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Abstract

CD44 is a widely distributed adhesion molecule implicated in a variety of physiological and pathological processes. CD44 proteins function as a molecular switch between cell growth and inhibition of proliferation by interacting with ERM (ezrin/radixin/moesin) and merlin proteins through their cytoplasmic tail. The CD44 ERM/merlin complexes must be tightly regulated to attain the optimal signaling capacity, suggesting that other intracellular components are probably part of this complex.

In an attempt to understand intracellular signaling triggered by CD44, I tried to identify intracellular components associated with CD44 under conditions of growth inhibition by co-immunoprecipitation with CD44. By MALDI-MS analysis, importin ß and importin 5 were identified as such intracellular components associated with CD44. The interaction between importins and CD44 was confirmed by co-localization experiments. The importins are probably involved in the nuclear translocation of the CD44 intracellular domain (CD44ICD) which is an intracellular cleavage product. Evidence for such an involvement is deduced from the observation that wild-type CD44ICD is located in the nucleus, while mutant CD44ICD to which importin cannot bind is located in the cytoplasm.

The CD44ICD is generated from CD44 by intracellular cleavage by presenilin (PS)-dependent γ-secretase. 12-O-tetradecanoylphorbol 13-acetate (TPA) treatment, hyaluronic acid (HA) treatment or serum starvation induced the intracellular cleavage of CD44. HA induced generation of CD44ICD required the activation of Rac signaling pathway. Interestingly, a mutant of CD44s to which importins do not associate undergoes cleavage much less efficiently as compared to wild-type CD44s. The nuclear CD44ICD regulates the expression of several genes, as revealed by microarray analysis. Among those genes are genes encoding interferon inducible proteins that have anti-proliferative and apopototic effects.

Cytoplasmatische Domäne von CD44 wirkt als nuklearer Transkriptionsregulator

Zusammenfassung

CD 44 ist ein weit verbreitetes Adhäsionsmolekül, das an einer Vielzahl von physiologischen und pathologischen Prozessen beteiligt ist. Das CD44 Protein funktioniert als molekularer Schalter, der zwischen Zellwachstum oder Proliferationshemmung entscheidet, indem sein zytoplasmatischer Teil mit ERM (ezrin/radixin/moesin) bzw. Merlin interagiert. Die CD44 – ERM, bzw. CD44/Merlin Komplexe werden strikt reguliert, um eine optimale Signalkapazität zu erreichen. Daher wird vermutet, dass noch weitere intrazelluläre Komponenten Teil dieser Komplexe sind.

Um intrazelluläre Signaltransduktion durch CD44 besser zu verstehen, habe ich versucht, durch Ko-Immunopräzipitationen intrazelluläre Komponenten zu identifizieren, die unter Bedingungen der Proliferationshemmung mit CD44 assoziieren. Durch MALDI-MS Analyse wurden Importin β und Importin 5 als intrazelluläre Bindungspartner von CD44 identifiziert. Die Interaktion zwischen CD44 und den beiden Importinen wurde durch Ko-lokalisationsexperimente bestätigt. Die Importine spielen wahrscheinlich eine Rolle bei der nukleären Translokation der intrazellulären Domäne von CD44 (CD44ICD), einem intrazellulär abgespaltenen Produkt. Einen Beleg für diese Hypothese liefert die Beobachtung, dass Wildtyp-CD44ICD im Nukleus lokalisiert ist, während eine Mutante, die nicht mit Importin interagiert, im Zytoplasma zu finden ist.

Die Abspaltung der CD44ICD von CD44 erfolgt intrazellulär durch die Presenilin-abhängige γ-Sekretase. In der Zellkultur kann die intrazelluläre Spaltung von CD44 durch Behandlung mit 12-o-tetradecanoylphorbol-13-acetat (TPA), Hyaluronsäure (HA) oder durch Serumentzug induziert werden. Die HA-induzierte CD44-Spaltung benötigt die Aktivierung des Rac-Signaltransduktionsweges. Interessanterweise wird eine zur Bindung von Importin unfähige Mutante von CD44 mit wesentlich geringerer Effizienz gespalten als Wildtyp-CD44. Die im Kern lokalisierte CD44ICD reguliert,

wie Microarray-Analysen gezeigt haben, die Expression verschiedener Gene, darunter auch solche Gene, die für Interferon-induzierbare Proteine codieren, die wiederum antiproliferative sowie apoptotische Effekte vermitteln.

INDEX

Abbreviations

APS	ammonium persulfate
APP	amyloid precursor protein
BSA	bovine serum albumin
°C	degrees celsius
CAMK II	calcium/calmodulin dependent protein kinase II
CD	cluster of differentiation
CD44ICD	CD44 intracellular domain
CD44s	CD44 standard
CD44v	CD44 variant
CI	Contact inhibition
cm	centimeter (10^{-2} meter)
CS	chondroitin sulfate
DAG	diacylglycerol
DMEM	Dulbecco´s modified eagles medium
DMSO	dimethylsulfoxide
DNA	deoxyribonucleic acid
DSL	Delta/Serrate/Lag-2
DSP	Dithiobis [succinimidy propionate]
DTT	dithiothreitol
ECL	enhanced chemiluminescence
ECM	extracellular matrix
EDTA	ethylendiamine-N,N-tetracetate
EGF	epidermal growth factor
ERM	ezrin/radixin/moesin
ERMAD	ezrin/radixin/moesin association domain
EXT	extracellular domain
FACS	fluorescence-activated cell sorting
FCS	foetal calf serum
FERM	domain 4.1, ezrin, radixin, moesin-like domain
FGF	fibroblast growth factor
FITC	fluorescein isothiocyanate
g	gram

G418	geneticin
GAGs	glycosaminoglycans
GAP	GTPase-activating protein
GDP	guanosine diphosphate
GEF	guanine-nucleotide exchange factor
GEMs	glycolipid-enriched microdomains
GFP	green fluorescent protein
GST	glutathione S-transferase
GTP	guanosine triphosphate
HA	hyaluronic acid, hyaluronate
HB-EGF	heparin binding-epidermal growth factor
HGF	hepatocyte growth factor
HGF/SF	hepatocyte growth factor/scatter factor
hr	hour
HRP	horseradish peroxidase
HS	heparan sulphate
ICAM	intercellular adhesion molecule
ICD	intracellular domain
IFI 16	interferon inducible protein 16
Ig	immunoglobulin
kDa	kilodalton
LRP	LDL receptor-related protein
m	milli
M	molar
mAb	monoclonal antibody
MALDI-MS	matrix-assisted laser-desorption/ionization mass spectrometry
merlin	moesin, ezrin, radixin-like protein
mg	milligram (10-3 gram)
min	minute
ml	milliltre (10^{-3} l)
MT	mutant
MW	molecular weight
NF2	neurofibromatosis type II

NICD	Notch intracellular domain
NLS	nuclear localization signal
OD	optical density
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PDGF	platelet-derived growth factor
PI	phosphoinositide
PI3K	phosphatidylinositol 3-kinase
PIP2	phosphatidylinositol 4,5-bisphosphate
PKA	cAMP-dependent protein kinase A
PKC	protein kinase C
PMA	phorbol 12-myristate 13-acetate
PMSF	phenylethylsulphonyl fluoride
PS	presenilin
SDS	sodium dodecyl sulphate
SDS-PAGE	sodium dodecyl sulphate polyacrylamide gel electrophoresis
sCD44	soluble CD44
sec	second
Tiam1	T lymphoma invasion and metastasis 1
TEMED	N,N,N´,N´-tetramethylethylenediamine
TPA	12-O-tetradecanoylphorbol 13-acetate
TRIS	tris-(hydoxymethyl)-aminomethane
UDP	uridine diphosphate
UV	ultra violet
WT	wild type
w/o	without
ß-APP	ß-amyloid precursor protein

PART ONE
INTRODUCTION

CD44 is a family of type I transmembrane glycoproteins that are broadly expressed in many cell types and tissues of the body. They are involved in various physiological and pathological processes such as cell-matrix adhesion, hematopoiesis, lymphocyte homing, tumorigenesis and metastasis.

1.1 The discovery of CD44

CD44 is the cluster designation assigned by the Third International Workshop on Leukocyte Differntiation Antigens (Cobbbold and Hale et al, 1987) for a p80 glycoprotein recognized by a variety of different antibodies on leukocytes and many other cells. During the discovery of CD44, different names were used by different groups for some particular CD44 family members. The first CD44 protein was identified in 1980 by Dalchau and colleagues as brain-granulocyte-T lymphocyte antigen (Dalchau et al, 1980). The other proteins were independently characterized which are now known to be members of the CD44 family. These members include the extracellular matrix receptor type III (ECMRIII) (Carter and Wayner, 1988); GP85 (Kalomiris and Bourguignon, 1988); HCAM (Picker *et al.*, 1989); Hermes antigen (Haynes *et al.*, 1983; Jalkanen *et al.*, 1986); HUTCH-1 (Gallatin *et al.*, 1989); In(Lu)-related p80 (Telen *et al.*, 1983) and Pgp-1/Ly-24 (Hughes *et al.*, 1981; Trowbridge *et al.*, 1982). Finally, it turned out that all these proteins are identical or closely related members of the CD44 family (Zhou et al, 1989; Picker et al, 1989).

1.2 The gene structure of CD44

The CD44 gene is located on the short arm of chromosome 11 in human (Goodfellow et al, 1982) and chromosome 2 in mouse (Colombatti et al, 1982). All CD44 family members are encoded by a single gene that consists of 20 exons (Screaton et al., 1992, 1993). Due to alternative splicing of 10 so called variable exons located in the center of the gene and different post-translational modification, a large number of CD44 isoforms are generated. The smallest form of CD44 is the standard one (CD44s), which lacks the entire variable region. The extracellular domain of CD44s is encoded by exon 1-5 and 15-16, the transmembrane domain is encoded by exon 17, and the

cytoplasmic domain is encoded by exon 18 and 19. The variant isoform contains this core structure with the additional inclusion of sequences encoded by one or more of ten variant exons between exon 5 and 15. The exon 5a-14 is commonly denoted variant exons 1-10 (v1-v10). As both the standard and variant isoforms usually contain exons 1-5 and 15-17, these regions are sometimes referred to as the 5' and 3' constant regions respectively (Idzerda et al., 1989; Notterburg et al., 1989; Zhou et al., 1989; Aruffo et al., 1990; Wolffe et al., 1990; Bosworth et al., 1991; Harn et al., 1991; He Q et al., 1992).

At least 20 different CD44 transcripts have been identified, for example the CD44E (CD44v8-10) (Stamenkovic *et al.*, 1991) and CD44v3-10, a 230 kDa isoform which is expressed in keratinocytes (Hofmann *et al.*, 1991). In general exons v3-10 are the main variant exons used in CD44 splice isoforms (reviewed Lesley *et al.*, 1993) and CD44 isoforms containing v1 are 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). The most widely expressed isoform appears to be CD44s. The expression of the variant isoforms is tightly controlled and restricted to a limited number of cell types such as epithelial cells, leukocytes and tumor cells. Transient, regulated expression of variant isoforms can however be observed during several physiological processes for example upon activation of lymphocytes (Arch *et al.*, 1992).

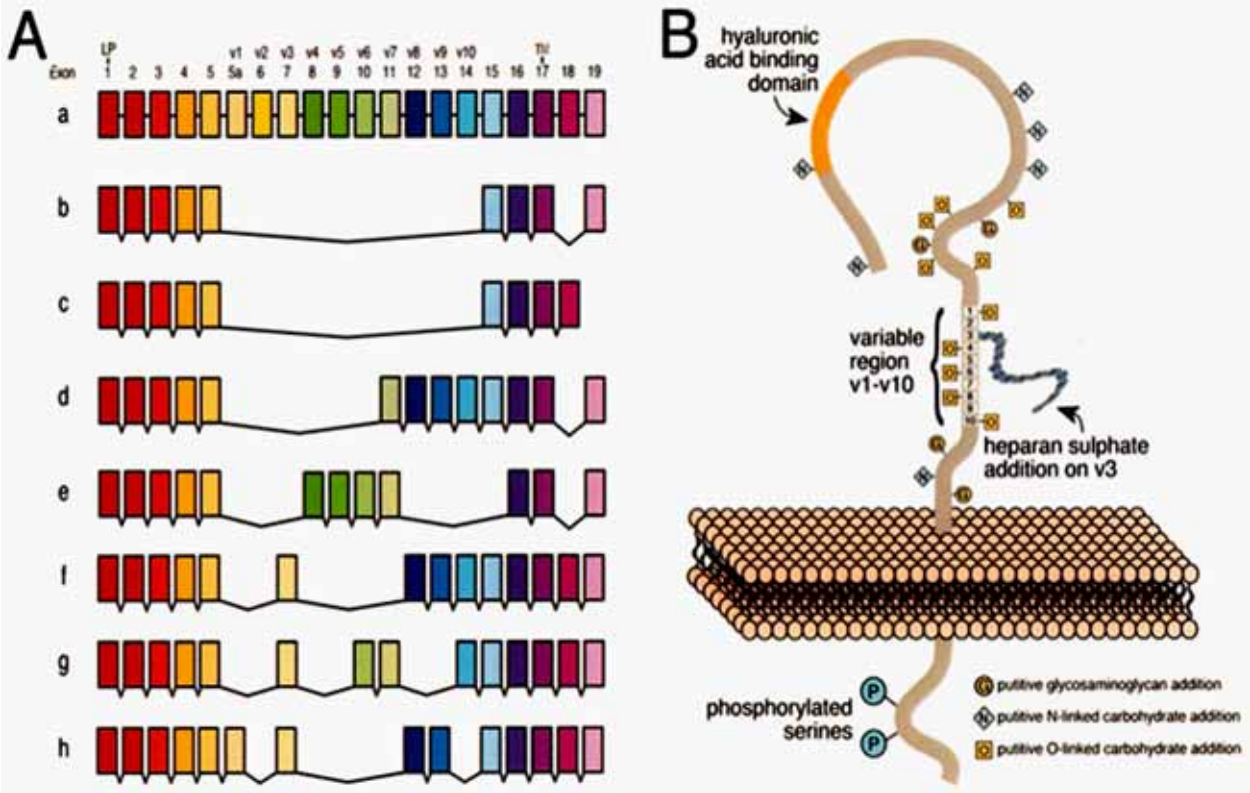


Fig. 1. (A) Genomic structure and multiple transcription products of the CD44 gene. The genomic structure is shown at the top (a) with the leader peptide (LP) and transmembrane domain (TM) indicated. The variant exon nomenclature is notated above exons 5a–14. Below (b–h) are some (but not all) observed transcripts from the CD44 gene. (B) Schematic drawing of the CD44 protein. Figure adapted from Ponta et al. (1998)

1.3 The protein structure of CD44

Early studies employing techniques such as cell surface iodination and trypsin cleavage of proteins from intact cells revealed that CD44 was expressed on the cell surface (Hughes *et al.*, 1981; Trowbridge *et al.*, 1982; Kalomiris and Bourguignon, 1988). Further biochemical studies, including detergent extraction analysis, liposome incorporation studies and ^{32}P-orthophosphate labeling demonstrated that the molecule was also a transmembrane protein and had a phosphorylated cytoplasmic domain (Isacke *et al.*, 1986; Carter and Wayner, 1988; Kalomiris and Bourguignon, 1988). It is now known that CD44 proteins have a high degree of cross species conservation and form a distinct family that are structurally unrelated to other families of cell surface molecules such as the integrins, cadherins, selectins, syndecans and immunoglobulins (Ig) superfamily.

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 the CD44s protein with a 19 amino acid signal peptide, cleavage of which yielded a putative type-I transmembrane protein of 341 amino acids. 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 (Figure 1).

1.3.1 The extracellular domain

The amino terminal ~ 180 amino acids of CD44 are relatively conserved among mammalian species (~85% homology). As indicated in figure 1, it contains six cysteine residues and five conserved N-glycosylation consensus sequences (referred to as N1-N5, for Asn residues at positions 25, 57, 100, 110 and 120, respectively). The cysteine residues are conserved throughout all examined species except the rat which has five and are possibly utilized to form a globular domain by the 3 disulfide bonds (Günthert et al., 1991). Included in this region is a stretch of approximately 90 relatively hydrophobic residues (amino acids 32-123) which shows 80-90% sequence similarity between species. It is this domain, which is known as the “link” domain, which enables CD44 to bind to hyaluronic acid (HA) as well as other

glycosaminoglycans (GAGs) (Sherman et al, 1994; Sleeman et al, 1997). Consequently, this domain has considerable homology (~30%) to domains of other hyaluronic acid binding proteins such as aggregating proteoglycan (aggrecan) (Doege et al., 1987) and the cartilage link protein (Deak et al., 1986, Neame et al., 1986, Stamenkovic et al., 1989).

The membrane proximal region of the extracellular domain is less conserved than the amino-terminal domain, showing only 35-45% similarities between mammalian species. It contains potential sites for numerous carbohydrate modification of CD44 (Nottenburg et al, 1989; Dasgupta et al, 1996; Brown et al, 1991; Takahashi et al, 1996) and the site of alternative splicing for the insertion of 10 variable exons of the CD44 (Screaton et al, 1992; Tolg et al, 1993; König et al, 1998; Weg-Remers et al, 2001).

Over one half of the molecular weight of CD44 can be attributed to post-translational additions in the form of carbohydrates. Amino acid sequence analysis shows that the extracellular domain of CD44 contains numerous clusters of serine and threonine residues together with prolines and multiple acidic residues which are commonly found in O-linked glycosylation sites (Marshall, 1974, Russell et al., 1985, Yamamoto et al., 1984). Some studies found that the inhibition of O-glycosylation enhanced HA binding (Dasgupta et al, 1996), while other studies did not observe the effect on HA binding as a result of inhibition of O-glycosylation (Lesley et al. 1995; Zheng et al.1997). Also located within extracellular domain are five N-glycosylation sites (Asn-X-Ser/Thr, where X is any amino acid except proline) and four serine-glycine motifs that represent potential glycosaminoglycan attachment sites (Doege et al., 1987). All five N-glycosylation sites appear to be utilized in murine and human cell lines (English et al, 1998; Bartolazzi et al, 1996), and studies have shown that differences in N-glycosylation can modulate the ligand binding function of CD44. The serine-glycine motifs can be modified by the glycosaminoglycan (GAG) heparin sulfate (HS) (Brown et al., 1991; Tanaka et al., 1993) or by chondroitin sulfate (CS) (Jalkanen et al., 1988; Stamenkovic et al., 1989, 1991), thereby converting the molecule to a proteoglycan with possibly altered ligand specificity (Faassen et al., 1992; Jalkanen and Jalkanen, 1992).

Alternative splicing of variant exons, as well as differential posttranslational modification, may increase the optional functions of CD44 molecules. The entire CD44 variable region (exon v1 to exon v10) reveals four additional potential N-glycosylation sites and a large number of O-glycosylation sites. In addition, one serine-glycine motif for insertion of GAG has also been detected in the variable region (Screaton et al., 1993; Bennett et al., 1995a). CD44 variants containing the exon v3 product can be decorated by heparan sulphate (HS) through the GAG attachment site (Ser-Gly-Ser-Gly), and thereby can bind heparin-binding growth factors and chemokines (Bennett et al, 1995b; Tanaka et al, 1993).

1.3.2 The transmembrane domain

The single transmembrane domain composed of 23 amino acids is encoded by exon 17 and shows almost 100% sequence homology between all examined species (Isacke, 1994). A proportion of CD44 transmembrane domain is subject to post-translational modification by palmitoylation (Bourguignon *et al.*, 1991; Guo *et al.*, 1994). Such lipid modification may act to regulate protein:protein interactions and protein:lipid interactions (Mcllhinney, 1990). There are two cysteine residues that might serve as substrates for palmitoylation: Cys286 in the transmembrane domain and Cys295 in the cytoplasmic domain. The transmembrane domain can also facilitate receptor dimerization via cysteine interactions which are mediated through Cys286 in the human CD44 sequence (Liu and Sy, 1996, 1997). Mutation of this cysteine residue prevented HA binding in CD44 transfected Jurkat cells in response to activating CD44 antibody or TPA (12-O-tetradecanoylphorbol 13-acetate), suggesting that disulfide bond formation through Cys286 may serve to stabilize aggregates of CD44, thus mediating ligand binding.

1.3.3 The cytoplasmic domain

The CD44 cytoplasmic domain shows more than 85% conservation of amino acid sequence among all examined species (Isacke, 1994). It has a total length of seventy amino acids, the first three encoded by exon 17 and the remainder by exon 19 (Screaton et al., 1992). In the majority of all observed CD44 proteins, sequences encoded by exon 18 have been removed by alternative splicing (Screaton et al, 1992). The isoforms generated by the inclusion of exon 18 contains a stop codon at position

195, resulting in a short form of the cytoplasmic tail. CD44 proteins with truncated cytoplasmic domain are expressed at very low levels (Goldstein et al, 1989, 1990) and there is no evidence that expression of exon 18 is physiologically relevant.

1.3.3.1 Phosphorylation

Phosphorylation takes place on serine residues in CD44, no phosphorylation on threonine or tyrosine have been observed (Lokeshwar et al, 1992; Isacke et al, 1986; Pure et al, 1995). There are seven serine residues in the cytoplasmic tail of human CD44. Of the serine residues in the human CD44 cytoplasmic domain, 4 (Ser291, Ser316, Ser323 and Ser325) are completely conserved between species. Ser325 is the major residue to be phosphorylated, accounting for approximately 90% of the phosphorylation on CD44. The phosphorylation at Ser325 is known to be important for the ability of CD44 to mediate cell migration on hyaluronic acid. This has been determined using both phosphorylation incompetent receptors where the Ser325 phosphorylation site has been mutated (Peck and Isacke, 1996) and CD44 blocking peptides phosphorylated at Ser325 (Peck and Isacke, 1998). Interestingly, the CD44 Ser325 kinase has recently been identified as calcium/calmodulin dependent protein kinase II (CAMK II) (Lewis and Isacke, unpublished results). 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 forms a putative PKC consensus sequence (Pearson and Kemp, 1991) and can be phosphorylated by PKC *in vitro* (Kalomiris and Bourguignon, 1989; Lewis and Isacke, unpublished results). The sequence around Ser316 also forms a putative protein kinase A phosphorylation site but there is no evidence that phosphorylation occurs at this site. Legg et al found that the phosphorylation of CD44 cytoplasmic tail could increase the binding of ERM proteins (ezrin, radixin, and moesin) to CD44. This binding was found to be necessary for cell migration (Legg JW et al, 2002; Clark RA, et al, 1996).

1.3.3.2 Interaction with cytoskeleton

The CD44 cytoplasmic domain can associate indirectly with actin-cytoskeleton via interactions with intracellular protein partners. The first intracellular partner of CD44 was identified as ankyrin, a protein that mediates contact with the actin-cytoskeleton via spectrin. The ankyrin binds to the CD44 cytoplasmic domain between Asn304 and Leu318 (Lokeshwar et al, 1994). Although the importance of CD44-ankyrin

interaction is not entirely understood, the interaction is implicated in mediating HA-dependant cell adhesion and motility (Lokeshwar et al, 1994).

ERM (ezrin, radixin, moesin) proteins and the related protein merlin (neurofibromatosis 2, NF2, moesin-ezrin-radixin-like-protein) have also been identified as CD44 intracellular partner proteins (Morrison et al, 2001; Sainio et al, 1997, Tsukita et al, 1994). The ERM binding domain of CD44 consists of several clusters of basic amino acids and is closer to the cell membrane than the ankyrin binding site (Legg and Isacke, 1998; Yonemura et al, 1998). ERM proteins act as linkers between CD44 and filamentous actin (F-actin). This interaction of CD44 to actin cytoskeleton via ankyrin and (or) ERM proteins accounts not only for cell-shape determination but also for membrane protein localization, membrane transport and signal transduction (Crepaldi et al, 1997; Yonemura et al, 1999; Dard et al, 2001; Bretscher et al, 2000; Paglini et al 1998; Ng et al, 2001; Takahashi et al, 1997, 1998). Merlin can also bind to the ERM-binding domain, however merlin does not contain the conserved F-actin binding region in the C-terminal domain as found in ERM proteins. However, interaction of merlin to the cytoskeleton has been proposed via another region in the N-terminus (Xu et al, 1998; James et al, 2001; Brault et al, 2001).

1.3.3.3 ERM proteins and Merlin

1) introduction

Among the ERM proteins, ezrin, the p81 substrate of the EGF receptor tyrosine kinase, was first purified from epithelial intestinal brush border microvilli (Bretscher et al, 1983; Pakkanen et al, 1987; Gould et al, 1986; Berryman et al, 1993). Radixin was isolated from liver-cell adherens junctions (Tsukita et al, 1989), and moesin was identified as a heparin-binding protein (Lankes et al, 1988). Merlin, also named schwannomin, was identified by genetic approaches aiming to characterize the molecular defect of neurofibromatosis type 2 (NF2), which is characterized by the development of nervous system tumors (Trofatter et al, 1993; Rouleau et al, 1993).

Collectively, merlin and ERM proteins are part of the band 4.1 superfamily of proteins, whose members all share a common homologous ~300-amino-acid domain named the FERM domain (Four-point one, ezrin, radixin, moesin) (Chishti et al, 1998). The secondary structure of these proteins consists of a globular NH2-terminal

domain which is called N-ERMAD (ERM association domains), a central α-helix region and a COOH-terminal domain called C-ERMAD.

2) The functions of ERM and merlin proteins

ERM proteins function as linkers between cytoskeleton and transmembrane proteins such as CD44 (Legg et al, 1998, Tsukita et al, 1994), CD43 (Allenspach et al, 2001; Shaw AS et al, 2001; Delon et al, 2001), intercellular adhesion molecule- 1, 2, and 3 (ICAM-1, ICAM-2, and ICAM-3) (Helander *et al.*, 1996; Serrador *et al.*, 1997; Heiska *et al.*, 1998). This interaction controls cell morphogenesis, adhesion and motility and also transduces growth signals for proliferation and survival. ERM proteins were found to be involved in the formation of actin rich structures such as microvilli, filopodia, lamellipodia and microspikes (Yonemura et al, 1999; Lamb *et al.*, 1997; Bonilha et al, 1999; Dard et al, 2001). Inhibition of the expression of ERM proteins caused loss of attachment of the cells from the substratum and from each other (Takeuchi *et al.*, 1994), and an increased motility (Hiscox and Jiang, 1999). ERM proteins control cell growth through different pathways. For instance, ezrin activation is required for the transformation of NIH3T3 cells by the Rho exchange factors Net and Db1 (Tran Quang et al, 2000), and ezrin is involved in cell survival through the phosphatidylinositol 3-kinase (PI3-k)/Akt pathway (Gautreau et al, 1999; Poullet et al, 2001). Increased ezrin expression has also been correlated with increased cell proliferation and immortalisation (Kaul *et al.*, 1996) and aberrantly high ezrin expression has been observed in some tumors (Bohling *et al.*, 1996).

Merlin is known to be a tumor-suppressor (Kinzler, et al, 1993; Rouleau et al, 1993; Trofatter el al, 1993; McClatchey et al, 1997 and 1998). Homologous knockout mice are embryonic lethal, whereas heterozygous mice are viable but prone to develop a variety of tumors such as osteosarcomas, but did not develop schwannomas or other tumor types seen in NF2 patients (McClatchey et al, 1997 and 1998). The conditional homozygous *Nf2* knockout mice with Cre-mediated excision of *Nf2* exon 2 in schwann cells did show the development of schwannomas as seen in NF2 patients (Giovannini et al, 1999). In the RT4-D6P2T and JS1 rat schwannoma cell lines as well as fibroblast cell lines over expression of wild-type merlin inhibits growth (Bianchi et at, 1994; Lutchman and Rouleau, 1995; Sherman *et al*., 1997; Tikoo *et al*., 1994). Reduction of merlin expression by antisense technique in a schwannoma cell

line (STS26T) resulted in increased cell proliferation (Huynh and Pulst, 1996). In addition, Morrison et al have proposed a model of cell contact inhibition in which merlin restricts cell proliferation through CD44 (Morrison et al, 2001).

3) Regulation of ERM-merlin activity

Several studies have shown that the bulk of ERM-merlin proteins in the cytoplasm are maintained in the closed conformation through an intramolecular N-/C-ERMAD interaction. It has been reported that phosphorylation of the conserved C-terminal Thr (Thr567 in ezrin, Thr564 in radixin, Thr558 in moesin) plays a major role in regulating ERM conformation. The phosphorylation of ERM proteins drastically reduces the N-/C-ERMAD interaction in vitro (Matsui et al, 1998; Nakamura et al, 1999) and correlates with their cytoskeletal association (Simons et al, 1998). In various cell lines, ERM association with the membrane-cytoskeleton always correlates with an enhanced state of ERM phosphorylation (Bretcher et al, 2000; Oshiro et al, 1998; Shaw et al, 1998a; Matsui et al, 1999).

Although the open form of ERM proteins represents the active state of the molecules, it seems that this is not the case for merlin. The phosphorylation of merlin takes place at Ser518 and not at the conserved threonine residue equivalent to Thr567 in ezrin. This phosphorylation of merlin at Ser518 also inhibits the N- and C-ERMAD interaction (Shaw et al, 1998b, 1998c and 2001). However, it is the dephosphorylated form of merlin that interacts with CD44 cytoplasmic tail and inhibits cell growth (Morrison et al, 2001), indicating that a closed, dephosphorylated merlin is in fact the active form of this protein. Furthermore, phosphorylated merlin can interact with ERM proteins in cell proliferation; however the function of phosphorylated merlin is presently unknown.

Phosphatidylinositol-4, 5-bisphosphate (PIP2) has also been implicated in ERM activation (Hirao et al, 1996; Nakamura et al, 1999). In vivo, the microinjection of NEOMYCIN, which titrates out polyphosphoinositides, results in loss of cell-surface microvilli (Hirao et al, 1996). Over expression of phosphatidyl inositol 4-phosphate 5-kinase, which increases the level of PIP2, enhances ERM phosphorylation (Matsui et al, 1999). PIP2 is also required together with phosphorylation to maintain moesin in an active conformation (Nakamura et al, 1999). PIP2 binds to the FERM domain of

ERM proteins, and this interaction involves three clusters of lysines (K63, K64, K253, K254, K262, K263) that are part of a groove in the FERM domain (Barret et al, 2000; Hamada et al, 2000). Mutagenesis of these residues abrogates the binding of PIP2 to the FERM domain and the membrane localization of ezrin *in vivo*. These results suggest that PIP2 in addition to phosphorylation is required for ERM protein activation.

1.4 Ligands of CD44 extracellular domain

1.4.1 Hyaluronic acid is the principal ligand of CD44

The extracellular matrix (ECM) component hyaluronic acid (HA) is a linear polymer made up of repeating disaccharide units D-glucuronic acid and N-acetyl-D-glucosamine and usually has a molecular weight of several million Dalton (10^6-10^7) (Laurent and Fraser, 1992). The evidence that CD44 is a principal receptor of HA has been established by several approaches. The amino-terminal domain of CD44 displays about 30% sequence homology with the HA binding region of other known HA binding proteins (cartilage link protein and proteoglycan core protein) (Goldstein et al, 1989; Stamenkovic et al, 1989). The binding of HA to CD44-expressing cells can be prevented by some (but not all) anti-CD44 antibodies, an excess of soluble HA or pretreatment of HA with hyaluronidase (Lesley et al, 1990, 1992; Miyake et al, 1990a; Bennett et al, 1995; Pure et al, 1995; Zahalka et al, 1995). Soluble CD44-IgG fusion protein binds to lymph node high endothelial cells, and this binding can be blocked by the inclusion of low concentrations of HA, but not of other GAGs, or by pretreatment of the endothelial cell with hyaluronidase (Aruffo et al, 1990). CD44 negative cells transfected with CD44 cDNA acquire the ability to bind to lymph node high endothelial cells, which can be inhibited by anti-CD44 antibody, soluble HA, or pretreatment with hyaluronidase (Stamenkovic et al, 1991; Lesley et al, 1992).

1.4.2 Other ligands of CD44 of the CD44 extracellular domain

In addition to HA, CD44 can also bind other molecules via interactions with its extracellular domain. CD44 can adhere to the ECM components collagen (Faassen et al, 1992), fibronectin (Jalkanen and Jalkanen, 1992), and laminin (Jalkanen and Jalkanen, 1992). The other ligands include mucosal addressin (Picker *et al*., 1989), MIP-1β (Tanaka *et al*., 1993), the chondroitin sulfate (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).

A relatively large number of growth factors and cytokines such as basic fibroblast growth factor (FGF), and heparin binding-epidermal growth factor (HB-EGF) (Jackson *et al*., 1995), hepatocyte growth factor (HGF) (van der Voort *et al*., 1999) have been shown to bind CD44. The binding of these growth factors or cytokines to CD44 is dependent on the inclusion of the CD44 variant exons v3 (Sherman *et al*., 1998; Jones *et al*., 2000).

1.5 Signal transduction via CD44

The role of many transmembrane receptors is to transduce signals from the outside of the cell to the inside of the cell. Such extracellular signals usually involve adhesion to extracellular matrix or binding of growth factors and are transmitted into the cell typically by phosphorylation events in the transmembrane receptor cytoplasmic domain.

There are numerous reports that binding of ligands to CD44 activates several signaling pathways. In lymphocytes, CD44 antibodies can trigger a signal transduction pathway similar to the one of activated T-cell receptor/CD3-complex in T-cells. In particular the tyrosine kinases $p56^{Lck}$ (Lck) (Taher *et al.*, 1996; Rozsnyay *et al.*, 1999) and Fyn (Ilangumaran *et al.*, 1998) 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 in glycolipid-enriched microdomains (GEMs). CD44 has been demonstrated to partition to the same membrane fraction as Lck and Fyn (Ilangumaran *et al.*, 1998), and stimulation with various CD44 antibodies such as MEM-85 (Ilangumaran *et al.*, 1998), Hermes-3 and J173 (Taher *et al.*, 1996) induces an increase of tyrosine phosphorylation of these molecules and phosphorylation of the Lck substrate ZAP-70 in human T lymphocytes.

Recently, hyaluronic acid binding by CD44 has been demonstrated to induce the activation of the small GTP-binding protein Rac1 (Oliferenko *et al.*, 2000). Addition of hyaluronic acid to EpH4 mammary epithelial cells induced the formation of lamellipodia which was inhibited by an anti-CD44 antibody or micro-injection of a dominant-negative mutant of Rac. Furthermore, the mechanism via which Rac is activated may be via recruitment of Tiam1 (T lymphoma invasion and metastasis 1), a Rac-specific GEF (guanine nucleotide exchange factor) (Bourguignon *et al.*, 2000). A direct association between CD44 cytoplasmic domain and Tiam1 has been demonstrated. In addition, Tiam1 from cells stimulated with hyaluronic acid had an increased ability to activate Rac *in vitro*.

1.6 CD44 functions in cellular growth control

1.6.1 CD44 in cell proliferation

CD44 plays an important role in the regulation of cell proliferation. The data from our lab showed that CD44 containing v6 exon is required for the hepatocyte growth factor/scatter factor(HGF/SF) dependent activation on its receptor c-Met, promoting cell proliferation through the Ras signaling pathway (Orian-Rousseau et al, 2002). In a growth-permissive state, the cytoplasmic tail of CD44 was found to form a complex with phosphorylated ERM and merlin proteins (Morrison et al, 2001). Moreover, the over-expression of the cytoplasmic tail of CD44, which could compete for the ERM binding, severely inhibited the HGF mediated signaling, whereas the over expression of CD44 cytoplasmic tail mutated in the ERM binding site failed to interfere with HGF dependent signaling (Orian-Rousseau et al, 2002).

1.6.2 CD44 in cell contact inhibition

The interaction between CD44 and merlin was also found to be involved in cellular growth control (Morrison et al, 2001). When cells grew to confluency, merlin was activated via dephosphorylation. Active merlin binds to CD44 cytoplasmic tail leading to the inhibition of growth through interference with growth factor activation of Ras. Furthermore, the addition of high molecular weight HA to the cells during logarithmic growth activates merlin and retards cellular growth.

Taken together, CD44 functions as a molecular switch that modulates cell

proliferation as well as growth inhibition. These actions are triggered by extracellular ligands binding to CD44, and determined by complexes associated with the CD44 cytoplasmic tail e.g. ERM and merlin proteins that influence signal transduction.

1.7 The cleavage of CD44

1.7.1 Shedding of the extracellular domain

The extracellular domain of CD44 is subject to proteolytic cleavage. Shedding of CD44 was observed from cells constitutively, but enhanced release can be induced by TPA, ionomycin (DeGrendele et al, 1997), cytokines (Campanero *et al*., 1991; Ristamaki et al, 1997), as well as bacterial- and leukocyte-derived proteinases (Cichy et al, 2002; Lazaar et al, 2002). Release of sCD44 (soluble CD44) is also likely to be ligand inducible, as antibody cross-linking CD44 leads to shedding of CD44 (Camp et al, 1993; Shi et al, 2001). The protease(s) responsible for cleavage of CD44 have not been identified, although some studies have implicated a metalloproteinase, since the cleavage could be inhibited with specific inhibitors of these enzymes (Okamoto *et al*., 1999a). This cleavage of CD44 can be regulated by intracellular signaling pathways. Shedding of CD44 has been reported to be induced by Ras, and the effect of Ras on CD44 shedding is mediated by members of the Rho family of GTPases (Cdc42 and Rac1) (Okamoto et al, 1999b; Kawano et al, 2000; Shi et al, 2001).

1.7.2 Intracellular Cleavage

Besides the cleavage of the CD44 extracellular domain, CD44 also undergoes the proteolytic cleavage in its intracellular domain, resulting in the release of CD44 intracellular domain (CD44ICD) (Okamoto, et al, 2001). The generation of CD44ICD requires a presenilin (PS)–dependent γ-secretase activity (Lammich S., et al, 2002; Murakami D., et al, 2003). This scenario is remarkably similar to the cleavage of some other proteins. These proteins include ß-amyloid precursor protein, Notch, LDL receptor-related protein, E-cadherin and ErbB-4 (Cao et al, 2001; Wong et al, 1997; De Strooper et al, 1999; May et al, 2002; Marambaud et al, 2002; Ni et al, 2001; Lee et al, 2002). All of these type-1 transmembrane proteins have been demonstrated to undergo two cleavage events. The first occurs outside the membrane, often in response to ligand binding, and results in a conformational change that triggers a second intramembraneous cleavage event that occurs at the transmembrane domain of

the protein, which releases an intracellular domain fragment. The ICD fragment then translocates to the nucleus and activates gene expression. Presenilin-dependent proteolysis of CD44 has been reported to occur at two sites: one close to the cytoplasmic border, leading to the liberation of CD44ICD, and another site within the transmembrane domain, resulting in the extracellular release of the so-called CD44ß peptide. The latter is reminiscent of the presenilin-dependent processing of ßAPP leading to the release of AßP implicated in Alzheimer's disease (Lammich et al, 2002). CD44ICD generated from the intramembraneous cleavage is targeted to the nucleus where it might regulate genes containing TPA-responsive elements through coactivator CBP/p300 (Okamoto, et al, 2001). No specific genes were identified so far. What is unclear is whether this transcriptional activity is a property of the CD44ICD or of other proteins that associate with the tail. Thus, the intracellular cleavage and nuclear translocation of CD44ICD might result in a direct activation of targets genes. Taken together, the intracellular cleavage of CD44 represents a novel aspect of signal transduction via CD44.

Aim of the project:

The principle goal of this thesis was to identify protein partners that associate to the CD44 cytoplasmic tail together with merlin. Based on the previous findings of our laboratory, CD44 plays a role in regulating cellular growth by associating with either merlin or ERM proteins with its cytoplasmic tail. The identification of other protein partners associating to the CD44 cytoplasmic tail should further elucidate the role of CD44 in intracellular signaling. For this purpose, I made use of a cell line in which merlin binds to CD44 at high cell density and performed co-immunoprecipitation of CD44, in order to identify the protein partners that associate with CD44 cytoplasmic domain.

PART TWO
MATERIALS AND METHODS

2.1 Materials

2.1.1 Chemicals

All general chemicals were, unless otherwise stated, purchased form *Carl Roth GmbH & Co* (Karlsruhe), *Merck* (Darmstadt) or *Sigma Chemie GmbH* (Deisenhofen) and were of the highest quality. All radiochemicals were obtained from *Amersham Pharmacia Biotech, Freiburg.*

2.1.2 Oligonucleotides

All the oligonucleotides were made by MWG Biotech GmbH and were all of an HPLC-purified grade.

2.1.3 Primary Antibodies

Name	**Reference/Supplier**
5G8 (pan-CD44), mouse monoclonal	Sleeman et al., 1996
Myc-tag (9E10), mouse monoclonal	Santa Cruz
NF2 (A-19), rabbit polyclonal	Santa Cruz
NF2 (C-18), rabbit polyclonal	Santa Cruz
Actin (I-19), goat polyclonal	Santa Cruz
Importin ß (Nuclear Transport Factor p97), mouse monoclonal	ABR (affinity bioreagents, INC)
Importin 5, rabbit polyclonal	Santa Cruz

2.1.4 Secondary Antibodies

Name	**Supplier**
HRP conjugated rabbit anti goat IgG	DAKO (Glostrup, Denmark)
HRP conjugated goat anti mouse IgG	DAKO (Glostrup, Denmark)
HRP conjugated goat anti rabbit IgG	DAKO (Glostrup, Denmark)
FITC conjugated goat anti mouse IgG	Dianova (Hamburg)
TRITC conjugated goat anti mouse IgG	Dianova (Hamburg)
TRITC conjugated goat anti rabbit IgG	Dianova (Hamburg)

2.1.5 Enzymes

All restriction endonucleases and other modifying enzymes were purchased from Invitrogen GmbH (Karlsruhe, Germany), Promega (Mannheim, Germany) or New England Biolabs (Beverly, USA) unless otherwise stated.

2.2 General Methods

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

2.2.1 Preparation of chemically competent E. coli

A single colony of E.coli DH5α was taken to inoculate 5 ml of LB medium (10g/l tryptone, 5 g/l yeast extract, 5 g/l NaCl) and allowed to grow overnight at 37°C with shaking (220 rpm). 4 ml was removed and added directly to 400 ml of LB medium. The bacteria were grown to an OD590 of 0.375 before incubating the bacteria on ice for 10 min. The bacteria were sedimented by centrifugation without brake at 3600 g for 7 min at 4°C 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 CaCl2 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°C.

2.2.2 Preparation of electrocompetent bacteria

As above, a single colony of E.Coli DH5α was taken to inoculate 1 ml of YENB (7.5g/l Bacto yeast extract, 8.0g/l Bacto Nutrient broth) medium and the culture grown overnight at 37°C 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°C with shaking, cells were harvested between an OD600 of 0.5 to 0.9. To harvest cells, a flask was chilled on ice and spun at 4000 g for 10 min at 4°C. 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 1.5-3 $\times 10^{10}$ 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°C. Thawed on ice before use. These competent cells are good for 1-2 years.

2.2.3 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 10000g 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).

2.2.4 Ethanol (or 2-propanol) precipitation of nucleic acids

In order to recover nucleic acids from solution, the salt concentration 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°C or 15 min at -80oC (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.

2.2.5 Determination of nucleic acid concentration

The concentration of nucleic acids was determined by measuring their optical density (OD) at 260 and 280 nm. An OD260 = 1 is equivalent to 50 µg/ml double stranded DNA or 40 µg/ml RNA or 20 µg/ml single stranded oligonucleotide. The OD280 is used as an indication of the purity of the nucleic acid; it should be approximately 50% of the OD260

2.2.6 Total RNA isolation from cells or tissue

Total RNA was prepared from the cells when they were 60-80% confluent in 15 cm culture dishes (*Greiner, Frickenhausen*). The medium was completely removed and cells were lysed by adding 1 ml of peqGOLD RNA Pure solution (*Peqlab Biotechnologie GemH, Erlangen*). To lysis tissue-derived cells, 100-mg snap-frozen tissue was placed directly into 1ml peqGOLD solution and homogenized using an ultra-Turrax T25 (*IKA-Labortechnik*) homogenizer for 3-5 min. after an incubation

period for 5 min at room temperature, 0.2 ml chloroform was added to the lysed cells or to the homogenized tissue mix and vortexed vigorously. Following a 3-10 min incubation at room temperature, the mixture was centrifuged for 5 min at 4°C at 13,000 rpm and the top aqueous RNA-containing phase was removed and transferred to a fresh tube. Again 0.2 ml chloroform was added and the extraction repeated as above. After one more round of the chloroform extraction (altogether 3 rounds were done), the RNA was precipitated by addition 1 ml isopropanol, subsequent vortexing, incubation for 5-10 min at room temperature and 15 min centrifugation at 4°C at 13,000 rpm. The RNA pellet was washed twice with 75% ethanol and re-suspended in 50 µl H_2O for determination of the RNA concentration. The RNA preparation was stored at -80°C.

2.2.7 Polymerase Chain Reaction (PCR)

All PCR reactions were performed in a total volume of 20ul in the presence of 250uM dNTPs, 1-2 pmol of primers, 0.25U of Tag polymerase, 1 × supplier's buffer and 2mM $MgCl_2$. the reaction were carried out in a PCR thermocycler (Perkin Elmer, Norwalk, USA), using the following cycling parameters: 94°C, 1min; 55°C, 1min; and 72°C 1min for a total of 30 cycles. Analysis of the PCR products was performed by a gel electrophoresis.

2.2.8 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 37oC (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.

2.2.9 DNA Ligation

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

2.2.10 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.

2.2.11 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: 90mM Tris-base, 90 mM boric acid, 2.5 mM EDTA, pH 8.3). Ethidium bromide was added at a concentration of 0.3µg/ml. The molten gel was poured into a horizontal (13.5 × 8 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 visualized by transillumination with 302 nm ultraviolet radiation.

2.2.12 Isolation/purification of DNA from agarose gels

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

2.2.12.1 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 centrifuged at high speed for 5-10 min. The upper aqueous phase was transferred to a new reaction tube and precipitated with ethanol.

2.2.12.2 DNAeasy kit (Biozyme) DNA isolation from agarose gels

As above with the exception that the gel strip containing the DNA was added to 3x its weight to volume of "salt buffer" (all reagents provided in the kit). The gel piece was melted in the buffer by incubation at 650C 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 H2O.

2.2.12.3 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°C with shaking. After two extractions with phenol/chloroform, the DNA was precipitated with ethanol, air dried and dissolved in an appropriate volume of water.

2.2.13 Transformation of E.Coli

2.2.13.1 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°C for 90 seconds before rapidly 30 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 MgSO4 and 20 mM glucose) the bacteria were transferred to a shaker and incubated for 45 min at 37°C. The cells were then pelleted lightly by a short centrifugation (3 min at 1000x g) and 1 ml of the supernatant was removed before resuspending the cells in the remaining 200 µl. A volume of 50-200 µl was plated out on LB-agar plates supplemented with the correct antibiotic and the plates incubated 18-24 hours at 37°C.

2.2.13.2 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 manufacturer's 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 re-suspended, transferred to a polypropylene tube (17 x 100 mm)

and incubated with or without shaking at 370C for 1-3 hours. Cells were plated on selective medium plates.

2.2.14 Mini-prep plasmid preparation from E.Coli

2.2.14.1 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°C until a stationary phase had been reached upon which 1.5 ml was removed and the bacteria pelleted by centrifugation at 4000 g for 5 min. The pellet was resuspended in 100 µl of solution I (50 mM Glucose, 25mM 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 10,000 g for 10 min before the aqueous supernatant was removed. Extraction of the supernatant 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 H2O.

2.2.14.2 Wizard Mini-prep kit (Promega)

Resuspension, lysis and neutralizing of the bacterial pellet was carried out according to and using the manufacturer's buffers provided. The plasmid DNA in the retained supernatant 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 H2O. This method was primarily used for the production of sequencing-grade plasmid DNA.

2.2.14.3 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 5g/lt NaCl) medium supplemented with the relevant antibiotic was inoculated with a single bacterial colony and incubated with shaking (220 rpm) at 37°C overnight till the bacteria had reached a stationary growth phase. The bacteria were pelleted by centrifugation in a fixed angle rotor at 4000 g for 10 min and the pellet resuspended in 10 ml of solution (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 (200mM 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 40C in a fixed angle rotor. The retained supernatant 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°C.

2.2.15 Sequencing of double-stranded template DNA

2.2.15.1 Automated (fluorescence) DNA sequencing method

The VISTRA Thermo Sequenase Pre-mixed Cycle Sequencing Kit (amersham Pharmacia Biotech) was used to routinely sequence cloned DNA on both strands. Briefly, 1 µg DNA was mixed with 4 pmol of an appropriate Texas-red-labelled primer (e.g. T7 Forward universal primer). From this mixture 6 µl was aliquoted into each termination vial (G, A, T, C) containing 2 µl of the corresponding ddNTP mix (i.e. ddGTP, ddATP, ddTTP and ddCTP) containing all the necessary reaction components such as polymerase and dNTPs plus the actual ddNTPs. The reaction was cycled in a PCR machine using the following parameters: 94°C, 1 min, 1 cycle; 94°C, 30 sec, 50°C, 30 sec, and 72°C, 30 sec for a total of 25 cycles. Upon completion, 3 µl loading buffer was added to each reaction mix and the volume of the reaction was reduced to 3 µl by drying in a vacuum centrifuge. The samples were loaded on a RapidGel-XL-6% gel (Amersham) and run with TBE buffer in a DNA Sequencer 725 (Molecular Dynamics & Amersham) for 12 hours. The sequencing data were analyzed using Molecular Dynamics softwere.

2.2.15.2 Manual (radioactive) DNA sequencing 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 α33P-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 20µl. 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°C, 30sec, 55°C, 30 sec and 72°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 run had reached the desired length, the gel was removed, dried on Whatmann 3MM paper at 80°C for 2 hours on a vacuum gel dryer before autoradiography. Films were developed after 18-36 hours exposure.

2.2.16 Stable and transient transfection of cells

Cells used for transfection were split and reseeded 24 hours before transfection commenced. Usually, stable transfections were performed in 6 well petri dishes containing 3×10^5 cells, giving a confluency of 60-80%. Transfection was performed using the liposomal transfection reagent DOTAP or FuGENE 6 (Roche 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 transient transfections, the transfectant cells were culture without antibiotic selection and 24-36 hours post-transfection cells were lysed.

2.2.17 Purification of immunoglobulins using protein G

Supernatant from hybridomas was collected and centrifuged at 4-5 K for 10 minutes to remove cellular debris. Antibodies were precipitated by the addition of ammonium sulphate to a final concentration of 50%. The solution was incubated overnight at 4°C with gentle stirring. The precipitate was collected by centrifugation at 5-6 K for 15

minutes. The pellet was resuspended in 10-20 ml 150 mM NaCl, 20 mM phosphate pH7.0 and dialyzed for several hours against the same buffer. After dialysis, the protein solution was centrifuged at 10 K for 10 minutes and the resulting supernatant added to 2 ml of 50% slurry of Protein G plus agarose for each 50 ml of supernatant. The mixture was incubated for 2 hours or over-night at 4°C with rotation. After incubation, the beads were centrifugated at 3 K for 10 minutes and the supernatant discarded. The beads were then washed 4 times with 20 mM phosphate pH7.0, 150 mM NaCl. After the final wash, the beads were taken up in 10 ml of wash buffer and transferred to an Econopak column and the excess fluid allowed to drain off. The antibodies were eluted with 100 mM glycine pH2.5 and ten 0.5 ml fractions collected. Each aliquot was neutralized by adding 50 µ1 M Tris pH9.6. a further 8 fractions was collected using 0.5 ml aliquots of 100 mM sodium bicarbonate pH10.8. each aliquot was neutralized by the addition of 87 µ1 1M sodium citrate pH5.0. protein concentration was estimated from the O.D.280 (Harlow and Lane, 1988), and the fractions containing significant amounts of antibodies were pooled and dialysed against PBS. The purified antibodies were stored in aliquot at -20°C.

2.2.18 Cell extracts preparation

2.2.18.1 Whole cell extracts for Western Blot analysis

Cells were either lysed directly in the SDS-PAGE sample buffer (120mM Tris-HCl, pH 6.8, 4%SDS, 50mM DTT, 20% v/v glycerol and 0.01% bromophenol blue) before a sonication to break down the chromosomal DNA, or lysed in buffer (20mM Tris-HCl, pH 7.4, 150mM NaCl, 0.5% NP-40, 1 × protease inhibitor cocktail), and the resulting cell extract was mixed with equal volume of 2 × sample buffer. For bother preparations, samples were boiled for 5 min before loading in a SDS-PAGE gel.

2.2.18.2 Nuclear and membrane/cytosol extracts

Cells were washed and scraped in ice-cold PBS and centrifuged at 1,000g for 10min. After the supernatant was removed, the pellets were gently suspended in hypotonic buffer (20mM Hepes-KOH pH7.4, 1mM EDTA, 0.05% NP-40, and fresh added protease inhibitor cocktail) and incubated on ice for 5 min. Then the nuclei were pelleted by centrifugation at 200g for 5min (4°C); the supernatant was used as soluble fraction after centrifugation at 1000g for 10min. the nuclei was washed once in hypotonic buffer and centrifugation again at 200g for 5min. the pellet was used as

nuclear fraction.

2.2.19 Determination of the protein concentration

2.2.19.1 The Lowry method

Lowry-I reagent (2% Na_2CO_3 in 0.1M NaOH) was added to 5 µl of the measured protein in 250 mM Tris-HCl (pH7.5), or to the various amounts (from 0 to 60 µl) of 0.5 mg/ml BSA supplemented with 5 µl of 250 mM Tris-HCl (pH 7.5) up to a total volume of 150 µl. 250 µl of freshly prepared Lowry-IV reagent (Lowry-I with 1/100 vol of 2% K-Na tartate and 1/100 vol of 1% $CuSO_4$) was added to the above solution and mixture was left for 5min at room temperature. Then 30 µl of 50% Folin-Ciocalteu reagent was added, mixed and the colour reaction was developed for 30-90 min in the dark. 100 µl of each sample was transferred into a separate well of 96-well ELLSA plate and the OD_{660} was measured on the Titertek Multiskan® MKII. A standard curve was made by plotting the absorbance versus concentration of the BSA solutions. The protein concentration in the experimental sample was determined by fitting its OD_{660} value into the standard curve.

2.2.19.2 The Bradford method

Various amounts (from 0 to 30 µl) of 0.5 mg/ml BSA solution and the experimental protein solution were diluted with 0.15M NaCl up to final volume of 100 µl. one ml of Coomassie brilliant blue solution (see below) was added to each tube and vortexed. The probes were incubated for 2 min at room temperature and the absorbance was read at 595nm. The standard curve was made by plotting the absorbance versus concentration of the BSA solutions, and the concentration of the investigated protein was determined from this curve. The Coomassie brilliant blue solution was prepared as followed: 100mg of Coomassie brilliant blue G-250 was dissolved in 50ml of 95% ethanol and supplemented with 100ml of 85% phosphoric acid. The volume was brought to 1 liter with water. The solution was filtered and stored at +4°C.

2.2.20 Separation of proteins by polyacrylamide gel electrophoresis (PAGE)

Unless otherwise indicated for most applications a polyacrylamide separating gel of 10-12.5% 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 H20 (for a 12%

separating gel, the amount of acrylamide/bis-acrylamide added was 10 ml and the H20 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/bisacrylamide, 2.5 ml of 0.5 M Tris.HCl pH 6.8, 50 ml of 20% SDS, 5.65 ml of H20, 100 µl of10% APS and 7.5 µl of TEMED. The gel was then run in 1× laemmli-running buffer (25mM Tris-HCl pH 8.3, 0.2 M glycine and 0.1% SDS) until the desired distance had been reached.

2.2.21 Staining the SDS-PAGE gels

2.2.21.1 Silver staining

Gels were placed in a clean container and covered with 3 to 5 gel volumes of fixing solution (40% methanol, 12% acetic acid). Agitate slowly 1-2 hr on orbital shaker or rocking platform. After washing twice with 30% ethanol, gels were incubated in 0.02% $Na_2S_2O_3$ solution for 1 min, and then washed with ddH_2O for 3 × 30 sec. gels were then stained in the silver solution (0.2% AgNO3, 0.03% HCHO) for 20 min with vigorous shaking. Pour off silver solution and wash the gel with large amount of ddH_2O twice for 1 min. add developing solution (6% Na2CO3, 0.02% HCHO, 0.0004% $Na_2S_2O_3$) to gels and agitate slowly until desired level of staining is achieved. Stop staining by incubating gels with fixing solution.

2.2.21.2 Coomassie colloidal staining

Gels were fixed for 1 hour in fixation solution (40% methanl, 10% acetic acid), stained with staining solution for overnight (at least 3 hours), and then destained carefully only with water until clear background is obtained. The staining solution is prepared as following: mix 98% solution A (20 g 85% H_3PO_4, 100g $(NH_4)_2SO_4$ and add H_2O to final volume of 980 ml) and 2% solution B (5% w/v Coomassie brilliant blue G250), and shake it intensive overnight, the resulting solution is called solution C. the staining solution is obtained by mixing 80% solution C and 20% methanol and should be used at the same day of preparation.

2.3 Experimental Procedures

2.3.1 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); Protein G plus agarose (Santa Cruz, CA); Nonidet P-40 (NP40; Boehringer Mannheim).

2.3.2 Plasmid constructs

The plasmids used for transfection of RPM-MC cells express CD44s or CD44s mutated in the ezrin binding domain (alanine substitutions for arginines at positions 293 and 294 and for lysines at positions 298, 299 and 300) were in the pcDNA3 vector (Invitrogen, DeShelp). The CD44s or CD44s mutated in ERM binding domain with a C-terminal Myc epitope was generated by PCR using the following primers: CD44 forward, 5'-gtaagctttccggaccagtttatg-3' and CD44-Myc reverse, 5'-atctcgagtcaagaCAGATCCTCTTCTGAGATGAGTTTTTGTTCgaaaggtcccaccccaatctt catatc-3'. Capital letters denote the C-terminal Myc epitope tag. The PCR product was subcloned into the Hind III / Xho I sites of pcDNA3.1/Hygro(+) (Invitrogen).

2.3.3 Coupled transcription/translation in vitro

The TNT (*Promega*) coupled rabbit reticulocyte lysate system was used to determine the authenticity of the cloned full length or cytoplasmic domain of CD44. All reagents used were supplied in the kit and the assay was performed according to the accompanying outlines provided. Briefly, 1 µg of highly purified plasmid DNA was added to a reaction vial containing 25 µl of reticulocyte lysate, 2 µl reaction buffer, 1 µl of amino acid mix (without methionine), 1 µl RNAsin (40U/µl), 4 µl of [^{35}S]-methionine, 1 µl T7 or Sp6 RNA polymerase (10U/µl) in a total volume of 50 µl. the reaction was incubated at 30°C for 1 hr before the product was heat denatured and loaded on a 10% or 12% SDS-PAGE. Once the run was complete, the gel was dried and exposed to autoradiography.

2.3.4 Immunoflurorescence of fixed cells

Adherent cells were grown on coverslips placed on the bottom of a 6-well culture dish

for 24-48 hrs. Once the cells reached the desired density the medium was removed and the cells washed in PBS. For fixation the cells were incubated with freshly thawed 4% paraformaldehyde/PBS solution for 30 min at room temperature. Permeabilization of the cells was achieved by incubation with 0.5% NP-40/PBS solution for 5 min at room temperature. Alternatively, fixation and permeabilization was achieved in one step by incubation with methanol for 2 min at room temperature. After the permeabilization was washed out by PBS, Blocking solution (10% FCS/PBS or 4% BSA/PBS) was added to prevent unspecific background and the cells were incubated for at least 30 min at room temperature. Once blocking was complete, the cells were stained with the appropriate primary antibodies diluted in Blocking buffer for 1-4 hrs at room temperature. After extensive washing in PBS the cells were incubated with the appropriate FITC-coupled secondary antibodies diluted in blocking buffer (approx. 1:100) for 1 hr at room temperature. The cells were washed three times in PBS prior to mounting coverslips. When the nuclear staining was necessary the DNA was counterstained with 4 µg/ml Hoechst dye for 1-2 minutes and washed in PBS before embedding. The samples were viewed by fluorescence microscopy with the appropriate set of filters.

2.3.5 Cell cultures

RPM-MC cells were kindly provided by Dr. I. Stamenkovic (Boston). The RT4-D6-P2T schwannoma cell line was purchased from the European Collection of Animal Cell Cultures (Salisbury). 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 °C.

2.3.6 Immunoprecipitation

2.3.6.1 Preparation of antibody-conjugated beads

30 µl of 50% protein G-sepharose beads slurry (Oncogene) together with 5 µg the 5G8 antibody and 500 µl ice-cold PBS (w/o Ca^{2+}/Mg^{2+}) were tumbled end over end at 4°C for ≥ 4 hr in a tube rotator. The beads containing the 5G8 antibody were precipitated by centrifugation at 4°C for 10 sec. after aspiration of the supernatant, the beads were washed with 1ml of lysis buffer (see below) twice.

2.3.6.2 Cell lysis and pre-clearing

For the immunoprecipitation of CD44, RPM-MC transfectant cells were grown to confluency (2×10^6), washed once in ice-cold PBS before lysing the cells on ice with 1 ml of lysis buffer (50 mM Tris pH7.4, 100 mM NaCl, 3 mM MgCl2, 0.5% NP-40) containing 1× Complete^{TM} protease inhibitors. Lysed cells were scraped together and transferred to a reaction tube and incubated on ice for 30 minutes. After DNA was sheared through a 26-guage needle, the lysate was cleared by centrifugation for 5 min 10000 × g at 4°C. Supernatant was transferred to new reaction tube containing 30 µl of 50% protein G-agarose beads slurry, tumbled end over end at 4°C for 1 – 2 hr. the supernatant was recovered by centrifugation at 12,000g at 4°C for 5 min.

2.3.6.3 Immunoprecipitation

The antibody-conjugated beads was added to the precleared cell lysate, and rotated overnight at 4°C. Immuncomplexes were recovered by centrifugation and washed 4 × with cold lysis buffer. 50 µl of 2 × Laemmli sample buffer was added. Co-immunoprecipitation of endogenous merlin with CD44, at confluency, as described above with the following exceptions: Lysis buffer 20 mM Tris pH7.4, 50 mM NaCl, 3 mM MgCl2, 0.5% NP40. Lysate was pre cleared for 2 hours with protein G agarose (Oncogene Science) before addition of the CD44 antibody 5G8 conjugated beads and rotated overnight at 4°C.

2.3.6.4 Western Blotting

Proteins resolved by 10% SDS-PAGE were transferred to Immunobilon-PVDF membrane (Milipore) using BioRad Transfer chambers containing transfer buffer (24mM Tris base, 193mM glycine, and 10% methanol). Transfer was performed at 4°C overnight.

2.3.6.5 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 was in blocking buffer (for phospho-specific antibodies 5% BSA, 0.1% tween in TBS) for 1 hour room temperature (for

phospho specific antibodies overnight 4°C). Washed 3 times for 10 min in TBS 0.1% tween 20. Incubation with secondary antibodies was in blocking buffer for 1 hour room temperature. Washed 3 times for 10 min in TBS 0.1% tween. Blots were developed using enhanced chemiluminescence (Amersham International plc) and visualized with Hyperfilm-ECL film (Amersham Life Science).

2.3.6.6 Stripping Western blot membrane

To allow more than a single use of western blot membranes, the membranes were stripped in the following way. They were incubated with a Strip solution (62.5 mM Tris, pH 6.8, 2% SDS, 0.8% DTT) at 50°C for 30 min with shaking. The membranes were then washed twice in the TBST for 2 min each time, blocked as usual and used for the normal western blot probing protocol.

2.3.6.7 DSP crosslinking

To co-immunoprecipitate endogenous merlin with wild-type CD44, the RPM-MC cells were treated with DSP (Dithiobis[succinimidylpropionate]) for crosslinking before cell lysis. The cross-linking of merlin with CD44 was performed in 1 mL of PBS with final concentration of 2 mM DSP at 4°C for 1-2 hr. The reaction was quenched by adding stop solution (1 M Tris, pH 7.5) to the final concentration of 20-50 mM and incubating for 15 min at room temperature. The cells were then washed with PBS twice, and lysed with lysis buffer for immunoprecipitation.

2.3.7 Generation of CD44-ICD and CD44 ectodomain cleavage product

RPM-MC cells stably expressing wild type CD44s or mutant CD44 were plated in duplicate, 2×10^5 cells were added to 12 well plates. Following day cells were serum starved for overnight in DMEM containing 5 μM L-685, 458, 10 μM γ-secreatase inhibitor E (Calbiochem) or Me_2SO as vehicle. Cells were washed once in ice-cold PBS before lysing the cells on ice with 100 μl 2 × laemmli buffer (160 mM Tris pH6.8, 4% SDS, 16% glycerol, 100 mM DTT, and 0.005% bromophenol blue). After heating at 95°C for 3 minutes, the samples were loaded on 12.5% SDS-PAGE, and blotted with anti-Myc antibody 9E10.

For treatment of HA, RPM-MC transfectant cells were grown on 12-well plates at density of 2×10^5 cells. Following day cells were serum starved for overnight in

DMEM medium with FCS. Cells were stimulated for 5 minutes with 100 µg/ml HA, then the cells were harvested in 2x Laemmli sample buffer and cell extracts were separated on a 12.5% SDS-polyacrylamide gel. Proteins were transferred to membrane, and immunoblotted with anti-Myc antibody 9E10.

2.3.8 Real-time PCR

2.3.8.1 First strand cDNA synthesis

To create the first strand of cDNA, the M-MLV Reverse Transcriptase (H-)(*Promega*) was used. Total RNA (20µg) was mixed with 2µl Oligo(dT)15 primer (0.5µg/µl) and bi-distilled water to give a 17µl reaction volume. After incubation of the mixture at 70°C for 5 min and quick chilling on ice for 5 min, 5 µl 5 × Reaction Buffer, 2 µl 10 mM dNTP mix (10mM each dATP, dGTP, dCTP and dTTP, *Peqlab*) and 1 µl Reverse Transcriptase were added to the reaction. Finally, the reaction was incubated at 42°C for 60 min. To inactivate the reaction, the tube was heated at 70°C for 15 min.

2.3.8.2 Amplification of first strand cDNA by real-time PCR

Real-time PCR was performed according the company introduction. Components of the reaction were mixed together as shown in Table 2.1

Components	**Volume, µl**
2 × SYBR-Green mix	10
Forward and reverse primers (10µM each)	2
Template DNA	2
Distilled water	6

Table 2.1 Composition of real-time PCR reaction

PART THREE
RESULTS

CD44 has been implicated in a wide variety of physiological and pathological processes including lymphocyte homing and activation, hematopoiesis, embryogenesis, tumor development and metastasis. This broad spectrum of functions suggests that CD44 can transduce multiple intracellular signals involved in growth, survival, differentiation, cell adhesion and motility. How CD44 is linked to multiple signal transduction pathways might be explained by the identity of intracellular complexes that are associated with CD44. However, to date only a few proteins associated with CD44 have been identified, for example, interaction between CD44 and ERM or merlin forms a molecular switch, specifying either growth promotion or growth inhibition (Morrison H. et al., 2001, Orian-Rousseau V. et al., 2002). When cells are at high cell density, the binding of high molecular weight HA to CD44 leads to the binding of activated merlin (dephosphorylated) to the CD44 cytoplasmic domain. This binding causes cell growth inhibition through interference with Ras dependent signaling. Since the complex contains dephosphorylated merlin, protein phosphates(s) might associate with the complex. When cells are at low cell density and growth permissive, CD44 cytoplasmic domain forms a complex containing phosphorylated ERM proteins. This suggests that protein kinases probably associate with the CD44 bound complex in the growth-permissive state. In both growth inhibitory and growth permissive states, the CD44-bound complexes might contain other protein partners that attain optimal signaling capacity to regulate cell proliferation.

To identify proteins that associate with CD44 specifically, I choose to work with the RPM-MC cell line. The RPM-MC cells are a human cell line that have no endogenous CD44 expression (Thomas, et al, 1992), but express merlin. The cells transfected with a construct encoding CD44s enhance HA binding (Peck et al, 1996), moreover HA treatment leads growth inhibition of the cells transfected with wild-type CD44s, but not the cells transfected with a CD44s mutated in ERM-merlin binding domain that prevented merlin binding (Legg et al, 1998; Morrison et al, 2001). I would perform the immunoprecipitation of CD44 from the transfectant cells at confluent conditions,

where active merlin associates with CD44. By compassion of co-immunoprecipitated proteins from wild-type CD44s transfected cells to those from mutant CD44s (CD44s with point mutations in the merlin-ERM binding domain) transfected cells, it should distinguish specific proteins that associate with CD44 from unspecific associating proteins that are always found in the immunoprecipitation. I transfected the RPM-MC cells with wild-type CD44s, or mutant CD44s as a negative control. Before performing co-immunoprecipitation, I examined whether CD44 has interaction with merlin in the cells. To do so, I examined whether merlin could undergo dephosphorylation at high cell density or by the treatment of HA at low cell density, and whether dephosphorylated merlin bind to CD44. If CD44 functionally interacts with merlin in the cells, I would perform co-immunoprecipition to identify proteins specifically associating with the CD44 cytoplasmic domain together with merlin.

3.1 CD44s interacts with merlin in RPM-MC cells

3.1.1 HA treatment induces merlin activation that is CD44 dependent in RPM-MC cells

As a principal ligand of CD44, high molecular weight HA can induce merlin activation (dephosphorylation) in the RT4-D6P2T cells and other cell lines. In addition it was shown that the RPM-MC cells transfected with wild-type CD44s, but not the cells transfected with mutant CD44, showed growth inhibition at low cell density upon HA treatment (Morrison et al, 2001). To determine whether CD44 functionally interacted with merlin, I treated the RPM-MC transfectant cells with 100 μg/ml high molecular weight HA for 5 min at low cell density. The cells were lysed and the lysates were subjected to 10% SDS-PAGE. Western blotting was performed with an anti-merlin specific antibody, and two merlin bands could be resolved (Fig.1A). The faster migrating band represents a dephosphorylated form, and the slower migrating band a phosphorylated form. HA treatment showed an increase of dephosphorylated merlin in RPM-MC cells expressing wild-type CD44s, but not in cells expressing the mutant defective in merlin-ERM binding (Fig 1A). This result indicates that CD44 functionally interacts with merlin in the RPM-MC cells.

3.1.2 CD44 triggers merlin dephosphorylation at high cell density

HA treatment mimics the effects of high cell density when cells are at low cell density, I therefore examined the phosphorylation status of merlin in the transfectant cells at different cell densities. RPM-MC transfectant cells at low and high cell density were lysed. The lysates were subjected to 10% SDS-PAGE and Western blotting was performed with an anti-merlin specific antibody. As shown in the Fig. 1B, from low to high cell density, the level of dephosphorylated merlin increased in all 3 transfected cells. This was expected, since the RPM-MC cells are contact inhibited, probably through transmembrane proteins other than CD44. However, there is more dephosphorylated merlin in the cells transfected with wt CD44 when cells are at high cell density, compared to mutant CD44 or empty vector transfected cells. This indicated that high cell density, as HA treatment at low cell density, can induce merlin dephosphorylation via CD44.

3.1.3 Co-immunoprecipitation of dephosphorylated merlin with wt CD44s, but not mt CD44, in the RPM-MC cells

It was shown previously that dephosphorylated merlin binds to CD44 when cells are at high cell density. Since merlin is activated (dephosphorylation) in the RPM-MC cells at high cell density, I investigated whether dephosphorylated merlin could also bind to CD44s. The RPM-MC transfectant cells were cultured at high cell density, treated with the crosslinker DSP, and then lysed and immunoprecipitated with the CD44 antibody 5G8 (Sleeman et al, 1996). The immunoprecipitates were subjected to 10% SDS-PAGE under reducing conditions, and Western Blotting was performed with an anti-merlin specific antibody (C18). As expected, I detected that dephosphorylated merlin was co-immunoprecipitated with wild-type CD44s, but not with CD44s with mutations in the merlin-ERM binding domain (Figure 1C).

Since wild type CD44s, but not mutant CD44, triggered merlin dephosphorylation and formed a complex with dephosphorylated merlin when cells are at high cell density, and HA binding to wtCD44s induced merlin dephosphorylation, I used this cell system to identify CD44 intracellular partners under high cell density conditions where active merlin was bound to CD44.

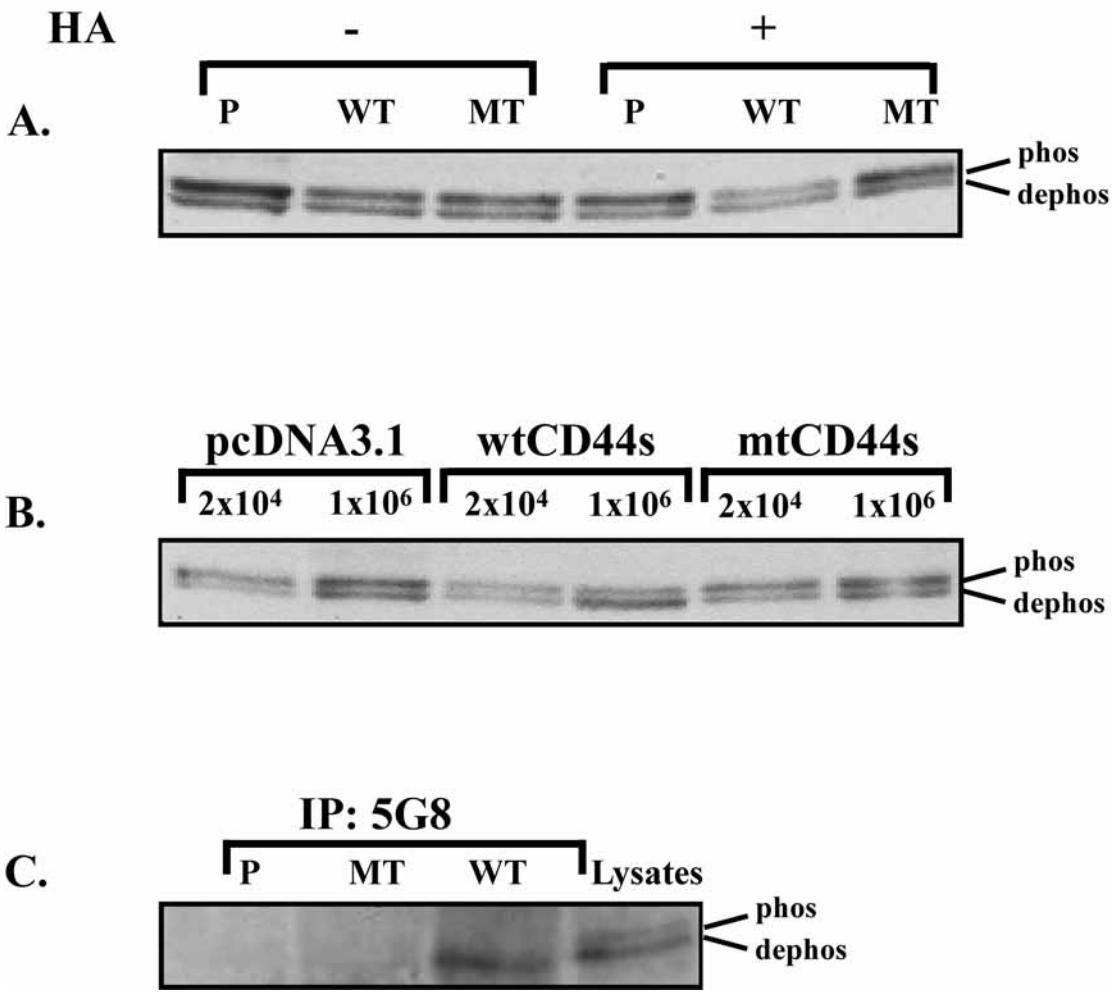


Figure 1. **Wild-type CD44s, but not mutant CD44, interacts with dephosphorylated merlin in RPM-MC cells.** (A). The RPM-MC were stably transfected with expression constructs encoding either a wild type CD44s (WT) or CD44 with point mutations in ERM binding domain (MT), and control cells were transfected with empty vector pcDNA3.1 (P). 1×10^5 RPM-MC transfectant cells were plated in 12-well plate, and were treated with 100 μg/ml HA for 5min. The cell lysates were subjected to 10% SDS-PAGE and probed with an anti-merlin specific antibody (C-18). (B).The cells were plated at low cell density or high cell density in 12-well plate (as indicated). The cells were lysed and the lysates subjected to 10% SDS-PAGE and Western Blotting using an anti-merlin specific antibody (C-18). (C). The RPM-MC were stablely transfected with wild type CD44s, CD44 with point mutations, and empty vector pcDNA3.1. The cells were seeded at high cell density and treated with a crosslinker DSP. The cell lysates were immunoprecipitated with CD44 antibody 5G8, and immunoprecipitates and whole cell lysates were then subjected to 10% SDS-PAGE. Western Blotting was performed using an anti-merlin specific antibody (C-18).

3.2 Identification of importin ß and importin 5 as CD44 associated proteins

To identify proteins that associate with the CD44 cytoplasmic domain, the following experiments were performed. The wild-type or mutant CD44 transfectant RPM-MC cells were cultured at high cell density and lysed. CD44 was immunoprecipitated with the 5G8 antibody. The immunoprecipitates were resolved by 8% SDS-PAGE, and the co-immunoprecipitated proteins were revealed by silver staining (Figure 2). In the immunoprecipitation of wild-type CD44, there were an additional 4 protein bands that were visualized in the silver staining, compared to the immunoprecipitation from mutant or empty vector cells. I performed this experiment several times and the same result was obtained every time. This indicated that these four proteins could be putative partners associating with CD44 cytoplasmic domain under growth inhibitory conditions.

To get enough protein for identification by matrix-assisted laser-desorption/ionization mass spectrometry (MALDI-MS) analysis, immunoprecipitation from 1×10^7 cells was performed. Proteins with immunoprecipitates were resolved by SDS-PAGE under reducing condition. After Colloidal Coomassie Blue Staining, the protein of 95kDa and 125kDa that were co-immunoprecipitated with wild-type CD44 were cut from the gel, and sent for identification. The protein identifications were kindly done by Dr. Lothar Jänsch (GBF, Braunschweig). The protein of 95kDa was identified as importin ß and the protein of 125kD as importin 5. The other proteins could not be identified since I could not get enough protein for identification.

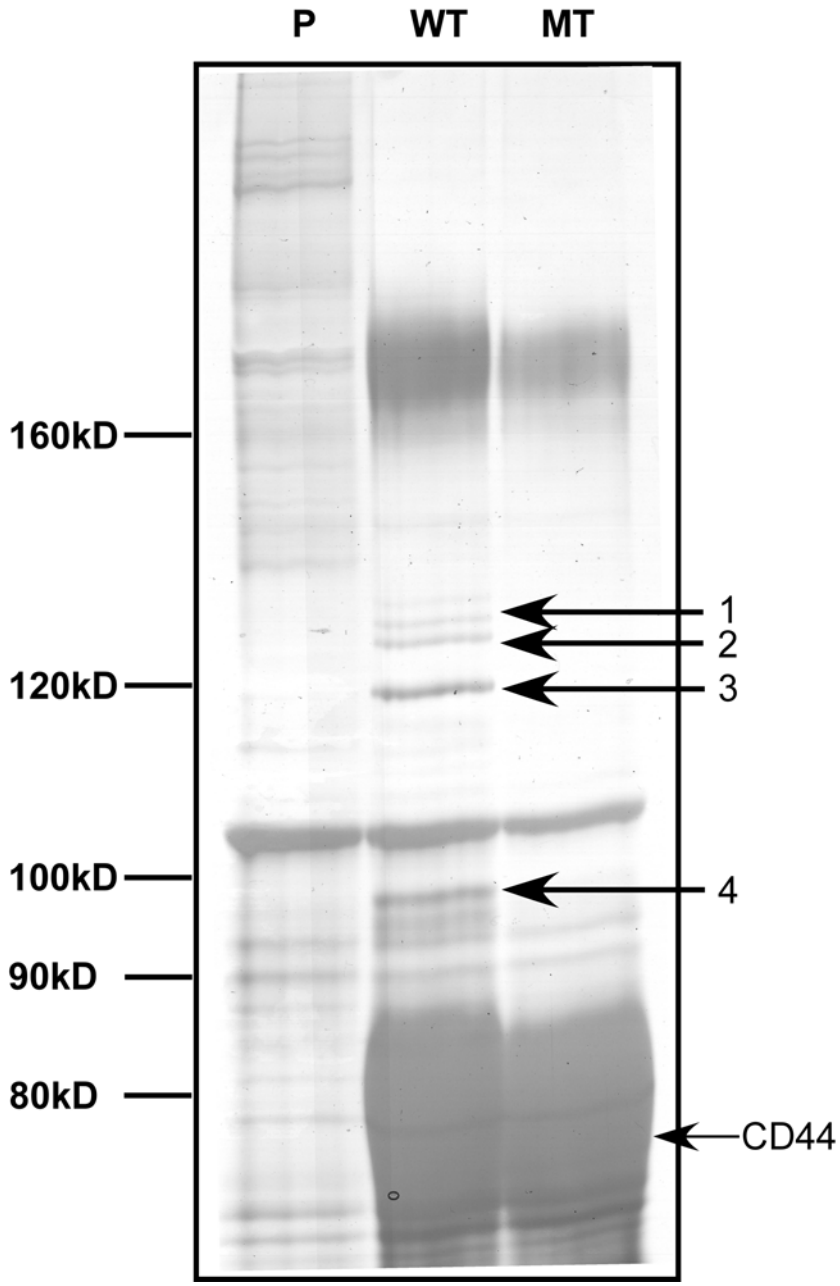


Figure 2. **Proteins were co-immunoprecipitated with wild type CD44s, but not CD44 mutant.** The RPM-MC cells transfected with empty vector pcDNA3.1 (P), wild-type CD44s (WT), or mutant CD44 (MT) were immunoprecipitated with CD44 antibody 5G8. The immunoprecipitates were subjected to 8% SDS-PAGE, and revealed with silver staining. Band 3 was identified as importin ß, and band 4 as importin 5. I performed the experiment more than three times and obtained the same results.

3.3 Importins associate with CD44 in both RT4-D6P2T and RPM-MC cells

3.3.1 Co-immunoprecipitation of importins with CD44 on RPM-MC cells

To confirm the results that importin ß and importin 5 associate with the CD44 cytoplasmic domain, I used CD44 specific antibodies to perform co-immunoprecipitation. The RPM-MC transfectant cells were lysed and immunoprecipitated with the CD44 antibody 5G8. The immunoprecipitated proteins were subjected to 10% SDS-PAGE and Western blotting was performed with importin ß or importin 5 antibodies. The immunodetection with importin ß or importin 5 antibodies showed that importin ß or importin 5 was co-immunoprecipitated with wild-type CD44s, but not with mutant CD44. At the same time, I also performed the co-immunoprecipitation experiment in the other direction. The RPM-MC transfectant cells were lysed and proteins were immunoprecipitated with importin ß or importin 5 antibody. The immunodetection with The CD44 antibody 5G8 showed that wild-type CD44s was co-immunoprecipitated with importin ß or importin 5. The mutant CD44 could not be co-immunoprecipitated with importin ß or importin 5. These data indicate that importin ß or importin 5 are associated with wild-type CD44s (Figure 3).

3.3.2 Co-immunoprecipitation of importins with CD44 in RT4-D6P2T cells

To find out whether importins could associate with CD44 in other cell lines, the co-immunoprecipitation experiments by the CD44 antibody 5G8 or anti-importin antibody were performed in the RT4-D6P2T cells in both directions. As shown in Figure 4, importin ß or importin 5 bound only to wild-type CD44s, but not to mutant CD44 similar to the result obtained in the RPM-MC cells. This experiment showed that importins associate with CD44 in cell lines other than RPM-MC cells (Figure 4).

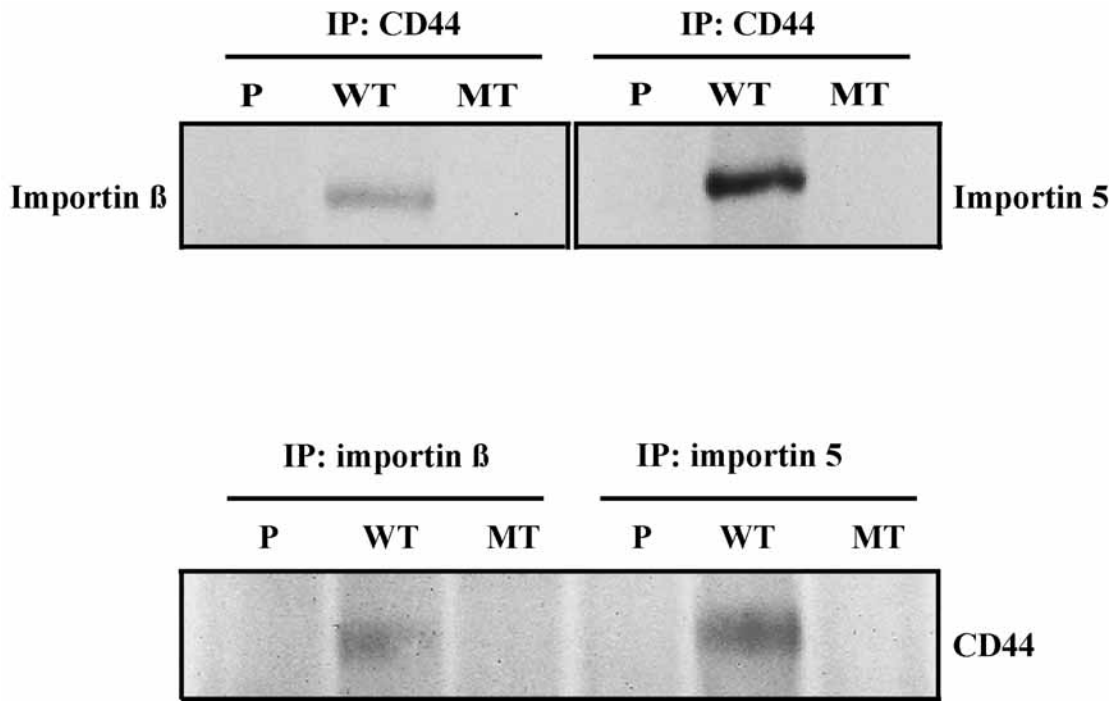


Figure 3. **Co-immunoprecipitation of CD44 with importin in RPM-MC cells.** RPM-MC cells transfected with empty vector pcDNA3.1, wild type CD44s (WT), or CD44 mutants (MT) were lysed and lysates were immunoprecipitated with either the CD44 antibody 5G8 or importin antibody. The lysates were subjected to 10%SDS-PAGE, and Western Blotting was performed using importin antibody, or CD44 antibody 5G8. The same results were obtained in 3 separate experiments.

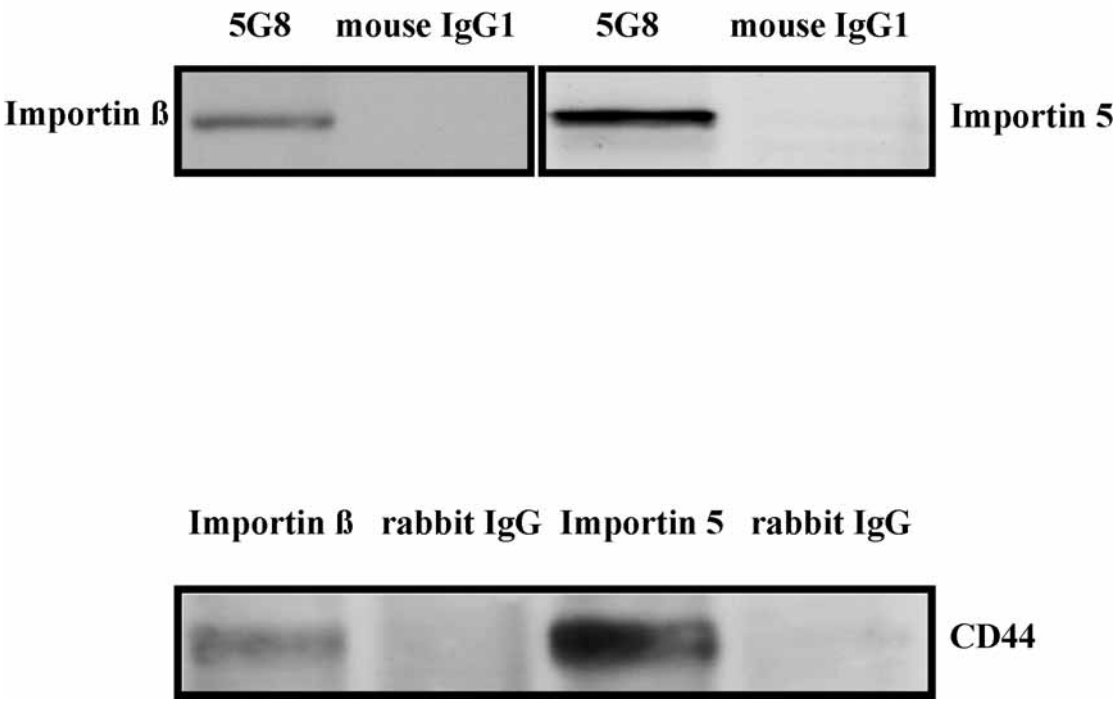


Figure 4. **Co-immunoprecipitation of CD44 with importin in RT4D6P2T cells.** RT4D6P2T cells transfected with empty vector pcDNA3.1 (P), wild type CD44s (WT), or CD44 mutants (MT) were lysed and lysates were immunoprecipitated with CD44 antibody 5G8 or importin antibody. The lysates were subjected to 10% SDS-PAGE, and Western Blotting was performed using importin antibody, or CD44 antibody 5G8. The same results were obtained in 3 separate experiments.

3.3.3 Co-localization of importins with CD44 in RPM-MC cells

To show that importin ß or importin 5 are associated to CD44s cytoplasmic tail *in vivo*, I investigated whether importins co-localized with CD44 in RPM-MC cells by immunofluorescence analysis. The RPM-MC transfectant cells were double labelled with the anti-CD44 antibody 5G8 and anti-importin ß or anti-importin 5 antibodies, and then immunofluorescence was detected by incubation with FITC-conjugated anti-mouse secondary antibody (green for CD44 indicated by arrow) and TRITC-conjugated anti-rabbit secondary antibody (red for importins indicated by arrow). CD44 proteins, as expected, were concentrated in the plasma membrane. Importin ß or importin 5 proteins mainly localized in cytoplasm and nucleus, but importin ß or importin 5 were also detected in the plasma membrane (Fig. 5). The overlay experiments showed that CD44s colocalized with importin ß or importin 5 at the plasma membrane.

3.3.4. Co-localization of importins with CD44 in RT4-D6P2T cells

To determine whether importin ß or importin 5 also co-localized with CD44 in RT4-D6P2T cells, I also checked the co-localization of importins and CD44 in these cells by immunofluorescence analysis. I obtained a similar result as observed in RPM-MC cells (Fig 6): CD44 colocalized with importin ß or importin 5 at the plasma membrane.

From the co-immunoprecipitation and co-localization experiments, I conclude that importin ß or importin 5 can interact with wild-type CD44s in both RT4-D6P2T and RPM-MC cells.

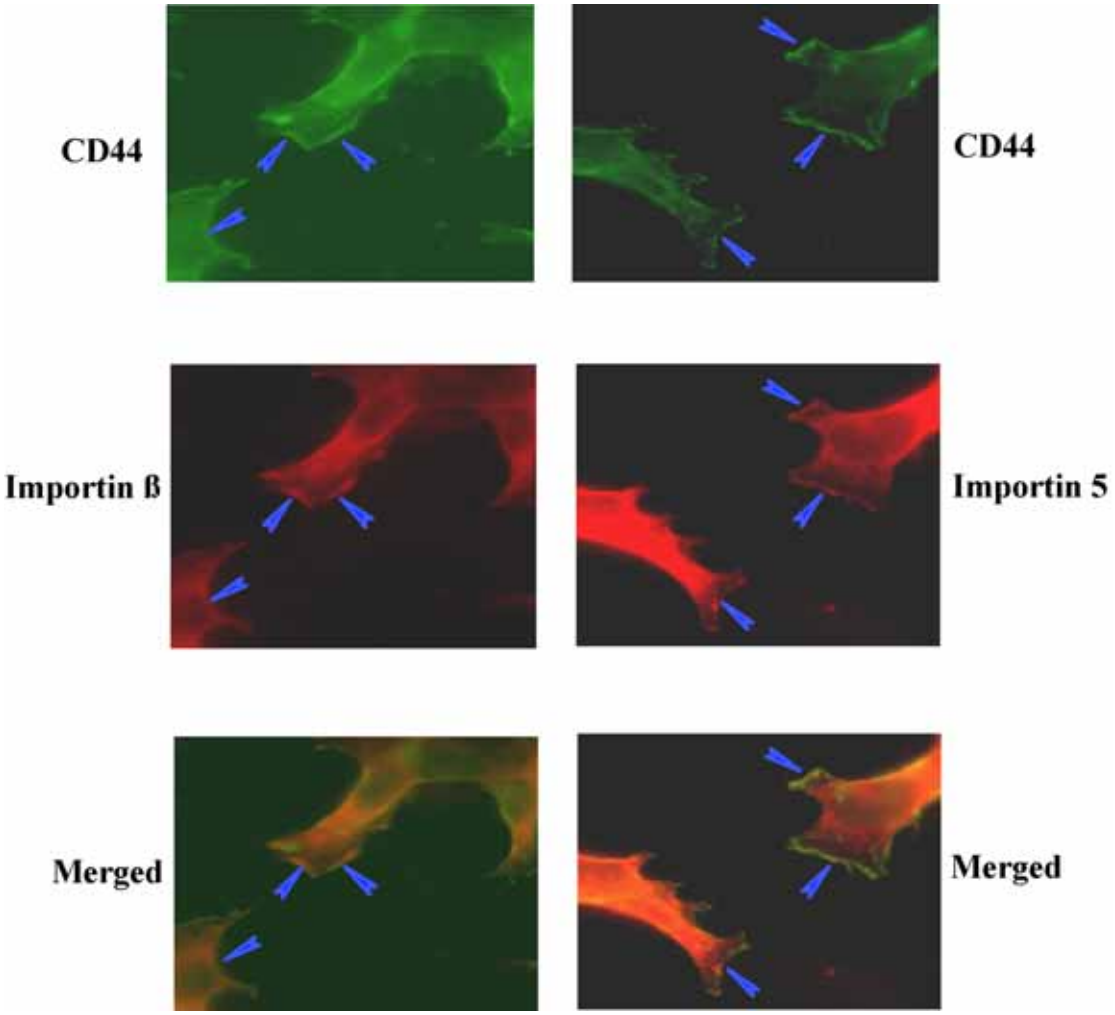


Figure 5. **Co-localization of importins with CD44 in RPM-MC cells.** RPM-MC cells transfected with wild type CD44 were fixed by 4% paraformaldehyde. Subsequently, cells were rendered permeable by 0.5% NP-40 treatment and stained with the 5G8 antibody (anti-CD44), importin ß or importin 5 antibody. Further, CD44 was stained with FITC-labeled anti-mouse IgG antibody (green for CD44 indicated by arrows), and importin ß or importin 5 were stained with TRITC-labeled anti-rabbit IgG antibody (red for importin ß or importin 5, indicated by arrows). The merged images showed the co-localization of CD44 with importin ß or importin 5 on plasma membrane structures of RPM-MC cells (yellow, indicated by arrows).

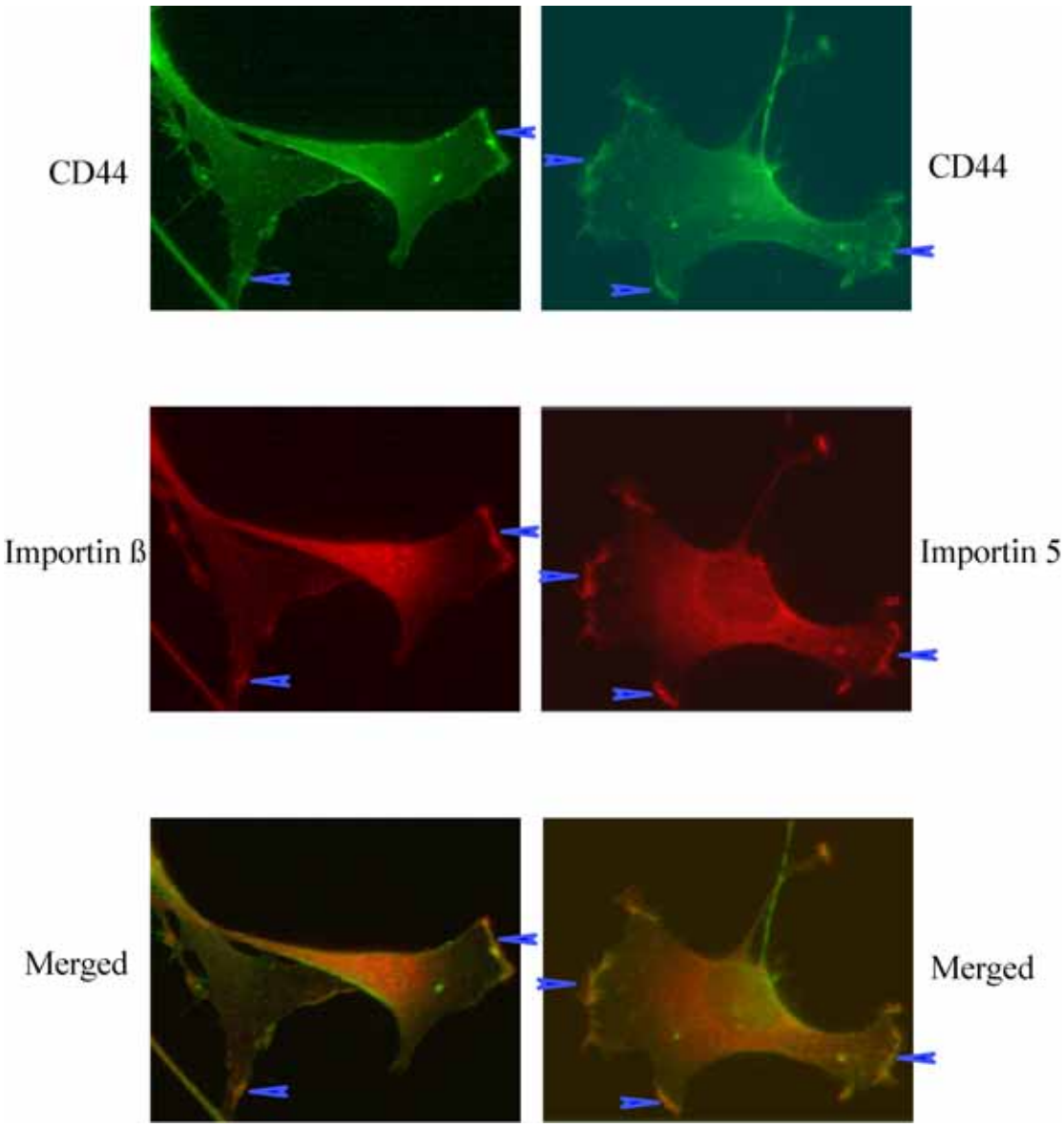


Figure 6. **Co-localization of importins with CD44 in RT4-D6P2T cells.** RT4-D6P2T cells were fixed by 4% paraformaldehyde. Subsequently, cells were rendered permeable by 0.5% NP-40 treatment and stained with the 5G8 antibody (anti-CD44), importin ß or importin 5 antibody. Further, CD44 was stained with FITC-labeled anti-mouse IgG antibody (green for CD44, indicated by arrows), and importin ß or importin 5 were stained with TRITC-labeled anti-rabbit IgG antibody (red for importin ß or importin 5, indicated by arrows). The merged images showed the co-localization of CD44 with importin ß or importin 5 on plasma membrane structures of RT4-D6P2T cells (yellow, indicated by arrows).

3.4 CD44 cleavage and nuclear translocation

Importin ß and importin 5 belong to one large family of homologous proteins. Members of this family mediate either nuclear import or nuclear export, and therefore are classified as importins and exportins. Importins recognize their substrates in the cytoplasm and transport them through nuclear pores into the nucleus. In contrast, exportins interact with their substrates in the nucleus and release them in the cytoplasm.

Although I found that importins associate to wild-type full length CD44s cytoplasmic domain, there was no evidence that full length CD44 translocates into the nucleus. In the immunofluroescence analysis using the 5G8 antibody, which recognizes the extracellular domain of CD44, no staining of full length CD44s was found in the nucleus. Interestingly, it had been previously reported that CD44 undergoes sequential proteolytic cleavage in the extracellular domain (EXT) and intracellular domain (ICD) (Okamoto et al., 2001). The first cleavage process generates a soluble NH2-terminal fragment released into the culture supernatant (soluble CD44) and a membrane-bound COOH-terminal cleavage product (CD44EXT, CD44 extracellular truncation). Following the ectodomain cleavage, the intramembraneous cleavage of CD44EXT occurs (the cleavage site are shown in Figure 7A), resulting in the release of CD44 intracellular domain (CD44ICD). The CD44 intracellular domain can translocate to the nucleus and it has been proposed that CD44ICD can regulate gene expression in the nucleus.

Normally, proteins that are transported into the nucleus by importin contain a nuclear localization signal (NLS) which is recognized by importin. The NLS consists of one or more cluster of basic amino acids. Interestingly, there are clusters of basic amino acids in the wild-type CD44 intracellular domain, suggesting these amino acids might function as NLS for the CD44 cytoplasmic tail. In the mutant CD44 protein that is unable to bind to merlin and ERM proteins, some of these basic amino acids were changed (Figure 7B), and these mutations abolished the association of importins to CD44. Therefore, I proposed that these amino acids serve as NLS for CD44 cytoplasmic tail and importins might play a role in the nuclear translocation of CD44ICD by association with the putative NLS of CD44.

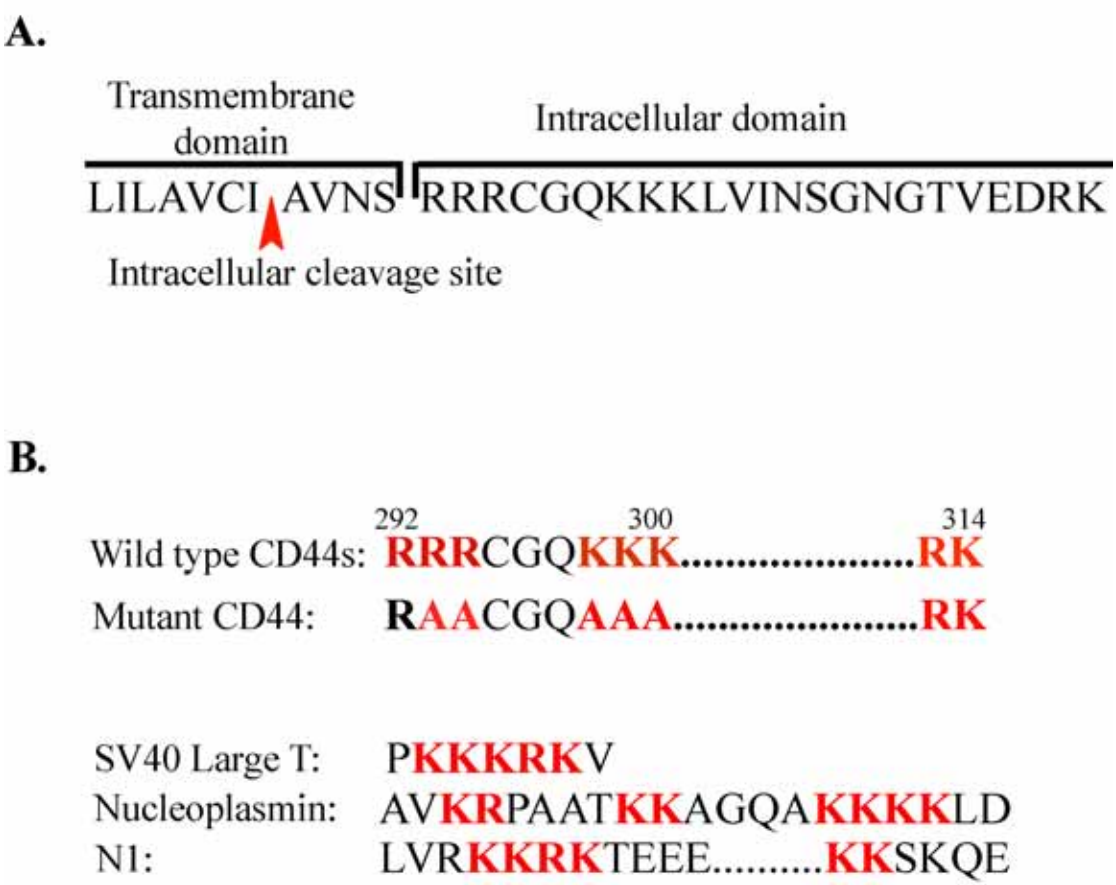


Fig.7 (**A) the cleavage site in CD44 intracellular domain**. Amino acid sequences in the transmembrane and intracellular domain of rat CD44 are shown by the single abbreviations. (B).**The putative nuclear localization signal (NLS) of CD44 comparing to the NLS of other proteins**. Wild-type CD44s contains several clusters of basic residues in its intracellular domain, and the mutation of some basic residues abolishes the binding of importins to CD44. Comparison of NLS of other proteins (SV40 Large T, Nucleoplasmin and N1 protein) to CD44 putative NLS. Amino acids important in nuclear targeting are boxed and in red type.

To test the assumption that importins mediate the nuclear translocation of CD44ICD, I examined whether CD44 undergoes intracellular cleavage in the RPM-MC cells, and whether the wild-type CD44ICD localizes in the nucleus, whereas the mutant CD44ICD localizes in the cytoplasm.

3.4.1 Translation of the CD44ICD protein *in vitro*

To get an idea of the size of untagged CD44ICD, expression constructs encoding wild-type or mutant CD44ICD were used as templates for *in vitro* transcription and translation. The translated protein was ^{35}S radiolabelled and subjected to 12% SDS-PAGE. A protein of a molecular weight of about 18kDa was observed for wtCD44ICD, and a protein of about 16kDa for mtCD44ICD. No product was observed in the empty vector pcDNA3.1 (Fig 8).

3.4.2 TPA induces CD44 cleavage and more cleavage products in wt CD44s than mutant CD44

To explore the cleavage of CD44ICD from full length CD44, I generated stable clones of RPM-MC cells that over-express wild-type or mutant CD44s with a C-terminal Myc epitope tag. Since it was previously shown that TPA can induce the cleavage of CD44ICD, I treated the transfected cells with TPA (100 ng/ml) for 30 min. By SDS gel electrophoresis and Western blotting with the Myc-specific antibody 9E10, a CD44ICD band could be resolved with a MW of 18kDa (Fig.10). TPA treatment could enhance the cleavage of wild-type CD44s. Interestingly the mutant CD44s underwent no cleavage compared to the wild-type CD44s. The difference was not due to the expression of CD44, since the expression levels of mutant CD44s was similar to wild-type CD44s as revealed by Western blotting with the anti-CD44 antibody (Fig 9).

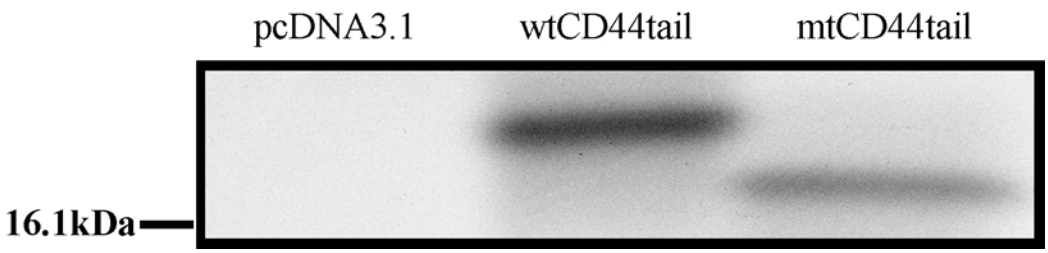


Fig. 8**. The *in vitro* transcription and translation of the CD44ICD protein**. The expression construction encoding wild-type CD44 intracellular domain (wtCD44ICD) or mutant CD44 intracellular domain (mtCD44ICD) were used as templates for *in vitro* transcription/translation. The empty vector pcDNA3.1 was used as negative control. The translated protein was ^{35}S-radiolabelled and subjected to 12% SDS-PAGE. After radiography, a protein of the molecular weight of about 18kDa was observed for wtCD44ICD, and a protein of about 16kDa for mtCD44ICD

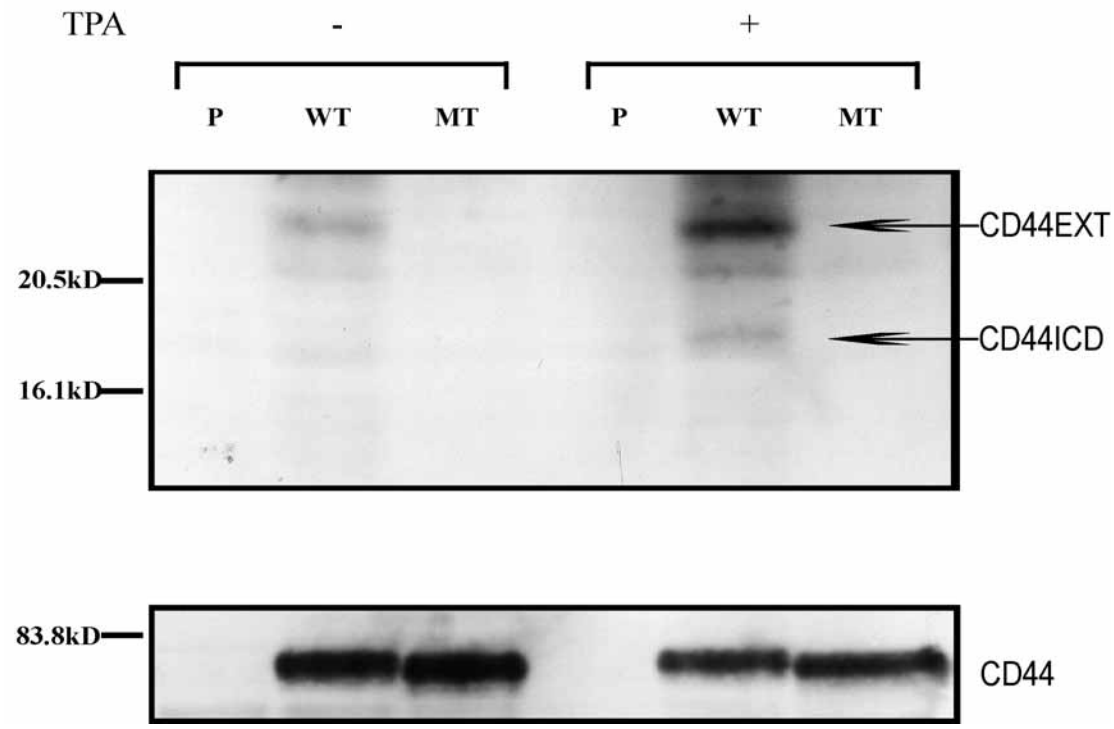


Figure 9. **Induction of CD44 cleavage by TPA treatment**. RPM-MC cells transfected with empty vector pcDNA3.1, wild type CD44s-Myc, mutant CD44-Myc constructs were plated (2×10^5 cells/well) in a 12-well culture plate. After incubation for 40min with TPA (100ng/ml), the cells were lysed, and subjected to 12.5% SDS-PAGE. (A).Western Blotting was performed by anti-Myc antibody (9E10). The upper band represents the CD44 ectodomain cleavage products, and the lower one the CD44ICD. (B) Western Blotting was performed by anti-pan CD44 antibody 5G8 to compare the expression level of wild-type and mutant CD44s. The same results were obtained 3 times.

3.4.3 Nuclear localization of wt CD44 cytoplasmic tail generated from full length CD44

To examine the localization of CD44ICD generated from full length CD44 upon TPA treatment, proteins of RPM-MC transfectant cells were separated into a membrane/cytosolic and a nuclear fraction. Histone H1 was used as nuclear marker and MEK1 as cytosolic marker. Immunoblot analysis with anti-Myc Antibody indicated that wild-type CD44ICD was found in the nuclear fraction. Since there was no ICD from the mutant CD44s, I could not detect the localization of mutant CD44ICD. The ectodomain cleavage product of both wild type and mutant CD44 were present in membrane/cytosolic fractions (Figure 10). This result indicated that importins might be involved in the nuclear translocation of CD44ICD, although I can not determine the localization of mutant CD44ICD.

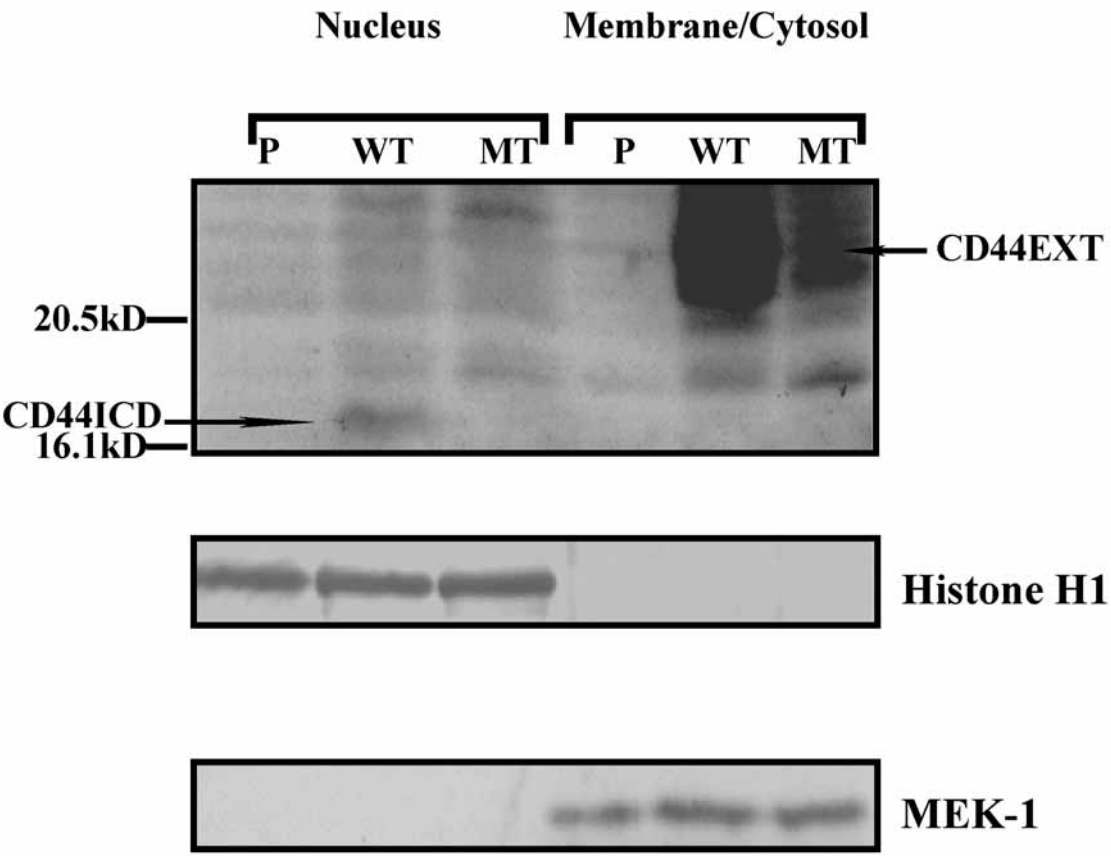


Figure 10. **Nuclear localization of wild type CD44ICD.** RPM-MC cells transfected with vector pcDNA3.1, Myc-tagged wild type CD44s, or Myc-tagged CD44 mutant constructs were treated with TPA (100ng/ml) for 40 minutes. Membrane/cytosol and nuclear fractions were analyzed by Western blotting with anti-Myc Ab (9E10), anti-MEK-1 Ab (cytosolic marker), and anti-Histone H1 (Nuclear Marker). The same results were obtained in 3 separate experiments.

3.4.4 Nuclear translocation of GFP tagged wild-type CD44 cytoplasmic tail

If importins mediate the nuclear localization of wild-type CD44 cytoplasmic tail, and importins cannot interact with mutant CD44, the mutant CD44 cytoplasmic tail should stay in the cytoplasm. Since there is no ICD cleavage from the mutant CD44s, this system can not be tested for the importins function. I therfore transiently transfected RT4-D6P2T or RPM-MC cells with GFP tagged wild-type CD44 cytoplasmic tail or mutant CD44 cytoplasmic tail. As shown in Fig.11, the GFP tagged wild-type CD44 cytoplasmic tails were extensively (about 90%) localized in the nucleus, whereas the mutant CD44 tails were mainly (about 60%) localized in the cytoplasm. These data suggest that importins might take part in the nuclear translocation of wild-type CD44 cytoplasmic tail. Moreover, the ERM binding motif containing clusters of basic residues is more than likely the nuclear localization signal.

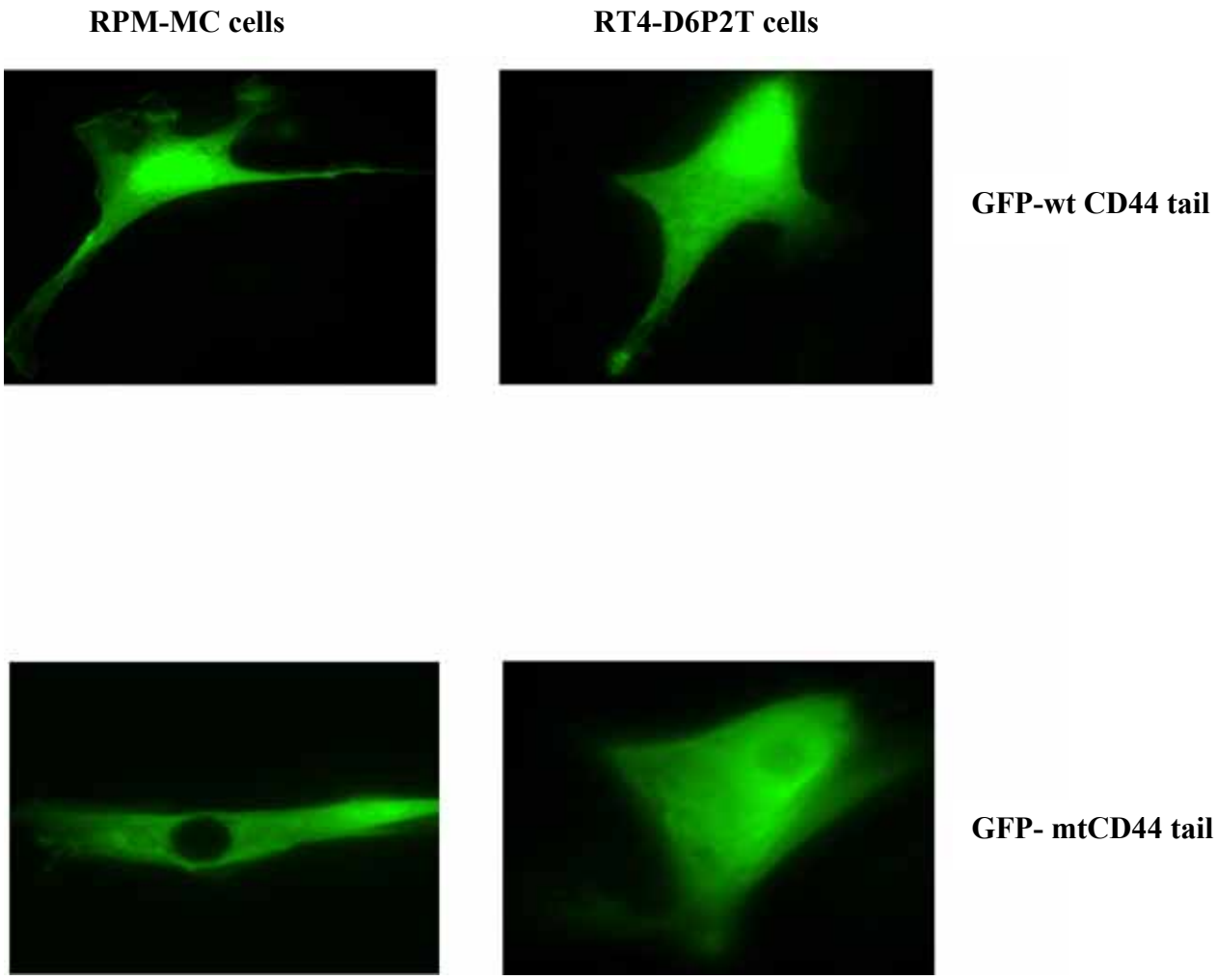


Figure 11. **Nuclear localization of GFP tagged wild type CD44 cytoplasmic tail in both RT4-D6P2T cells and RPM-MC cells.** The RPM-MC or RT4-D6P2T cells were transiently transfected with GFP-tagged wild type CD44 cytoplasmic tail (GFP-CD44s tail), or GFP-tagged mutant CD44 cytoplasmic tail (GFP-CD44m tail). After 24 hours, the cells were fixed by 4% paraformaldehyde, and examined by microscopy (Axiovert 135). In RPM-MC cells, there are 46 cells showing nuclear localization of wild-type CD44 tail in total 50 cells, and 32 cells showing cytoplasm localization of mutant CD44 tail in total 50 cells. In RT4-D6P2T cells, there are 45 cells showing nuclear localization of wild-type CD44 tail in total 50 cells, and 30 cells showing cytoplasm localization of mutant CD44 tail in total 50 cells

3.5 Analysis of the conditions for CD44 cleavage

It has been demonstrated that CD44 cleavage requires a presenin-dependent γ-secretase which is also involved in the intramembraneous cleavage of the ß-amyloid precursor protein (ß-APP), Notch, LDL receptor-related protein, E-cadherin, and ErbB-4 (Cao et al, 2001; Wong et al, 1997; De Strooper et al, 1999; May et al, 2002; Marambaud et al, 2002; Ni et al, 2001; Lee et al, 2002).

3.5.1 Generation of CD44ICD is γ-secretase dependent

To check whether the CD44ICD generation is dependent on the activity of γ-secretase in the RPM-MC cells, the cells transfected with wild-type CD44s were incubated with specific γ-secretase inhibitors, for example 10nM compound E (10nM) (Seiffert et al, 2000), or 10nM L-685, 458 (Shearman et al, 2000) overnight in serum-free medium. Both γ-secretase inhibitors prevented the production of CD44ICD and led to accumulation of CD44EXT production (Fig 12). This result indicated that the generation of CD44ICD is also dependent on γ-secretase activity in RPM-MC cells.

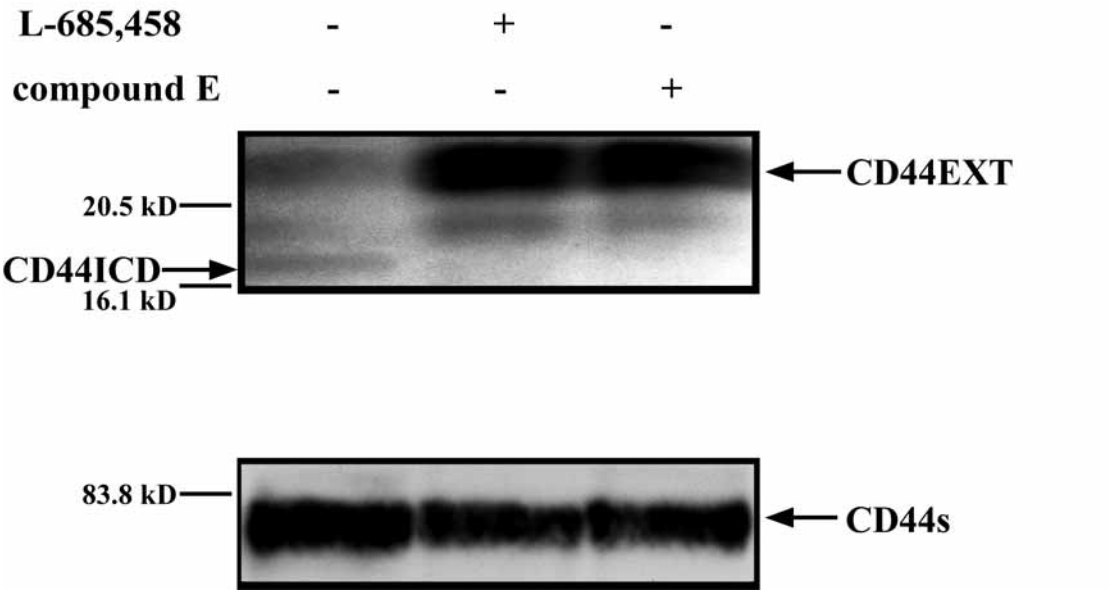


Fig. 12. **γ-secretase inhibitors inhibit production of CD44ICD and lead to accumulation of CD44EXT production.** RPM-MC cells transfected with wild type CD44s were treated with γ-secretase inhibitors L-685,458 or compound E (Calbiochem) or Me_2SO for 24 hours in serum-free medium, and Western blotting was carried out using anti-Myc antibody 9E10. The figure shows that the full length CD44s (CD44Full), extracellular domain cleavage production (CD44EXT), and intracellular cleavage of CD44 (CD44ICD). In the absence of inhibitors, CD44ICD was produced. Note that there more CD44EXT products in cells treated with inhibitors than ones in cells treated without inhibitors. Similar results were obtained in 3 separate experiments

3.5.2 HA treatment

1) HA treatment enhanced the cleavage of CD44ICD

The binding of ligands to cell-surface receptors such as Notch and ErbB 4 provoked the cleavage of the receptors, resulting in the release of the intracellular domain. Since the recruitment of merlin and the association of importins can be induced by HA treatment (Fig 1 & Fig 3), I examined whether the binding of HA to CD44 could induce the intracellular cleavage of CD44. The RPM-MC transfectant cells were serum starved for overnigh and then treated cells with HA for 5 minutes. The cells were lysed and the lysates were subjected to 12.5% SDS-PAGE. By Western blotting with the Myc-specific antibody 9E10, I found that HA can in fact trigger the proteolytic cleavage of CD44ICD in RPM-MC cells (Figure 13).

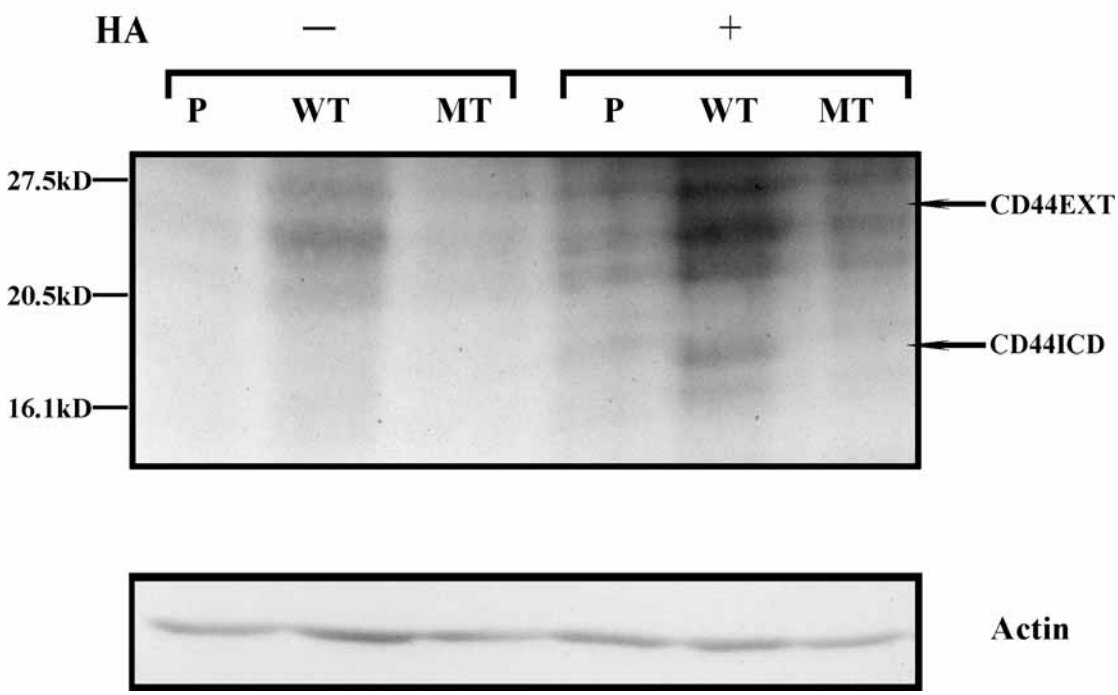


Figure 13. **HA stimulates the cleavage of CD44.** RPM-MC cells transfected with empty vector pcDNA3.1, wild type CD44s-Myc, and mutant CD44-Myc constructs were plated (2×10^5 cells/well) in a 12-well culture plate. Cells were serum starved in DMEM medium for overnight. After incubation for 5min with HA (100ug/ml), the cells were lysed, and subjected to 12.5% SDS-PAGE. Western Blotting was performed by anti-Myc antibody (9E10). The upper band represents the CD44 ectodomain cleavage products and the lower one the CD44ICD. For loading control the anti-actin antibody was used. Similar results were obtained for 3 times.

2) HA induces the cleavage of CD44ICD through a Rac-dependent pathway

There are several putative pathways activated by CD44 interaction with its ligand HA. HA treatment on logarithmically growing cells resulted in growth inhibition through inhibition of the Ras signaling pathway (Morrison H. et al, 2001). Binding of HA to CD44 activates Rho GTPase such as RhoA and Rac1 participating in the interaction between CD44 and cytoskeletal proteins (Schmits et al, 1997; Sy et al, 1991; Liu et al, 1996). To explore which signaling pathways are involved in the induction of CD44 cleavage by HA treatment, I treated the RPM-MC transfectant cells exposed to HA with toxin TcsL1522 which inhibits Rac activity. The effects of HA treatment were almost completely abolished with toxin TcsL1522 pretreatment. I got a similar result by using another chemical (TcdB 1470) which also inhibits Rac activity (data not shown). From this experiment, I conclude that the cleavage of CD44ICD by binding of HA to CD44 requires Rac signaling.

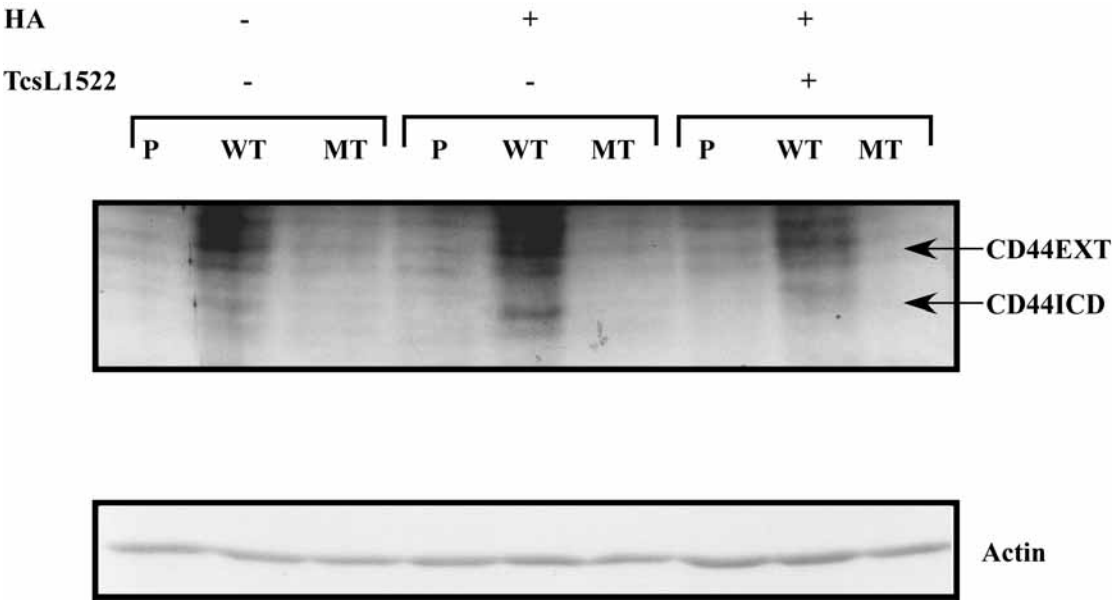


Fig. 14 **Inhibition of Rac activity prevents CD44 cleavage induced by HA treatment**. RPM-MC cells stably transfected with empty vector pcDNA3.1 (P), wild-type CD44s (WT), or CD44 mutant (MT) were serum starved in DMEM medium for overnight, and then treated with or without 200ng/ml TcsL1522 (inhibitor of Rac) for 1 hour before the cells were exposed to 100μg/ml HA for 5 min. The cell lysates were subjected to 12.5% SDS-PAGE, and Blots were probed with anti-Myc antibody 9E10. Western blotting with anti-actin antibody was used for a loading control. Similar data were also obtained with TcdB1470 which also inhibits Rac activity. Similar results were obtained 3 times

Treatment of cells with high molecular HA at low cell density could mimic the situation of cells at high cell density, leading to cell growth inhibition and merlin dephosphorylation. I therefore examined how various conditions influence cleavage of CD44. I observed no CD44 cleavage at low cell density, but found that serum starvation induced the proteolytic cleavage of CD44s (Figure 15).

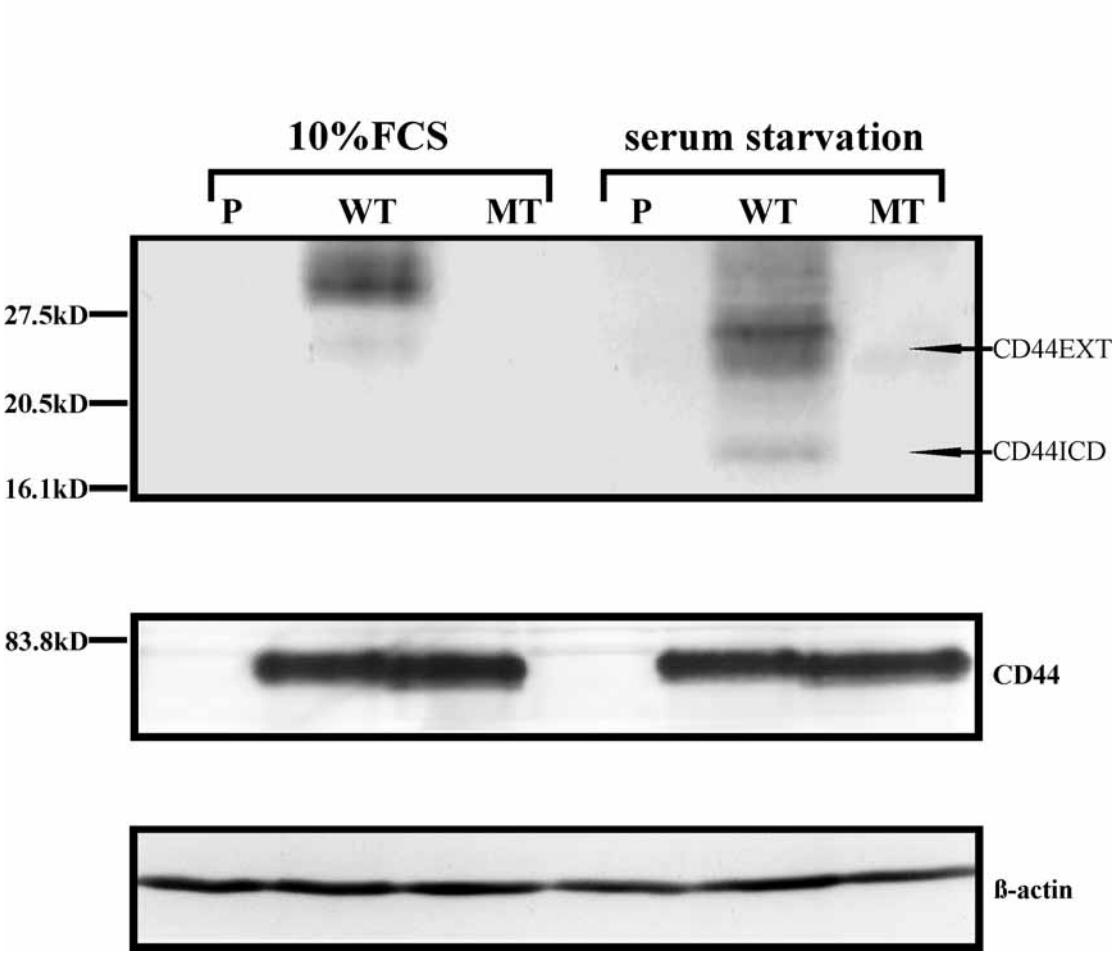


Figure 15. **Serum starvation induces CD44 cleavage**. RPM-MC cells transfected with empty vector pcDNA3.1, wild type CD44s-Myc, and mutant CD44-Myc constructs were plated (2×10^5 cells/well) in a 12-well culture plate. The cells were starved in DMEM medium without FCS overnight and lysed, and then the lysates were subjected to 12.5% SDS-PAGE. Western Blotting was performed by anti-Myc antibody (9E10). The upper band represents the CD44 ectodomain cleavage products and the lower one the CD44ICD. The loading were controlled by anti-actin antibody and anti-pan CD44 antibody 5G8. Similar results were obtained 3 times.

3.6 Induction of CD44ICD by TPA inhibits the proliferation of RPM-MC cells

The phorbol ester 12-O-tetradecanoylphorbol 13-acetate (TPA) is a potent activator of protein kinase C (PKC). The initial response to TPA – i.e., activation of PKC and induction of immediate early genes such as *fos*, *myc*, and *jun* – is observed in many different cell types. However, the overall biological response to TPA is quite disparate. It was shown that TPA can either stimulate or inhibit cell proliferation, depending on the cell type (L'Allemain et al, 1991; Nori et al, 1992; Choi P et al, 1990). Based on the finding that TPA could induce the cleavage of CD44ICD (Fig. 10) which is normally detected under the conditions of growth inhibition, it provokes the question whether TPA treatment in the RPM-MC cells stimulates or inhibits proliferation. The proliferation of RPM-MC cells upon TPA treatment was measured by BrdU incorporation. TPA treatment significantly inhibited BrdU incorporation in all RPM-MC cells transfected with empty vector, wild-type or mutant CD44s, however the wild-type CD44s transfectant cells were stronger inhibited than mutant CD44s or vector transfectant cells (Fig 16).

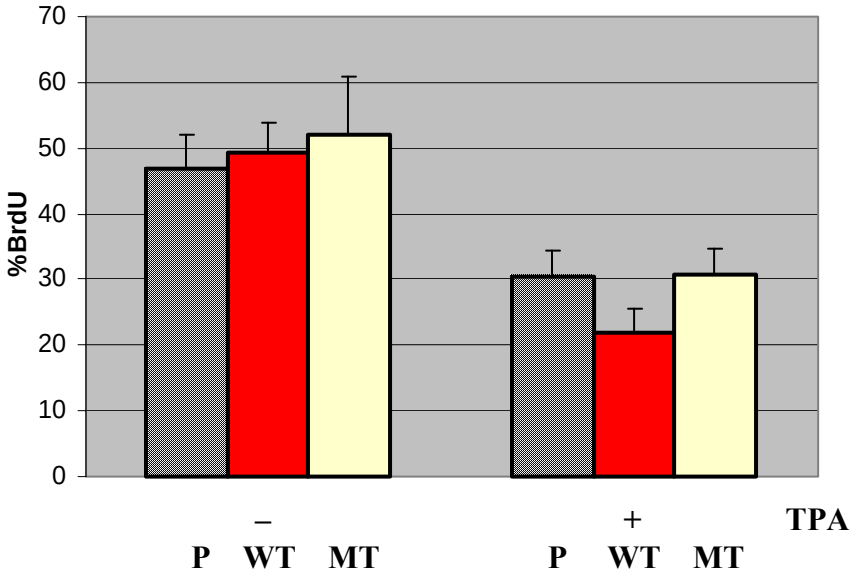


Figure 16 TPA inhibited RPM-MC cell proliferation, especially in the wild-type CD44 transfectant cells. 2×10^5 RPM-MC transfectants were seeded into 6-well plate, serum deprived for overnight, and then treated with 100ng/ml TPA for 30 min. After a further 12 hours culture, the cells were labelled with BrdU for 1 hr and then stained for incorporation using a biotinylated BrdU antibody. The mean percent cells that incorporated BrdU are plotted and standard errors are indicated. Similar results were obtained in 3 separate experiments.

3.7 Characterization of genes regulated by CD44ICD

3.7.1 Microarray assay

The release and nuclear translocation of CD44ICD is reminiscent to the release of cytoplasmic domains from Notch, ErbB4 and ß-APP. The cleavage and translocated peptides of these receptors regulate gene transcription in the nucleus. I questioned whether CD44ICD could also cause gene regulation when it is translocated into the nucleus. I used DNA microarrays to detect genes that are up or down regulated upon CD44ICD cleavage. RPM-MC cells stably transfected either with wild-type CD44s or mutant CD44s were treated with TPA to generate CD44ICD, and then the cells were cultured for an additional 12 hours. RNA isolated from the cells were reverse transcribed, labeled, and hybridized to a DNA microarray. Bound cDNA was detected using Cy3 (5-FU treated) or Cy5 (control) reporter dyes. The expression profile in the wild-type and mutant CD44s transfectant cells were compared, and the data analysis was performed by a software program developed by Matthias Bauer (ITG). The results are shown in table 1.

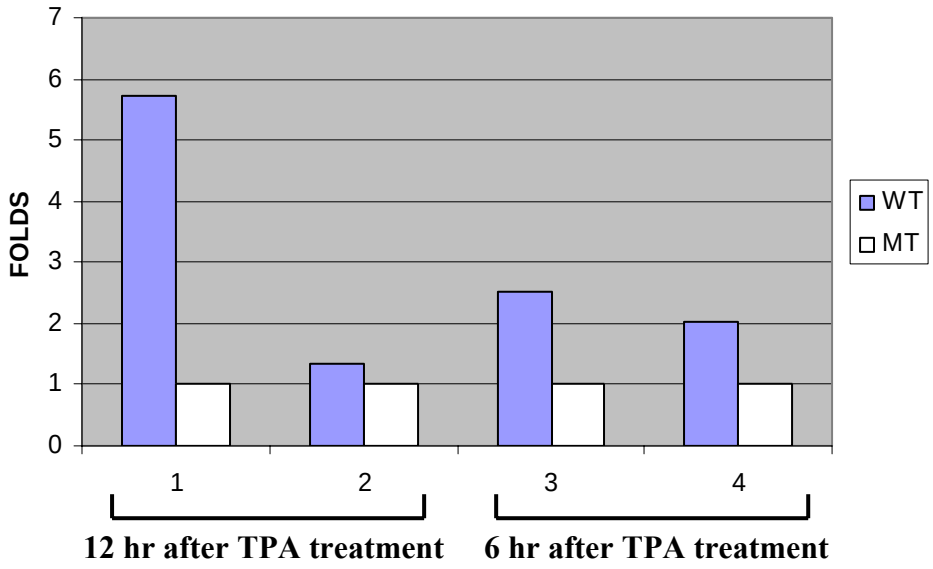
genes	Fold change
Upregulated genes	
Apolipoprotein precursor (APO-E)	2.478
Interferon-inducible protein	1.836
Interferon-induced transmembrane protein 3	1.770
Serine/threonine-proteins kinase 11	1.719
Fas-apoptosis inhibitory molecule	1.503
Antigen peptide transporter 1	1.386
Y-box protein MSY2	1.380
Neprilysin-like peptidase gamma	1.375
Interferon-inducible protein 16	1.371
Downregulated genes	
Structural protein FBF1	-3.575
KIAA 1413 protein	-3.300
Fatty-acid amide hydrolase	-2.852
Protein kinase C, theta type	-2.183
Type II Na/Pi cotransport system protein	-1.958
Myosin-binding protein C	-1.610
B cell linker protein	1.610

Table 1. **Comparison of genes upregulated or downregulated by CD44ICD.** 1 × 10^7 wildtype CD44s or mutant CD44s transfectant cells were cultured at 20 cm dish. Afte serum deprivation for overnight, the cells were stimulated with 100ng/ml TPA for 30 min. Following 12 hrs the cells were cultured at DMEM medium and the total RNA were isolated. For DNA microarray, the total RNA from mutant CD44s transfectant cells were used as negative control.

3.7.2 CD44ICD upregulates the expression of interferon inducible proteins

To confirm the microarray data, I performed real time PCR to examine the expression of some genes regulated by CD44ICD. I concentrated on the expression of interferon inducible proteins. Three interferon inducible proteins are upregulated in the microarray data, namely interferon inducible protein 16 (IFI 16), interferon induced transmembrane protein1, and interferon induced transmembrane protein3. This is particular interesting, because: 1) interferon inducible proteins have been implicated in transcription regulation. The interferon inducible proteins seem to interact with the transcriptional regulatory proteins p53 (Datta et al, 1996), pRb (Choubey et al, 1995), E2F (Choubey et al, 1996), c-Jun and c-Fos and NFκB (Min et al, 1996). 2) the interferon inducible proteins have profound effects on cell physiology leading to growth inhibition (Johnstone et al, 1998; Choubey et al, 1996; Liu et al, 1999). 3) HA treatment, TPA treatment and serum starvation cause growth inhibition, and my works showed that all these treatments could induce the generation of CD44ICD.

The RPM-MC transfectant cells were treated with TPA, and total RNA was prepared similar to the conditions for the microarray analysis. First strand cDNA was synthesized and real time PCR was performed in accordance with the manufacturer's instruction (Applied Biosystems). As shown in figure 17, after TPA treatment for 12 hours, I observed up-regulation of IFI 16 and interferon inducible-transmembrane protein 3 in the wt CD44s transfectants but not in the mt CD44s transfectants. Similar results were obtained after TPA treatment for 6 hours. The relative expression levels of mRNA were normalized against those of the actin gene in the same RNA preparation. Thus the results of real time PCR confirmed the microarray data.



1. interferon inducible protein 16
2. interferon inducible-transmembrane protein 3
3. interferon inducible protein 16
4. interferon inducible-transmembrane protein 3

Figure 17. **CD44ICD upregulates the expression of interferon inducible protein 16** Wildtype CD44s or mutant CD44s transfectant cells were serum starved overnight and then treated with 100ng/ml TPA for 30 min. After additional 12 hr (1 and 2) or 6 hr (3 and 4) culture in serum-free medium, total RNA were isolated and used for real-time PCR. Similar results were obtained in 3 separate experiments.

Conclusion:

In conclusion, I have found that importin ß and importin 5 formed a complex with CD44 cytoplasmic tail, and importins may be involved in the nuclear translocation of CD44ICD. Upon TPA treatment, HA binding or serum starvation, wild-type CD44s undergoes cleavage in extracellular and intracellular domains, while mutant CD44s undergoes extracellular domain cleavage quite less efficiently and no intracellular domain cleavage. The generation of CD44ICD requires γ-seretase activity in a Rac-dependent manner. CD44ICD were observed to upregulate the expression of IFI16 and interferon inducible-transmembrane protein 3 in the nucleus.

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PART FOUR
DISCUSSION

4.1 The goal of the study

CD44 is implicated in diverse biological processes such as hematopoiesis, lymphocyte activation and homing, limb development, wound healing and tumor progression. What is unclear is how CD44 can regulate such diverse processes. A striking finding from our lab is that CD44 functions as a cellular switch regulating cell growth by association with ERM/merlin proteins (Morrison H. et al., 2001; Orian-Rousseau V. et al., 2002). Since these complexes must be tightly regulated, it is likely that other intracellular components are a part of these complexes. Therefore, the goal of this study was to find such components. I focused on conditions where CD44 and merlin inhibit cellular growth.

4. 2 Importins and nuclear translocation

Four proteins that only associate with wild-type CD44s, but not with a CD44 mutant defective in merlin binding, were revealed by silver staining. Two of them could be sequenced and were identified as importin ß and importin 5 (RanBP 5 or karyopherin ß3) by MALDI-MS analysis. Since merlin is activated by protein phosphatase(s) under the conditions that I have identified these proteins, I expected to find protein phosphatase(s) that probably associate with the CD44-merlin complex. However, I could not identify a protein phosphatase. The explanation could be that the low abundance of the phosphatase made it undetectable with the silver staining method.

The function of importins is to transport proteins from the cytoplasm into the nucleus. The question is what might be the relationship between CD44 and importins? My data indicate that both importin ß and importin 5 associate with full-length CD44s. However, no full-length CD44s was detected in the nucleus so far. However, a recent report showed that CD44 undergoes sequential extracellular (EXT) and intracellular domain (ICD) cleavage, resulting in the release of CD44ICD which can translocate to the nucleus (Okamoto et al., 2001). Also in the RPM-MC cells that I have investigated the cleavage of CD44 occurs and I found that CD44ICD localizes predominantly in the cell nucleus. Interestingly this is only true for wild-type CD44ICD where importins can associate, but not for CD44ICD mutated in the merlin

binding site. The mutated CD44ICD stays mainly in the cytoplasm. Thus importins might be involved in the nuclear translocation of CD44ICD.

Import is a highly selective process triggered by binding of importins to nuclear localization signals (NLS) of the transported protein either directly (Adam et al, 1994; Görlich et al, 1994, 1995; Chi et al, 1995), or indirectly via adaptor proteins that recognize NLS (Görlich et al, 1996; Weis et al, 1996). Such NLS are typically composed of one or more clusters of basic amino-acid residues (Dingwall C., et al, 1991). CD44 has several basic amino-acid residues in its cytoplasmic domain, among which the basic residues in the CD44 ERM binding domain probably serve as NLS, since the mutation of these basic residues results in failure of nuclear translocation of CD44ICD. Since the presumptive NLS and the ERM binding domain overlaps, the question arises whether importins might associate to CD44 via merlin which serves as an adaptor protein, or whether they bind directly to CD44? I could not co-immunoprecipitate importins with merlin in the RPM-MC transfectant cells. Furthermore, if merlin would be the adaptor, it should be also transported into the nucleus for which no evidence exists. Therefore, I suggest that importins associate with CD44 directly without merlin serving as an adaptor protein. Since only a small portion of CD44 undergoes intracellular cleavage, only this portion of CD44 molecules might be bound by importins, while the major portion of CD44 is bound by merlin.

We find two importins associated with CD44. Do they bind independently or do they form a heterodimer? Both importin ß and importin 5 can bind directly to the NLS of target proteins (Görlich et al, 1999; Ross et al, 2003; Deane et al, 1997; Jäkel et al, 1998). Interestingly, importin ß can also form a heterodimer with importin 7, which significantly enhances the binding to their cargoes (Jakel et al, 1998; 1999). Thus it could be that both importin ß and importin 5 associate together with CD44 as heterodimers.

4.3 Intramembraneous proteolysis

The enzyme that accounts for intracellular cleavage of CD44 and several other cell-surface receptors is the protease γ-secretase. How is the activity of γ-secretase regulated? The generation of ICDs from various receptors can be induced by ligand binding. The first described and best characterized is the Notch receptor which undergoes intracellular cleavage upon the binding of its ligand from the Delta/Serrate/Lag-2 (DSL) family (Struhl et al, 1999, 2001; De Strooper et al, 1999; Mumm et al, 2000; Weinmaster et al, 1997). The ErbB4 receptor tyrosine kinase has also been shown to be cleaved by γ-secretase in response to binding of its ligand heregulin (Ni et al, 2001; Lee et al, 2002). Can the binding of CD44 ligand HA induce the intracellular cleavage of CD44? Indeed, high molecular weight HA binding to CD44 increased the cleavage of CD44ICD in the RPM-MC cells (Fig 13). Furthermore, the cleavage of CD44ICD induced by HA requires activation of the Rac pathway. This finding is reminiscent to the finding that the cleavage of ß-amyloid precursor protein (ß-APP) by γ-secretase is dependent of the Src-Rac pathway (Gianni et al, 2003). Interestingly, Rac activation also increases the cleavage of CD44EXT, the precursor of CD44ICD cleavage, by α-secretase (Kawano et al, 2000; Okamoto et al, 1999a, b; Elser et al, 2001). These results propose the hypothesis that extracellular signals that induce Rac could activate γ-secretase activities.

The cleavage of mutant CD44s is much less efficient than the cleavage of wild-type CD44s (Fig 10). What might be the reason for that? Is the interaction between ERM-merlin proteins or importins needed for the CD44 cleavage, or does the mutation change the conformation of CD44 that would influence the cleavage? Interestingly, growing evidence suggests that the cleavage could take place in lipid rafts. Consequently depletion of membranes of cholesterol, a main constituent of lipid rafts, completely inhibited the secretase activities (Simons et al, 2001; Ehehalt et al, 2003; Riddell et al, 2001; Wahrle et al, 2002). We examined whether the mutation in the CD44 cytoplasmic tail changed the distribution of CD44 in lipid rafts. We found that the distribution in lipid raft was similar for wild-type CD44s and mutant CD44s (HY Yu et al, unpubl.). So far I have not tested the distribution of wt CD44s or mt CD44s (full length or CD44EXT) under the conditions of CD44 cleavage.

4.5 The function of CD44ICD

What is the role of CD44ICD in the nucleus? ICDs from various other receptors function as transcription regulators in the nucleus. For instance, the Notch intracellular domain (NICD) was found to bind to transcription factors of the CSL (CPB/SuH/LAG-1) family (De Strooper et al, 1999; Struhl et al, 1999 & 2001). The ICD of ßAPP (ß-amyloid precursor protein) mediates the assembly of a transcriptionally active complex that contains the nuclear adaptor protein Fe65 and the histone deacetyltransferase TIP60, and might repress retinoid-responsive gene expression (Leissring et al, 2002; Cupers et al, 2001; Gao et al, 2001). Although CD44ICD has been reported to control gene expression through a TPA-responsive element (Okamato et al, 2001), no target genes were identified. The data presented in this thesis indicate that CD44ICD upregulates at least 30 genes and downregulates about 100 genes. Among the upregulated genes are ones encoding interferon inducible proteins. The interferon inducible proteins have anti-proliferative or pro-apopototic effects (Sen, 2000; Gutterman et al, 1999; Johnstone et al, 1998). This would fit with the finding that HA treatment, serum starvation, and TPA treatment conditions that lead to increase of CD44ICD cause cell growth inhibition. Whether interferon inducible proteins are direct CD44ICD target genes has to be proven. Chromatin IP of CD44ICD would be needed to be performed to examine whether CD44ICD associate with the promoters of interferon inducible proteins.

The conditions that induce CD44ICD cleavage (HA treatment or serum starvation) also cause merlin dephosphorylation, its binding to CD44 and finally inhibition of cell growth (Morrison et al, 2001). Does the growth inhibition caused by CD44ICD target genes complement the CD44-merlin dependent inhibition of cell growth? If the generation of CD44ICD and merlin binding to CD44 occurs in different cells, the CD44ICD target genes and merlin binding to CD44 probably inhibit cell growth independently and coordinately. To prove this hypothesis, cell proliferation needs to be examined under conditions where merlin is activated but no CD44ICD can be generated. For example, pretreatment with γ-secretase inhibitors pretreatment should not interfere with merlin activation (dephosphorylation) upon HA treatment. Under this condition, if HA treatment can not completely inhibit the cell growth compared to the HA treatment without γ-secretase inhibitors pretreatment, it would mean that the

generation of CD44ICD is required to complement for cell growth inhibition trigged by HA treatment.

4.6 Summary

In conclusion, this thesis reveals that CD44 cytoplasmic tail interacts with importin ß and importin 5. Most likely the importins mediate nuclear translocation of proteolytically cleaved CD44 intracellular domain. This cleavage can be induced by HA binding, serum starvation and TPA treatment, conditions that lead to growth inhibition. The CD44ICD appears to activate the expression of several proteins, among which are interferon inducible proteins. These genes might contribute to the growth regulation trigged by HA.

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