteins (13B). The CD44-merlin association under low cell density conditions is likely to be indirect through ERM proteins. Consistent with this interpretation, merlin immunoprecipitated with an anti-ezrin antibody (that also recognizes moesin) in lysates from low density cultures, while at high cell density no such association could be detected (Fig. 13C).

This result indicates that both merlin and ezrin activities are mutually exclusive and their function might depend on their interaction with CD44 (see Discussion). Merlin seems to be active at high cell density whereas ezrin might act under low cell denstiy. To test whether ezrin could block merlin function, I overexpressed full length ezrin in the dox-inducible cell lines. Full-length ezrin did not compete with merlin in the soft agar assay, suggesting that ezrin is indeed inactive under these growth conditions (Fig. 14). The N-terminal halves of merlin and ezrin are not dependent on such growth conditions for their activity. Consequently both exerted dominant-negative effects on merlin action (Fig. 14). This result could be interpreted that the N-terminal of both ezrin and merlin competed with full length merlin for the same binding site on CD44.

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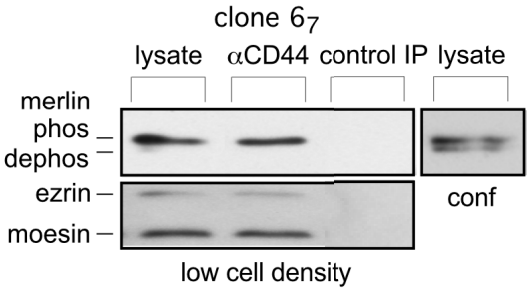

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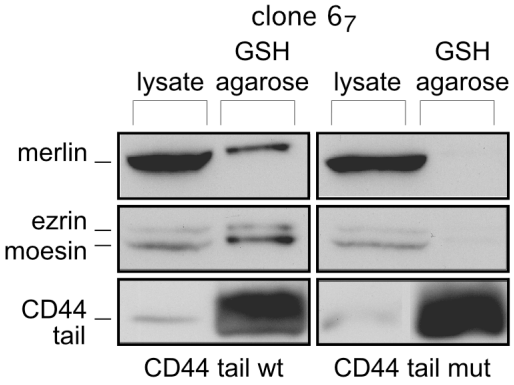

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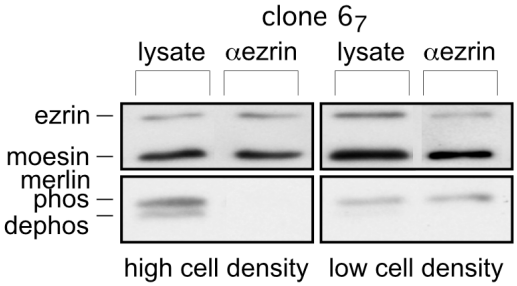

Figure 13. **Ezrin, moesin and doxycycline-induced phosphorylated merlin are coprecipitated with CD44** (A). For coimmunoprecipitation experiments clone 6_7 cells were seeded at low cell density and treated with dox for 8 hours. Lysates were incubated overnight with the CD44 antibody 5G8, then treated with protein A sepharose. Whole cell lysates and immunoprecipitates were then subjected to 8% SDS-PAGE (higher resolution gel). Lysates of clone 6_7 cells at high cell density was included to control for the resolution of merlin modifications. Western blotting was performed using either an anti-merlin antibody (C-18) or anti-ezrin/moesin antibody. “Control IP” represents treatment with protein A sepharose alone.

Overexpressed wild type cytoplasmic tail of CD44 pulls down merlin and ezrin from lysates of cells at low cell density (B). Cells of clone 6_7 transfected with either wild type or mutant CD44 tail constructs, were plated at low cell density. 8 hours after dox addition, the cells were lysed and the CD44 tails enriched by GSH agarose. Lysates and precipitates were subjected to 10% SDS-PAGE (minigel). Western blotting was performed using either anti-merlin, anti-ezrin/moesin or anti-CD44 tail antibodies.

Phosphorylated merlin coprecipitates with ezrin and moesin at low cell density ©. Clone 6_7 cells were plated at low or high density and treated with dox for 8 hours. Coimmunoprecipitation was performed as described in A, except for the use of an anti-ezrin/moesin antibody (C-19) for the precipitation. Lysates were subjected to 8% SDS-PAGE (higher resolution gel). Western blotting as in A.

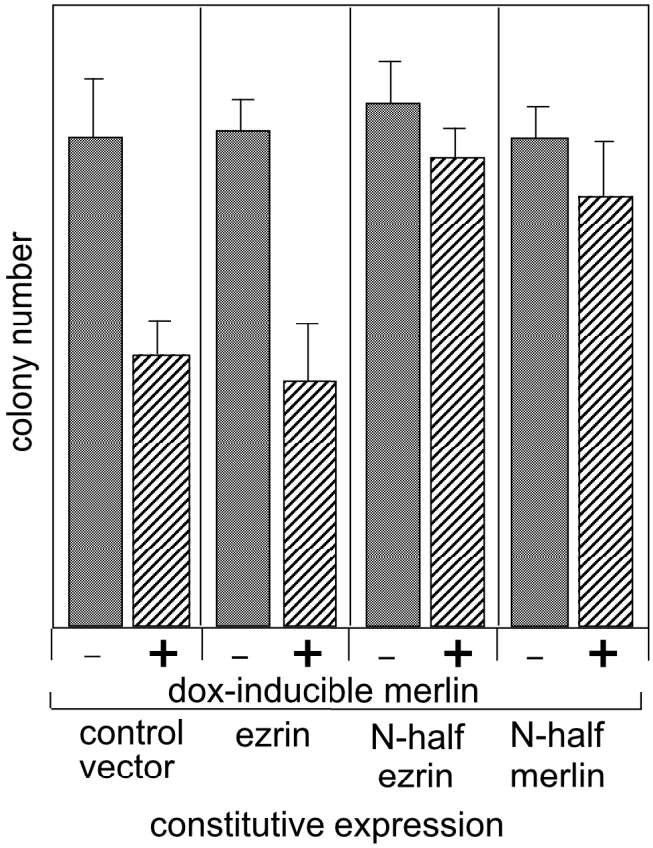

Figure 14. **N-terminal ezrin interferes with merlin function**. Dox- inducible clone 5_4 was stably transfected with either N-terminal merlin, N-terminal ezrin or full length ezrin expression vectors with an expression vector for hygromycin resistance. Hygromycin resistant clones were placed in soft agar in the presence or absence of dox.

HA is the ligand to CD44 that modulates merlin activity during contact inhibition

The finding that merlin activity depended on cell-cell contact or on embedding in semisolid media, provokes an obvious question regarding the structure of the transmembrane protein CD44. Is CD44 simply an anchor in the cell membrane? Or can merlin activity be modulated through ligands that bind to the extracellular portion of CD44. To test whether a ligand or ligands exist that bind CD44 and signal growth arrest, I added soluble CD44 protein consisting of the extracellular domain of the smallest CD44 splice variant, (solCD44s) to confluent dox-inducible cells. The rational was that the solCD44s would be a useful tool to sequester active ligands away from the endogenous CD44 protein. Confluent cultures induced to express merlin showed growth inhibition (Fig. 15) as was expected from previous experiments (Fig. 4). Upon treatment with solCD44s the merlin expressing cells continued to proliferate showing no growth inhibition (Fig. 15).

Glycosaminoglycans including HA are ligands to CD44 moreover, the residues within CD44 extracellular domain which mediate HA binding have been elucidated. To test whether glycosaminoglycans are the putative ligands I tested whether solCD44s mutated in the glycosaminoglycan binding domain could still block merlin action. In contrast to the wild-type solCD44s protein the mutant solCD44s protein could not block merlin dependent growth inhibition of the confluent cultures (Fig. 15). These data taken together suggest that for activation of merlin in confluent cultures, a ligand is required to interact with CD44 and this ligand is presumably a glycosaminoglycan.

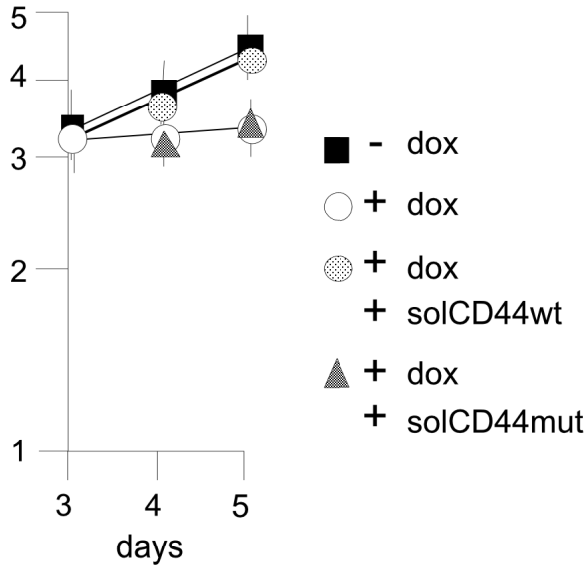

Figure15. **Block of activation of merlin by competing for endogenous extracellular ligands to CD44**. Dox-inducible cells from clone 5_4 were seeded in triplicate into 24-well plates and treated with dox to induce merlin expression. On day 3 (confluent cells) 10ng/ml of the soluble extracellular domain of CD44, either wild type (solCD44) or mutant for glycosaminoglycan binding (solCD44mut) were added. Control cells without dox were treated identically (only one data set is shown as the addition of solCD44 had no effect).

HA is a likely glycosaminoglycan candidate sequestered by the solCD44s. Previous studies have shown that HA binds to cell-bound CD44s (Sleeman *et al*., 1997). In addition it was shown that the RT4-D62T cells, and other schwannoma cell lines, synthesized HA and that CD44 was the major HA receptor in these cells. It is plausible that HA produced and accumulated locally by RT4-D6P2T cells mediating their contact inhibition when there is sufficiently high levels of merlin (Sherman *et al*., 1995). I examined whether HA serves as the natural ligand during the confluent growth by adding HAase to confluent cells and determined merlin activation by its inhibition of PDGF-dependent Erk phosphorylation. The HAase treatment abrogated the inhibitory effect of merlin (Fig 16), suggesting that the

interaction of CD44 with the glycosaminoglycan HA was responsible for the activation of merlin in confluent cultures.

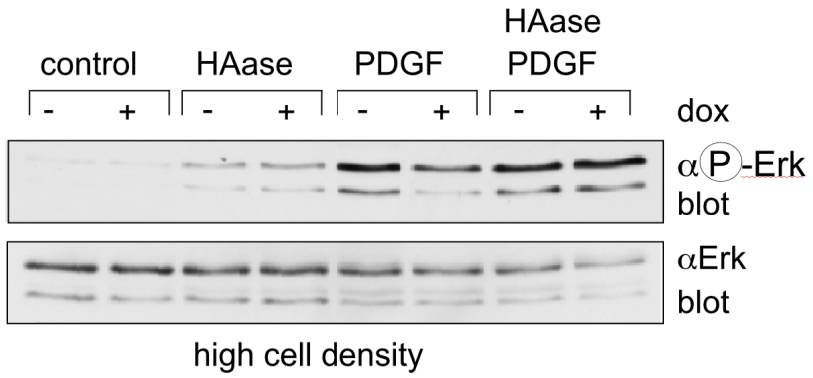

Figure 16. **HAase destroys the ligand responsible for merlin activation in confluent cells**. Dox-inducible cells from clone 5_4 were plated at a high density, serum starved for 24 hours and treated with dox for the last 8 hours (+) or not treated (-). Where indicated the cells were treated with HAase (5U/ml) for 2 hours then 5ng/ml of PDGF for 5 min. The lysates were resolved by SDS-PAGE and immunoblotted with phospho-Erk and Erk specific antibodies. Similar data were also obtained with clone 6_7.

Alternative ligands of CD44 present during logarithmic growth

So far the data suggest that HA induces the activation of merlin and causes growth arrest when cultures reach high cell density. In logarithmically growing cells CD44 could be free or occupied by a ligand that of course would not induce merlin activity. To test if there are alternative ligands bound to CD44, I added solCD44s and solCD44mutant, non HA binding, (as previously performed) to in logarithmically growing cells. Again, the assumption was that a putative ligand would be competed away from its active site. Induction of merlin in logarithmic cells did not show growth inhibition as previously shown (Fig. 4), however with addition of the solCD44s growth inhibition in the dox-inducible merlin expressing cells was observed (Fig. 17 A). Moreover, the solCD44mutant defective in HA binding induced growth inhibition as well (Fig. 17A). This suggests that in logarithmic cultures CD44 carries a ligand which represses merlin activity and this ligand is not HA. Removal of the inhibitory ligand by solCD44s induced immediate dephosphorylation of merlin (Fig 17B) and merlin´s immediate activation was measured in the blocking of PDGF-dependent Erk phosphorylation (Fig. 17C) and induction of p27 (Fig. 17D).

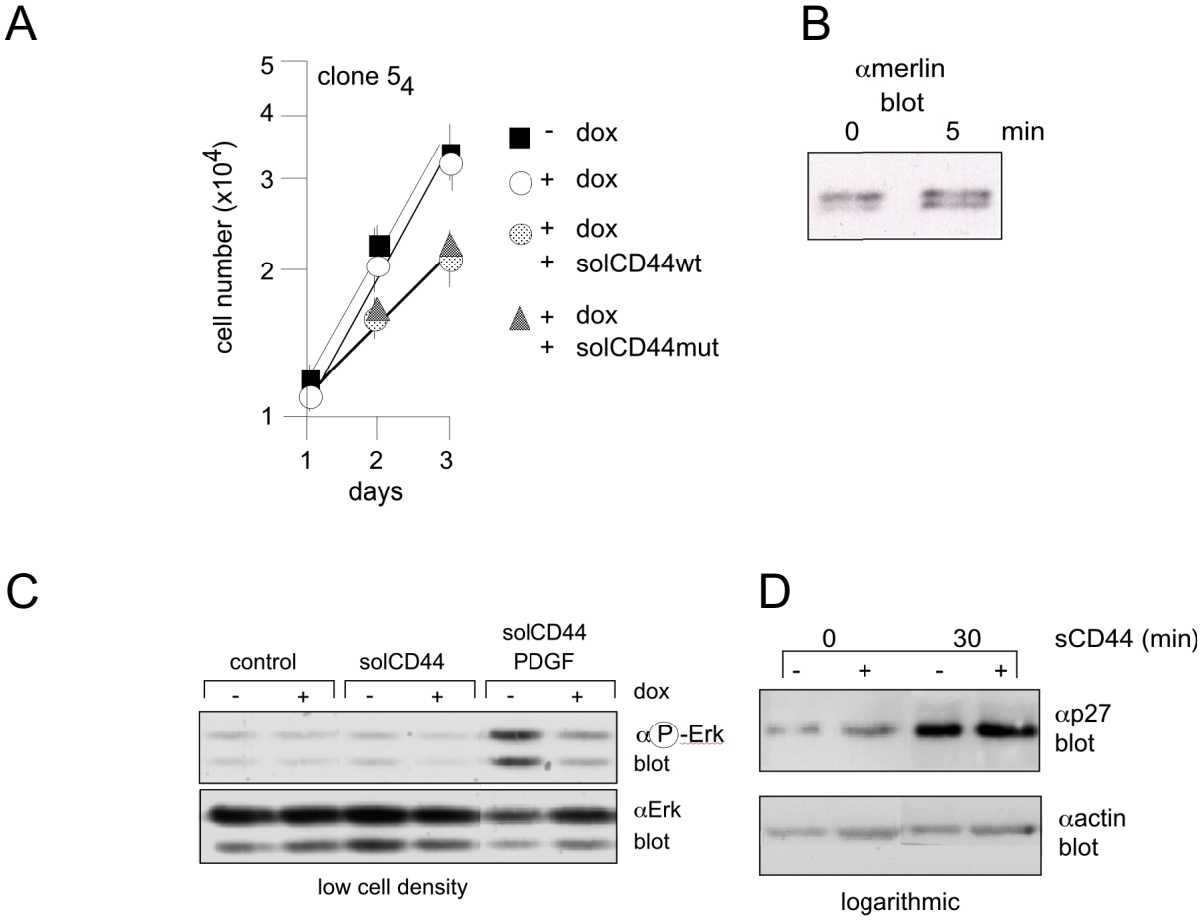

Figure 17. **Ligands that repress merlin activity are sequestered by the soluble extracellular domain of CD44 from logarithmically growing cultures.** (A) Dox-inducible clone 5_4 were seeded in triplicates, at a low density, into 24 well plates and treated with dox to induce merlin expression. 10ng/ml of solCD44s or solCD44mutant was added and each day for 3 days cells were counted. As a control cells without dox were treated identically. (B) Induced dephosphorylation of merlin in logarithmically growing cells in response to solCD44. Immunoblot detection of melrin in lysates of logarithmically growing cells of clone 5_4 treated with doxycycline for 8 hours. Compared to cells in the absense of solCD44 or addition of 10ng/ml of solCD44 for 5 min. (C) Activated merlin blocks PDGF-dependent Erk phosphorylation. Sermum starved and dox treated cells were grown at a low density and tested for PDGF dependent Erk phosphorylation after addition of solCD44 addition. Where indicated pretreatment of solCD44 (10ng/ml) was present for 10 min, PDGF (5ng/ml) for 5 mins. The lysates were resolved by SDS-PAGE and immunoblotted with phospho-Erk and Erk specific antibodies. These data was also obtained from clone 6_3. (D) Induction of p27 upon solCD44s addition. Dox-inducible cells from clone 5_4 were plated into 6 wells at a low density treated or untreated with dox. The following day 10ng/ml of solCD44s was added and the plates were incubated for 30 min. Lysates resolved of a SDS-PAGE gel and immunoblotted with an anti p27 specific antibody. For loading control an anti actin antibody was used.

HA competes with the alternative CD44 ligands present during logarithmic growth

During confluent growth these alternative ligands are either not present or HA prevents their binding. I tested whether HA could mimic these effects of contact inhibition in cultures of logarithmically growing cells by adding an excess of soluble HA to dox-inducible log cell cultures. Dox-inducible merlin cells treated with HA demonstrated significantly reduced growth prior to reaching confluency (Fig. 18A left panel) compared to no growth inhibition of log cells without addition of HA (Fig. 18A right panel). Further, cells treated with HA showed increased hypophosphorylated merlin (Fig.18B), inhibition of PDGF-induced Erk activation (Fig. 18C) and induction of p27 (Fig.18D).

Active merlin can affect the cytoskeleton organization

The simulation of “high cell density” conditions to logarithmically growing cells upon addition of HA allows to test another question of the antagonistic action of merlin compared to the ERM proteins Is merlin able to disrupt the ezrin-mediated link between cell membrane and cytoskeleton? Using fluorescence confocal microscopy I monitored the kinetics of the actin cytoskeleton dynamics in logarithmically growing dox-induced cells after addition of the merlin activator HA. Within 5 minutes merlin-expressor cells showed significant signs of actin cytoskeleton reorganization upon HA treatment (Fig. 19 lower panel) compared to dox-induced cells without HA. The numerous intact actin stress fibres observed in the absence of HA were dramatically reduced and the actin appears to be shortened and condensed into clumps (Fig 19 bottom, arrow. This effect did not change over time, the longest kinetic being 24 hours. Could the action on the cytoskeleton be linked to the effects seen on signal transduction?.

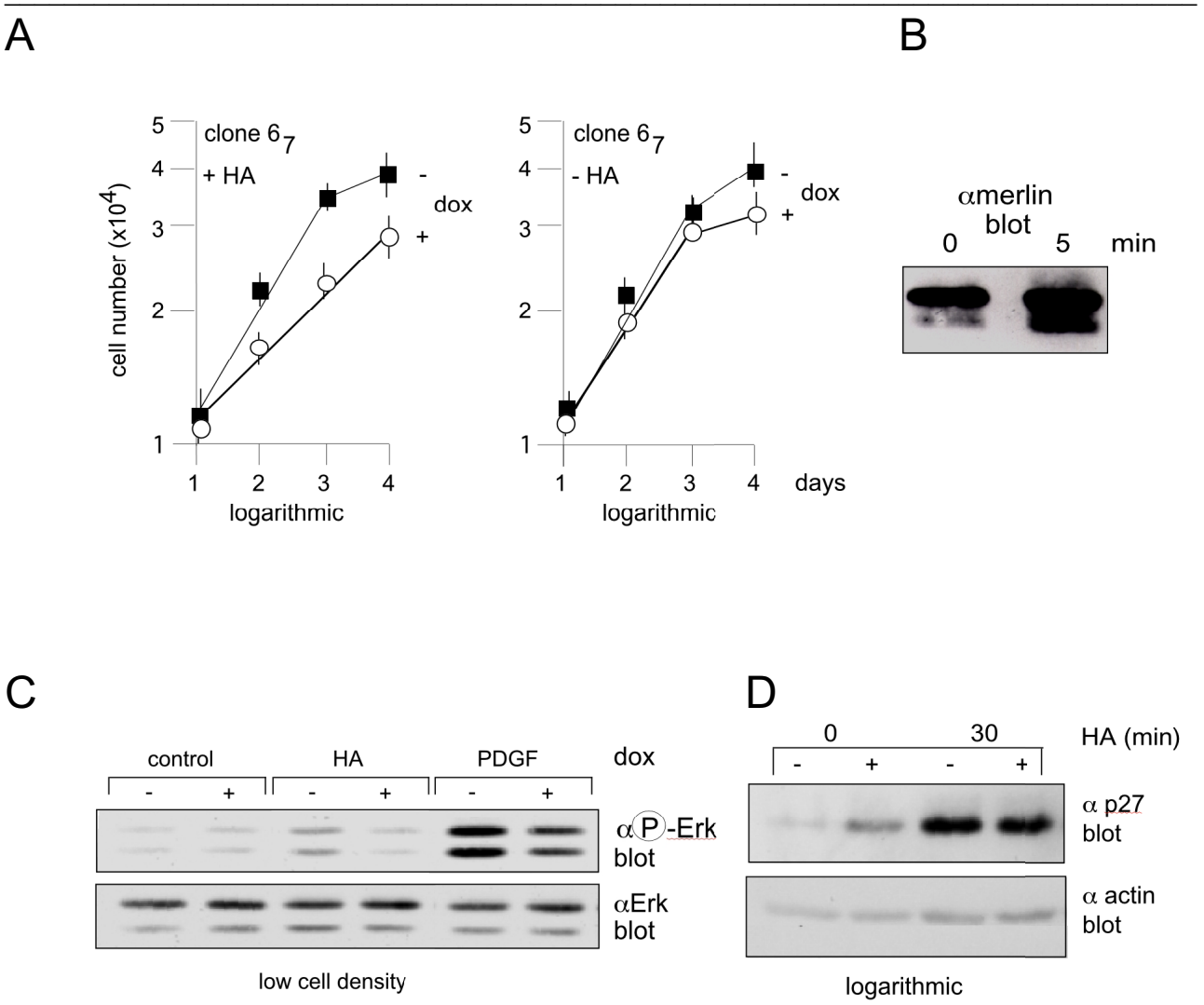

Figure 18. **HA competes with the alternative CD44 ligands present during logarithmic growth.** (A) Dox-inducible clone 5_4 was seeded in triplicates, at a low density, into 24 well plates and treated with (+) or without (-) dox. 100 μg/ml of HA was added, and each day for 3 days cells were counted. As a control cells were plated as described but without the addition of HA. (B) Induced dephosphorylation of merlin in logarithmically growing cells in response to HA. Immunoblot detection of merlin in lysates of logarithmically growing cells of clone 5_4 treated with dox for 8 hours. Compared to cells in the absense of HA or addition of 100 μg/ml of HA for 5 min. (C) Activated merlin blocks PDGF-dependent Erk phosphorylation. Cells were seeded at a low density, serum starved for 24 hours dox treated for 8 hours and then tested for PDGF dependent Erk phosphorylation after addition of HA. Where indicated pretreatment of hyaluronan (100 μg/ml) was present for 10 min, PDGF (5ng/ml) for 5 mins. The lysates were resolved by SDS-PAGE and immunoblotted with phospho-Erk and Erk specific antibodies. These data was also obtained from clone 6_3. (D) Induction of p27 upon HA addition. Dox-inducible cells from clone 5_4 were plated into 6 wells at a low density treated or untreated with dox. The following day 100 μg/ml of HA was added and the cells were incubated for 30 min. Lysates resolved of a SDS-PAGE gel and immunoblotted with an anti p27 specific antibody. For loading control an anti actin antibody was used.

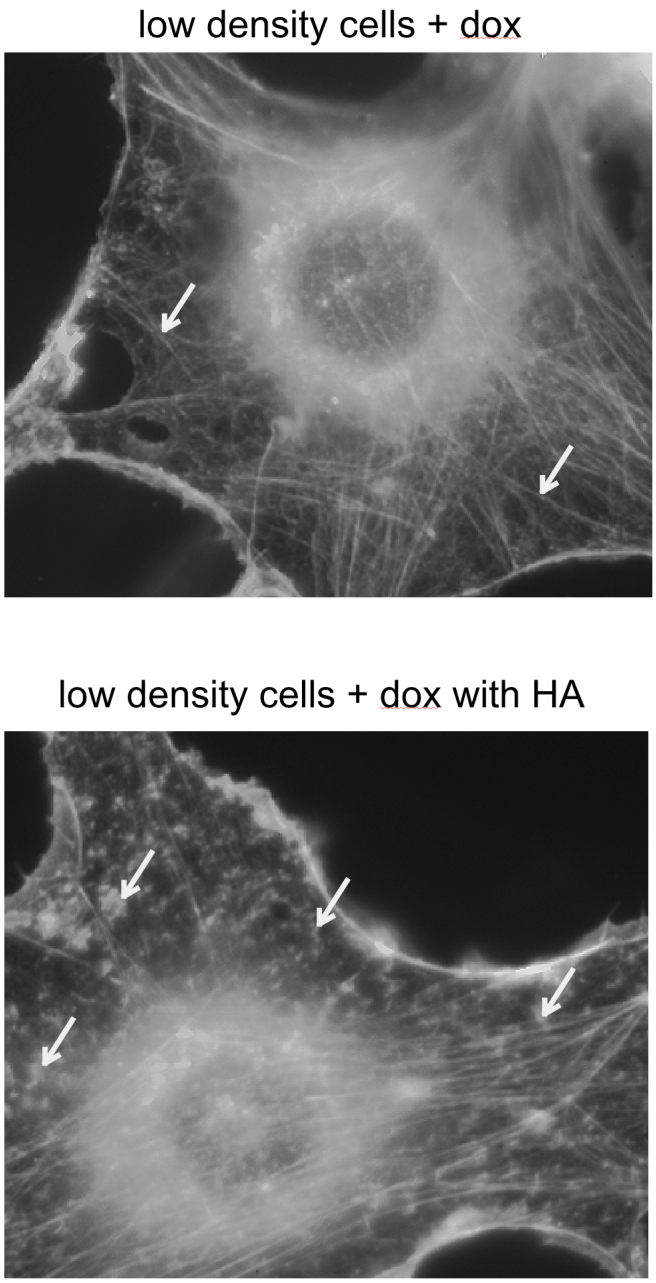

Figure 19. **Merlin activated by HA alters the actin cytoskeleton.** Dox-inducible cells were plated on glass slides at a low density. The following day cells were serum starved and dox was added for at least 8 hours. 100µg/ml of HA was added for 5 minutes. Cells were fixed and polymerised actin was detected using a fluroescently labelled phalloidin (Texas Red).

CD44 mediates merlin-dependent growth inhibition in several cell lines

To ascertain whether CD44 is the major mediator of merlin activity and also works in other cell types, I took two approaches: attempting to activate merlin by specific anti CD44 antibodies, and by studying the HA-dependent growth inhibition in a CD44-negative cell line prior to and after transfection with a CD44 expression plasmid. Growth inhibition of cells in log phase as determined by the incorporation of BrdU was not only established by the addition of HA, but also by the CD44 specific antibody IM7, in NIH3T3 cells (Fig. 20 top panel). An antibody directed against the HA-binding motif of CD44, KM81, also triggered growth inhibition of NIH3T3 cells (not shown). These data directly link CD44 to HA-induced growth inhibition through merlin, and indicate that CD44 and merlin function together in cell types other than the RT4-D6P2T cell line.

As a second test of the requirement for CD44 in mediating HA-induced activation of merlin's growth inhibitory function, I transfected both wild type rat CD44s and CD44s with a deletion in the ERM-binding domain into a human melanoma cell line (RP-MC) that lacks endogenous CD44 but which expresses endogenous merlin (not shown). HA had no effect on BrdU incorporation of logarithmically growing RP-MC cells transfected with empty vector or expressing mutant CD44 (Fig. 20 lower panel). However, HA significantly inhibited BrdU incorporation in RP-MC cells expressing wild type CD44 (Fig. 20 lower panel) but not the mutant defective in merlin and ERM binding. These data confirm that CD44 is absolutely required for HA-mediated activation of merlin's growth suppressive function.

I conclude that at least two specific and different ligands are bound to CD44. One characteristic for, and specifying logarithmic growth, the other occurring in confluent cells and mediating contact inhibition. The CD44 ligand of the logarithmic condition appears to be inhibitory for merlin activation as its removal by the soluble CD44 extracellular domain triggered merlin activation and this was independent of HA. In contrast, the CD44 ligand in confluent conditions where merlin is activated appears to be HA. Taken together the data show that CD44 confers extracellular influences on merlin activity. The type of ligand bound to CD44 determines whether a silencing, contact inhibiting complex or a growth-promoting complex is formed.

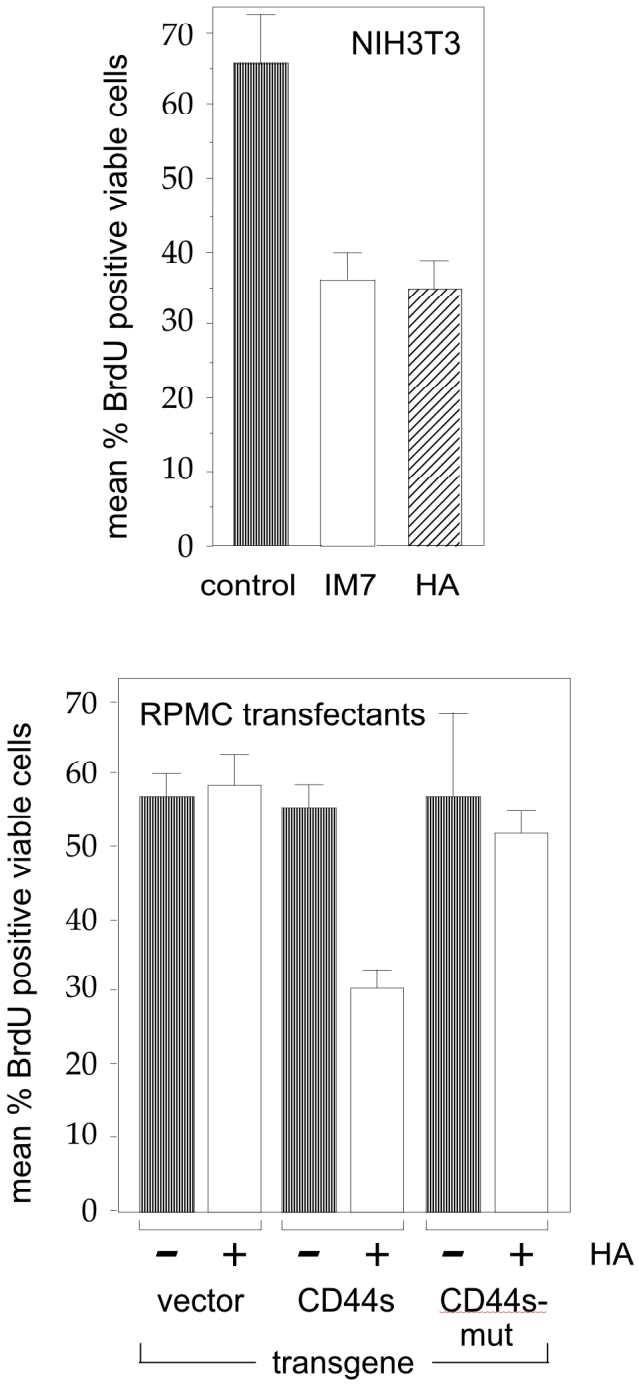

Figure 20. **In several cell lines inhibition of DNA synthesis is mediated by CD44.** The CD44 specific antibody IM7 activates merlin. NIH3T3 cells or cells of RP-MC transfectants were seeded into 8-well chamber slides at low density. Overnight either IM7 or Hermes-3 (control antibody) or HA (100 μg/ml) was present and dox was added where indicated (control without antibody). Cells were labelled with BrdU and stained for incorporation using a biotinylated BrdU antibody. The mean percent cells that incorporated BrdU, are plotted and standard errors are indicated.

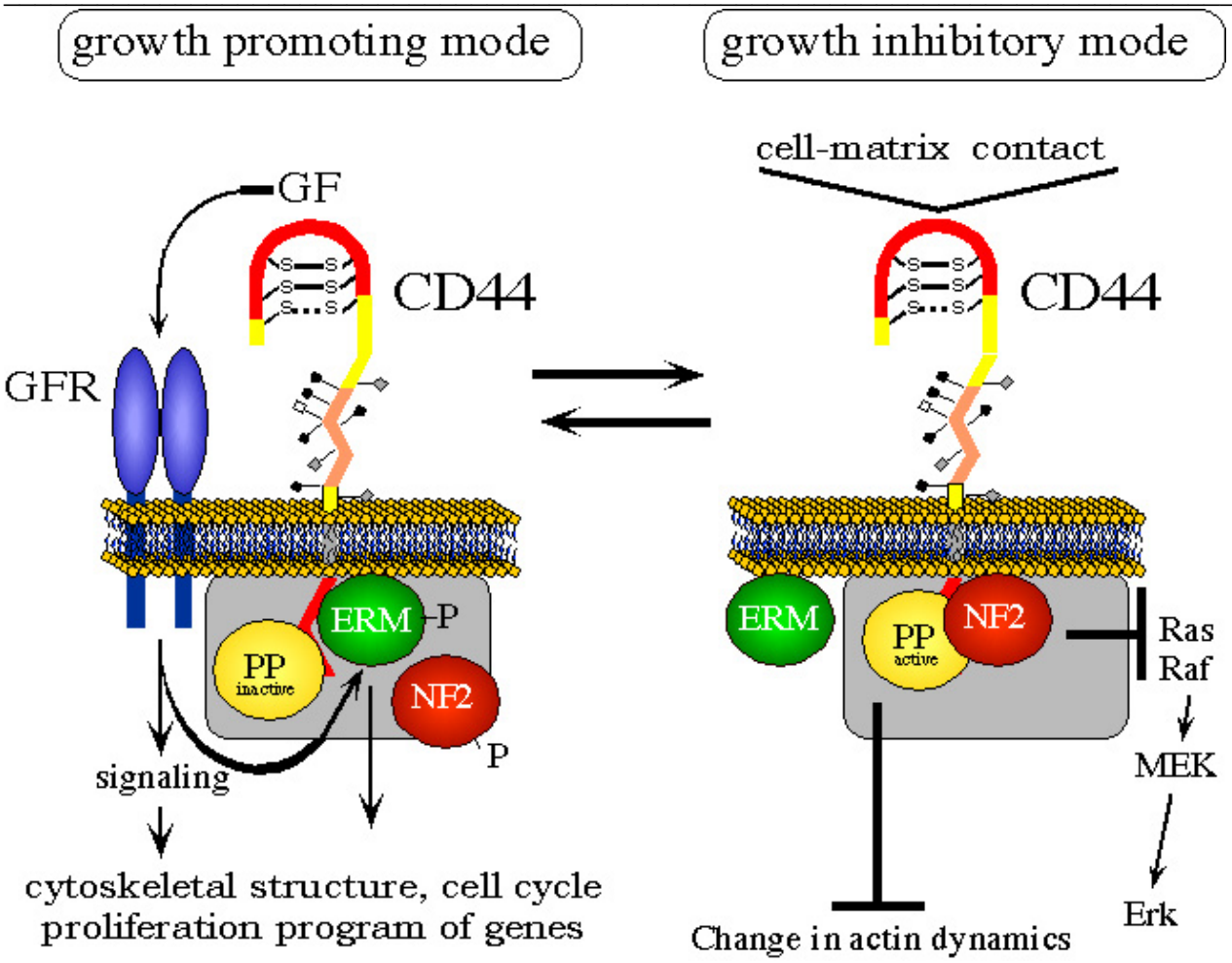

Figure 21. **Model of CD44 action in logarithmic and confluent growth conditions**. Specific ligands determine two functional states of CD44 which influence cytoplasmic complexes. The ligands of the growth mode have not been defined. It is, however, known that CD44 and particularly the larger splice variants serve as platform for the activation of growth factors (GF; Bourguignon et al., 1997; Sherman et al., 1998; Van der Voort et al., 1999). PP = protein phosphatase. Additional components are likely associated with the CD44-bound complexes (grey boxes).

DISCUSSION

The process of contact inhibition demands the existence of one or several cell surface sensors which communicate with the cell nucleus to inhibit cell cycle progression. Alternatively during a growth phase, these contact sensors must be silent or exist in another growth-permissive conformation, only becoming growth-inhibitory during cell-cell or cell-matrix contact. I have identified one such sensor complex, which can specify a switch between cellular growth and contact inhibition. I provide evidence that the transmembrane protein CD44 and three band 4.1 family members, merlin, ezrin, and moesin, are involved in a molecular switch that signals cellular growth or growth inhibition.

To study the functions and biochemical activities of merlin, I looked for a cell system in which merlin expression was either lost or aberrantly regulated and which was amenable to biochemical and molecular manipulation. There were several reasons in deciding to work with the ENU induced rat schwannoma cell line RT4-D6P2T. Unlike the human schwannomas from NF2 patients, (Hung *et al*., 1999; Pelton *et al*., 1998; Rosenbaum *et al*., 1998) the RT4-D6P2T maintain wild type merlin, but are transformed by mutations in a number of genes including an activating mutation of the neu protooncogene (Bargmann and Weinberg, 1988; Nikitin *et al*., 1991; Weiner *et al*., 1989). One advantage of these transformed cells, in comparison to the human schwannomas, is that they can be maintained in long-term cultures and are easier to use in biochemical and molecular studies. In addition, I found that merlin expression is aberrantly regulated in the RT4-D6PT cell line. For example at high cell density there was no increase in merlin expression when compared to primary rat Schwann cells. If increased endogenous merlin expression at high cell density contributes to growth arrest in normal cells, then increasing merlin expression in malignant rat schwannoma cells, to a degree similar to that observed in confluent primary rat Schwann cells, should inhibit cell growth. This assumption was valid since the inducible overexpression of merlin inhibited the rat schwannoma cell growth, indicating that RT4-D6P2T cells are an appropriate model system for the study of merlin function.

The tumor suppressor function of merlin upon overexpression in the RT4-D6P2T cells is documented by reduced schwannoma growth in the animal, by reduction in the number of agar colonies and by a corresponding change in cell cycle parameters. Merlin interfered

with growth factor dependent or Ras-dependent signal transduction to MAP kinase and the step of interference was located downstream of Ras and Raf and upstream of MEK. Moreover, several novel features of merlin function were identified. Only at high cell density overexpressed merlin was activated and growth inhibiting. Merlins growth inhibitory function was associated with dephosphorylation and depended on interaction with a basic amino acid motif in the cytoplasmic tail of CD44. Other 4.1 family members ezrin and moesin, which also bind to the same basic amino acid motif in CD44 (Legg and Isacke, 1998) were absent within this growth inhibitory complex (Fig 21). Alternatively in logarithmically growing cells, merlin was not growth inhibiting. It was hyperphosphorylated and found in a complex with ezrin and moesin. This growth permissive complex (Fig 21) was also associated with the cytoplasmic tail of CD44. Sequestering experiments with soluble extracellular CD44 proteins revealed that merlin activity and phosphorylation status depended on alternative extracellular ligands associated with the N-terminus of CD44. For example, treating logarithmically growing cells with soluble extracellular CD44 proteins (either wild type or mutated in the glycosaminoglycan binding domain) suggested that growth promoting ligands are associated with CD44 and that these putative ligands (or ligand) are not glycosaminoglycans. Alternatively, treating confluent cells with wild type but not mutant soluble CD44 proteins abrogated merlin growth inhibition, suggesting ligands (or ligand) binding to the glycosaminoglycan binding domain specifies a growth inhibitory mode. HA is the likely ligand in confluent conditions, as it could induce merlin dephosphorylation and inhibit growth in low density cultures. Further, removal of HA at high cell density by treatment with hyaluronidase abrogated the inhibition of MAP kinase activity.

Growth arrest in contact inhibited cells has been correlated with increased levels of the cell cycle inhibitors p21Cip1 and p27Kip1 leading to accumulation of hypophosphorylated Rb, a key regulator of cell cycle progression (Fuse *et al*., 2000; Yanagisawa *et al*., 1999). The rat schwannomas, typical for transformed cells, grow independently of contact inhibition and maintain cell cycle profiles similar to proliferating cells. However, merlin overexpression inhibited the cell cycle in the G1 phase in these cells, at confluency, leading to accumulation of hypophosphorylated Rb. I therefore expected that the expression of cell cycle inhibitors would be significantly higher in merlin overexpressors. However, at confluency even in the untransfected cells both cyclin-dependent kinase inhibitors p21Cip1 and p27Kip1 are strongly upregulated, even though the growth arrest

was not proportional to that of merlin overexpressors. So why is the p27 increase not sufficient to induce cell cycle arrest? One explanation could be that the cell cycle inhibitors are inappropriately located. Interestingly, when the subcellular distribution of cyclin-Cdk complexes and their inhibitors in normal fibroblasts was compared to transformed fibroblasts (Orend *et al*., 1998), the transformed cells maintained anchorage-independence by excluding the majority of p21 and p27 from the nucleus and thereby compromising the association of nuclearly localized inhibitors with cyclin E-Cdk2 complexes. In normal cells the Cdk inhibitors would be nuclear and the result would be loss of Cdk2-associated kinase activity leading to growth arrest. It is plausible that transformed schwannoma cells, although responding to the cell-cell contact by increased expression of inhibitors, circumvent the contact inhibiting growth control, by maintaining cell-cycle relevant p27 in the cytoplasm. It is likely therefore that in the transformed rat schwannomas the interruption of signal transduction by active overexpressed merlin causes transfer of p27 to the nucleus.

Loss of contact inhibition is a common feature of transformed cells, and it is more than likely linked to the activation of particular oncogene-driven signalling cascades. A previous report indicated that merlin overexpression reversed anchorage-independent growth in soft agar of v-Ha-Ras-transformed NIH3T3 cells (Tikoo *et al*., 1994). Consistent with these findings, I found that increasing merlin expression in the transformed RT4-D6P2T cell line at high cell densities induced dephosphorylation of merlin with subsequent growth inhibition. This suggested that an active merlin can reverse the dominant growth signals observed in these transformed cells. Further, active merlin inhibited growth factor dependent MAP kinase signalling. Growth factors themselves have been shown to alter the phosphorylation status of merlin: For example Shaw et al correlated the appearance of a hypophosphorylated form of merlin in cells starved of serum and showed that addition of serum rapidly induces merlin phosphorylation with the complete loss of the hypophosphorylated form (Shaw *et al*., 1998). By analogy these growth promoting factors would induce phosphorylation of merlin and block its growth inhibitory activity in logarithmically growing cells. My data support this notion since only the phosphorylated form of merlin is observed in logarithmic cultures. Taken together this would indicate that merlin is both upstream and downstream of mitogen signalling pathways. Mitogens

suppress merlin activation during logarithmic growth while in confluent cultures an activated merlin blocks mitogen signalling.

Other 4.1 family members are also phosphorylated in response to growth factor stimulation (Gould *et al*., 1986; Jiang *et al*., 1995; Krieg and Hunter, 1992). Both epidermal growth factor (EGF) and hepatocyte growth factor/scatter factor (HGF/SF) lead to phosphorylation of ezrin on tyrosine 145, 353 and threonine 567 residues. Moreover, phosphorylation of ERMs on the conserved threonine residue, activates these proteins resulting in a conformational change with subsequent binding to membrane targets and actin cytoskeleton (Gary and Bretscher, 1995; Gonzalez-Agosti *et al*., 1999; Gronholm *et al*., 1999; Hirao *et al*., 1996; Magendantz *et al*., 1995; Tsukita and Yonemura, 1997). This ERM targeting is proposed to mediate actin dynamic structures such as the formation of lamellipodia, microvilli and cell-matrix adhesion sites (Tsukita and Yonemura, 1997), The small GTP binding protein Rho has been implicated in regulating the association of ERM proteins with their transmembrane targets. Growth factor stimulation evoked a Rho dependent activation and redistribution of these ERM proteins (Hirao *et al*., 1996; Shaw *et al*., 1998). Morever a Rho kinase has been identified and was necessary and sufficient for ERM phosphorylation. Similar to ERMs, merlin is targeted to the membrane localizing in various areas of membrane remodelling, particularly membrane ruffles (Gonzalez-Agosti *et al*., 1996). There is correlative evidence suggesting that merlin also plays a role in Rho and Rac signalling pathways, since inhibiting these pathways in human schwannoma cells from NF2 patients reversed the aberrant organization of the actin cytoskeleton (Pelton *et al*., 1998). Taken together I propose that merlin is both upstream and downstream of Rac and Rho signalling, such that a putative Rac or Rho kinase would phosphorylate and inactivate merlin during logarithmic growth. Then during contact inhibition, an active merlin would in turn negatively regulate Rac and Rho dependent signalling.

The N-terminus and C-terminus of the ERMs interact strongly with each other resulting in inactive forms in which the binding sites for membrane components and F-actin are masked. Activation of these proteins releases this intra- and inter-molecular binding, exposing the N-terminus and C-terminus for subsequent binding to its targets. Merlin has also intra- and inter-molecular binding properties similar to the ERMs. However, merlin has a unique mechanism of inhibiting cell proliferation via self association where the N- and C-termini interact producing an active molecule (Sherman *et al*., 1997). I showed that the N-

terminal and C- terminal domains of wild type merlin were necessary for its function and that indeed the N- and C- terminal halves can coimmunoprecipitate. There are however, no data so far to show that a closed conformation is functionally relevant. Further studies should identify whether N- and C- terminal halves of merlin are interacting while bound to the tail of CD44 and identification of point mutations in merlin that disrupt this N- and C- interaction should give some functional relevance to this complex.

The overexpression of N- terminal halves of merlin did not suffice to inhibit growth, suggesting that not only merlin must be localized via its N- terminus to the transmembrane protein CD44 but that the C- terminus is functionally distinct. The N- terminal half of merlin shows the greatest degree of similarity to ezrin, whereas the C- terminus of both proteins is less homologous. For example, merlin lacks the consensus actin binding domain and the phosphorylation sites between these two proteins are not conserved. By analogy then the N- terminus would direct both proteins to their appropriate active sites, such as binding to the cytoplasmic tail of CD44, and their alternative function would then be defined by their C- terminus. This idea that ezrin and merlin are targeted to the same sites in the membrane but are functionally distinct presents a novel antagonism within this family of proteins. Since merlin lacks the proposed F-actin binding domain found in the ERMs, an active full length merlin should by antagonism with ezrin interrupt the ezrin-mediated link between cell membrane and cytoskeleton. My experiments suggest the formation of the actin cytoskeleton is disrupted with an activated merlin, since HA addition to logarithmically growing cells activated merlin with subsequent loss of stress fibre formation. These findings are consistent with previous observations where merlin overexpression in rat cells (Gutmann *et al*., 1999) or lack of expression in human schwannoma cells resulted in altered cytoskeletal organisation (Pelton *et al*., 1998; Rosenbaum *et al*., 1998).

Alteration in actin dynamics and inhibition of signal transduction via merlin may depend on common targets. The small GTPases Cdc42, Rac and Rho are not only involved in the formation of actin-dependent structures in response to mitogens but also contribute to cell cycle progression and transformation (Olson *et al*., 1995; Ridley, 1995). Cross talk between Raf-1 and the small GTPases on cellular transformation has been observed. For example, Rho, Rac1 and Cdc42 have been implicated to cooperate with Raf-1, via PAK (p21-activated kinases) to activate the MAP kinase pathway (King *et al*., 1998; Tang *et al*.,

1999). If this coorperation is necessary in MAP kinase signalling then merlin's growth inhibiting function may depend on inhibiting signalling components, such as Rac and Rho. It is plausible that signalling components proximal to the membrane are dependent on a type of defined organization for their activity, and this specific organization may be dependent of the actin cytoskeleton. This may explain a mechanism by which active merlin negatively regulates the Ras MAP kinase pathway upstream of MEK. Perhaps MEK activity is dependent on the regulation of scaffolding proteins that controls the proximity of Raf to MEK, analogous to the Ste5 protein in yeast, where Ste5 has a critical structural role linking multiple kinases together for sequential activation within a MAP kinase cascade required for mating (Choi *et al*., 1994). Another link between cytoskeleton organisation and signal transduction has been suggested by the observation that in NIH3T3 cells at high cell density, a UV, PDGF or EGF dependent activation of Jun is inhibited (Lallemand *et al*., 1998). This inhibition depended on the integrity of the actin cytoskeleton, which was overcome by expression of constitutively active Rac or Cdc42.

ERM proteins and merlin bind to the same basic amino acid motif located in the cytoplasmic tail of CD44. However, in confluent cultures only active hypophosphorylated merlin was bound to CD44, whereas ezrin or moesin were bound to CD44 only in logarithmically growing cultures. This indicates that ezrin. moesin and merlin proteins are active under different growth conditions. In logarithmic cultures ezrin and moesin would be phosphorylated and in an active open conformation with an exposed N-terminus targeted to CD44 and exposed C-terminus for binding to F-actin. The overexpression of merlin in this growth permissive mode could not compete for the ezrin binding to CD44 since it is phosphorylated and inactive. Further, in soft agar assay where merlin is activated, full length ezrin could not compete since in this growth inhibiting mode ezrin is inactive, probably in a closed conformation, masking its membrane and F-actin binding sites. In agreement with the requirement for growth-condition-dependent activation, overexpresson of the N- terminal halves of either merlin or ezrin which do not require growth-condition activation exerted dominant-negative effects on merlin action.

Ezrin has been implicated by other groups to be an important protein for growth control (Kaul *et al*., 1996). Although the function of ezrin is not addressed here I would predict that its function is also likely dependent on its association with CD44 or a similar transmembrane protein. In view of these data when I kept the CD44 tail overexpressors in

low density culture for several days (in contrast to cells in confluence), I invariably lost tail expression suggesting that sequestering ezrin away from CD44 was not compatible with proliferation. My data also suggests that in logarithmically growing cells, ezrin, moesin and phosphorylated merlin are associated with CD44. It is most likely that merlin is complexed indirectly to CD44 through heterodimerisation with the ERMs. These merlin-ERM interactions are implicated by my ezrin-merlin coimmunoprecipitation experiments and this interpretation is consistent with a number of previous in vitro studies where merlin-ERM form heterodimers (Gronholm *et al*., 1999; Meng *et al*., 2000). However, the function of these heterodimers is unknown but it is likely that merlin is an inactive molecule and is in close association ready to be activated.

In my model, the switch from one mode to the other would be accomplished by a change in extracellular CD44 ligands. I have shown that HA is the ligand specifying contact inhibition in the RT4-D6PT cell line. The RT4-D6PT cell line is known to secrete HA (Sherman *et al*., 1995) but whether there is a transient overexpression at high cell density is not known. It is possible that HA is secreted constitutively and membrane bound only signalling growth inhibition when cells are in close contact. This would be consistent with HAs numerous roles in tissue formation where cells contact dense ECM and stop proliferating (Laurent *et al*., 1996). Intracellular signals transmitted upon HA binding to CD44 has recently been reported showing the direct activation of Rac, with actin cytoskeleton rearrangements (Oliferenko *et al*., 2000). This observation suggests a direct involvement of HA and CD44 mediating signalling to actin cytoskeleton components possibly involving ERM proteins. This is consistent with my observation that HA mediates signals activating merlin, although the consequence in activating merlin would be antagonistic to the ERM proteins. This discrepancy may be due to size differences in HA, for example low molecular weight HA has been shown to have an opposite influence on cell proliferation compared to high molecular weight HA (Forrester and Balazs, 1980; West and Kumar, 1989). In addition several laboratories have used HA to try and interfere with tumor growth. Tumor cells were reported to either profit, not profit or even die upon removal of HA (Bartolazzi *et al*., 1994; Dahl *et al*., 1985; Dasgupta *et al*., 1996; Knudson *et al*., 1989; Sleeman *et al*., 1996). Perhaps in my case high molecular weight HA predominantly activates merlin and lower molecular weight would differentially activate ERM proteins.

The growth-permissive mode could represent a default state. However, I found that the soluble extracellular domains of CD44, intended to sequester CD44 ligands from endogenous CD44, exerted an effect on logarithmically growing cells (merlin activation) that was opposite to that observed in confluent cells (where soluble CD44 abolished merlin activation). This ligand is different from the one acting in confluent cultures since, my results indicate that this merlin-inhibitory effect does not depend on the HA binding motif of CD44. Although I can not exclude the possibility that these soluble CD44 proteins modulate a structure of membrane-bound CD44 in an as of yet unknown fashion, I propose, that soluble CD44 sequesters an inhibitory ligand that prevents merlin activation during logarithmic growth. In support of this idea, merlin is rapidly dephosphorylated when cells are treated with trypsin (Shaw *et al*., 1998), with the assumption that trypsin will eliminate ligands bound to the cell surface. It is possible, that the states of ERMs and merlin are, in addition to CD44, influenced by other signal transduction pathways that become dominant if CD44 is not bound to a ligand

I have not yet identified what ligand the soluble CD44 protein sequesters from growing cells. Other types of experiments have yielded plausible candidates for extracellular ligands that might be responsible for the CD44-mediated complex on the inner side of the plasma-membrane. There is evidence that growth factors and other growth-promoting proteins utilize CD44 as a platform required for their action (Bennett *et al*., 1995; Lamb *et al*., 1997; Sherman *et al*., 1998; Tanaka *et al*., 1993; van der Voort *et al*., 1999; Yu and Stamenkovic, 1999). This platform function may be enhanced by clustering of the CD44 molecules (Sleeman *et al*., 1996; Yu and Stamenkovic, 1999). One way to enhance CD44 clustering appears to be the inclusion of variant exon sequences (Sleeman *et al*., 1996). Clustering can also be achieved by CD44-specific antibodies (Yu and Stamenkovic, 1999). This latter finding suggests that the platform function may involve other domains of CD44 in addition to the HA binding motif and that, in certain cases, the platform function and the activation of merlin can be dissociated.

The most interesting function of the platform concerns the activation of receptor tyrosine kinases. A function of CD44 for cell proliferation under physiological conditions has been observed in transgenic animals expressing antisense CD44 in keratinocytes where a severe loss of skin-regenerating potential is observed (Kaya *et al*., 1997) and in

embryogenesis where antibodies to a splice variant of CD44 inhibited limb outgrowth (Sherman *et al*., 1998). Irrespective of the exact nature of this "growth factor presentation", these data link the CD44 complex to growth-promoting signalling. From these sets of recent observations I conclude that CD44 plays a dual role, represented by the two conformations proposed in Fig 21. During tumorigenesis cells apparently develop mechanisms by which they overcome the tumor-suppressing function of CD44 while conserving other CD44 functions such as growth-promotion or migration. One could envision several ways that tumor cells could shift the balance of the CD44 complexes towards the growth mode, including inactiviation of merlin or alteration of CD44 alternative splicing, such that the balance of CD44 molecules on the cell surface no longer favours interactions with HA.

In addition to the CD44-merlin complex described in this report, other mechanisms are known to mediate cell contact-dependent inhibition of growth. For example other adhesion molecules, such as integrins interacting with ECM or cell-cell contact via cadherins have been shown to induce cell cycle arrest (Aoki *et al*., 1991; Giancotti and Ruoslahti, 1999; Kandikonda *et al*., 1996; Takahashi and Suzuki, 1996). Generally this suggests that adhesion-dependent signals have an important role in growth control, however the downstream signalling components utilized by these adhesion molecules appears to be different to CD44. For example, cell-cell adheren junctions of epithelial cells, the tumor-suppressor gene product adenomatous polyposis coli (APC) binds to β_2-catenin which is cytoplasmically associated with cadherin (Hulsken *et al*., 1994). ECM binding to integrins triggers the assembly of focal adhesion contacts with focal adhesion kinase (FAK) being a crucial signalling molecule for this assembly (Schwartz, 1997). Taken together it is possible that these mechanisms operate either together or independently mediating cellular contact to ECM or with other cells. In addition, integrins appear to play a dual role in growth control analogous to the bimodal function of CD44 described in this thesis where CD44 plays a dual role in either promotion or inhibition of growth. For example the fibronectin receptor $\alpha5\beta1$ integrin was described to be decreased in transformed cells, then upon restoration of $\alpha5\beta1$ integrin induced growth inhibition. Interestingly ligation of $\alpha5\beta1$ integrin on the same cells, by attachment to fibronection, led to activation of immediate early genes suggesting that $\alpha5\beta1$ integrin plays a role in regulating cell proliferation (Ben-Ze'ev, 1997; Rosales *et al*., 1995).

It is more than likely that other intracellular components are part of the growth-inhibitory or growth-promoting complex. During a change in mode the components of the complex are probably not totally disassembled/reassembled. Rather they remain in relative proximity, which would perhaps explain colocalization data reported for ezrin, merlin and CD44 (Sainio *et al*., 1997). A regulatory co-factor for sodium-proton exchange is a common interactor for merlin and the ERM proteins. It is not yet known how this interaction relates to merlin or ezrin function. Other merlin-binding proteins have been found by two-hybrid analyses and confirmed by coimmunoprecipitations (Murthy *et al*., 1998; Reczek *et al*., 1997). Components such as protein kinases must be in close proximity preventing merlin activation while maintaining a CD44-ERM association (Fig 21). A Rho-kinase has been shown to phosphorylate ERMs and In coimmunoprecipitation experiments, Rho-kinase was found in a complex with CD44 (Matsui *et al*., 1998). Merlin is not phosphorlylated via Rho kinase but it has been suggested, but not proven, to be phosphorylated by a putative Rac kinase. It is plausible that a putative Rac kinase may be associated in the complex maintaining an inactive merlin during a growth phase. This complex must be also controlled by one or several phosphatases that dephosphorylate and activate merlin. To further emphasize the importance of dephosphorylation the merlin mutant L64P was not dephosphorylated at high cell densities and was found to be inactive in several growth assays. The next challenge would be to find which membrane-located phosphatase and kinase are responsible in activating/inactivating merlin, and how the extracellular ligands, including HA, bound to their partner CD44 controls the activity of these putative partners. Apart from alternative intracellular components assembled at the CD44 cytoplasmic tail, merlin may bind other membrane proteins providing a similar contact sensing function. This notion is valid since membrane proteins such as ICAM-2 and CD43 have been shown to bind in vitro to ERM proteins (Legg and Isacke, 1998; Yonemura *et al*., 1998). These putative alternative membrane binding partners for merlin may be cell-type specific or perhaps dependent on alternative extracellular ligands.

The identification of a growth-inhibiting verses a growth-permissive complex originated while attempting to analyze the function of the nf2 tumor suppressor gene product, merlin. Loss of heterozygosity (LOH) at the nf2 locus is associated with neurofibromatosis type 2 (NF2), an inherited human disease predisposing individuals to develop Schwann cell tumors. Why loss of merlin function contributes to the formation of only Schwann cell tumors or why Schwann cells are susceptible to LOH is presently not known. In contrast

to human NF2 patients, mice hemizygous for the Nf2 gene do not develop schwannomas, the hallmark feature of neurofibromatosis type 2. Firstly homozygosity for each mutant allele results in embryonic lethality (McClatchey *et al*., 1997). Secondly, mice heterozygous for each mutant allele do not show features of human NF2. In particular they demonstrate a tumor spectrum that differs entirely from that observed in NF2 patients (McClatchey *et al*., 1998). My data, also showed that merlin acts as a tumor suppressor not only in schwannomas but also in NIH3T3 fibroblasts and RPMC cells, suggesting that merlin would function in several cell types. Consistent with this idea Nf2 mutations have also been reported in other tumors unrelated to NF2 disease, including mesotheliomas and breast carcinomas (Bianchi *et al*., 1994; Cheng *et al*., 1999; Deguen *et al*., 1998). In addition judging from the diversity of tumors arising from the heterozygote knock-out mouse, merlin appears to exert functions in a variety of cells (McClatchey *et al*., 1998). Several factors may explain the lack of overlap of the tumor spectrum in humans and mice. One hypothesis is that the tumor spectrum is modulated by the rate of the loss of the wild-type allele in specific tissues. The conditional somatic mutation of the Nf2 gene has addressed this hypothesis. The conditional homozygous Nf2 knockout mice with Cre-mediated excision of Nf2 in Schwann cells showed characteristics of neurofibromatosis type 2 (Giovannini *et al*., 2000).

In conclusion, I have found that merlin forms a complex with CD44 that is critical for matrix contact mediated growth inhibition. As cells proliferate and form tissues, their proliferation must be regulated such that at a particular cell-matrix density, they become quiescent (Holley, 1975). This growth regulatory circuit would be disrupted in schwannomas and other tumors with NF2 mutations, such that cells could not transduce signals mediated by CD44 ligands which normally indicate the conditions of high matrix density. One might expect that cells under these conditions would be at least weakly hyperproliferative, as is the case of the typically slow-growing tumors associated with NF2. These observations have significant implications for understanding the nature of CD44 function and how loss of merlin contributes to tumorigenesis of schwannomas and other tumors. In addition during logarithmic growth I found ezrin, moesin and phosphorylated merlin in a complex with CD44. This additional observation is novel since it identifies CD44 as having a bimodal function. Depending on the ligand bound CD44 would signal either growth arrest or proliferation. HA signals growth- arrest and a ligand for logarithmic growth is as yet not

identified. In addition to extracellular ligands, intracellular proteins proximal to the membrane, such as ezrin and merlin appear to have antagonistic functions, both binding to CD44 but with alternative downstream consequences. The fact that they appear to heterodimerise raises interesting possiblities of how these proteins regulate each other in masking or unmasking important phosphoylation sites or other relevant functional domains.

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