

Identification of ADAM8 substrates involved in neurodegeneration and immune response

Dissertation
zur Erlangung des Doktorgrades
der Naturwissenschaften

der Fakultät für Biologie
Universität Bielefeld

vorgelegt von

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Bielefeld, im April 2005

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Zusammenfassung

ADAMs sind Transmembranproteine mit einer Disintegrin- und Metalloprotease-Domäne (A Disintegrin and Metalloprotease). Entsprechend dieser funktionellen Domänen sind sie an Zell-Zell-Fusion und -Adhäsion sowie an der Proteolyse von Membranproteinen wie Rezeptoren, Zelladhäsions- und Signalmolekülen beteiligt. ADAMs erfüllen wichtige Funktionen bei der Fertilisation, Myogenese und Neurogenese und sind an der Metastasierung verschiedener Krebszellen beteiligt. Einige physiologisch relevante ADAM-Substrate sind bekannt. Es wurden bislang jedoch wenige systematische Analysen zur Bestimmung von ADAM-Substraten sowie zur Bestimmung von ADAM-Expressionsmustern in verschiedenen Geweben durchgeführt. Der Schwerpunkt der vorliegenden Arbeit liegt auf der Proteasefunktion des Familienmitglieds ADAM8 sowie der systematischen Suche nach neuen physiologisch relevanten Substraten.

Für die systematische Suche nach neuen ADAM8-Substraten wurden *in vitro* Spaltungstests mit fluoreszierenden Peptid-Substraten und löslichen, rekombinanten Formen von ADAM8-Protease durchgeführt. Etwa 40 Peptide wurden als potentielle ADAM8-Substrate ausgewählt. Diese Peptide wurden entweder von bekannten Peptid-Substraten anderer Metalloproteasen oder von membrannahen Bereichen möglicher Metalloprotease-Substrate abgeleitet. ADAM8 wird im Organismus durch entzündliche Stimuli aktiviert. Die Suche nach Substraten wurde deshalb schwerpunktmäßig mit Peptiden durchgeführt, die von Zytokinen oder anderen Molekülen, die an Entzündungsprozessen beteiligt sind, abgeleitet wurden. Durch *in vitro* Spaltversuche wurden neue Kandidaten-Substrate für ADAM8 bestimmt. Zusätzlich ergaben sich durch diese Tests Hinweise für bevorzugte Aminosäuren im Bereich der Spaltstelle von ADAM8 Peptid-Substraten. Bislang ist keine Konsensus-Sequenz für ein Spaltmotiv bekannt, es konnten jedoch in dieser Arbeit minimale Notwendigkeiten für die Spaltung bestimmt werden.

Als Beispiel einer sehr spezifischen Protease-Substrat Beziehung wurde das Zelladhäsionsmolekül CHL1 (Close Homologue of L1) als Substrat für ADAM8 identifiziert. Durch biochemische Studien wurde die physiologische Relevanz dieser Protease-Substrat-Beziehung bestätigt. Dabei stellte sich heraus, dass CHL1 ein physiologisches Substrat für ADAM8 ist. In *in vivo* Studien förderte die von ADAM8 abgespaltene lösliche Form von CHL1 das Neuritenwachstum und unterdrückte neuronalen Zelltod. Basierend auf den Ergebnissen der Peptid-Tests werden die zugehörigen Proteine gespaltener Peptide ebenfalls in weiteren physiologischen Tests auf die physiologische Relevanz als ADAM8-Substrate untersucht. Bislang konnte gezeigt werden, dass das Amyloid Precursor Protein (APP) in Ko-

Transfektionsexperimenten in Zellkultur ebenfalls von ADAM8 gespalten wird und dass der Tumor Nekrose Faktor Rezeptor 1 (TNF-R1) in Geweben von ADAM8 defizienten Mäusen in geringerem Umfang gespalten wird als in entsprechenden Wildtyp-Geweben.

In einem parallelen Projekt wurde eine Methode für die Detektion von ADAM-Aktivitäten in Zellkultur und in Lysaten von primären humanen Gehirntumoren entwickelt. Unter Verwendung von ADAM8 beziehungsweise ADAM19 spezifischen Peptid-Substraten wurden erhöhte Protease-Aktivitäten dieser ADAMs in bestimmten Gehirntumor-Typen nachgewiesen.

Summary

ADAM proteins are transmembrane multidomain proteins with A Disintegrin And Metalloprotease domain. Due to these functional domains, they are involved in cell-cell fusion or adhesion events as well as in proteolysis of membrane proteins such as receptors, cell adhesion and signalling molecules. They serve essential function in fertilisation, myogenesis and neurogenesis and have potential implication for metastasis of cancer cells. Although a number of ADAM substrates have been determined, comparatively little work was performed on the systematic substrate search and on determination of ADAM expression patterns in different tissues. In the work presented here, I have focused on the protease domain of ADAM8 by searching for new, physiologically relevant substrates.

A systematic method was established in order to find new ADAM8 substrates. A collection of fluorogenic peptides was designed for *in vitro* cleavage assays with soluble forms of ADAM8. The peptides were derived from known peptide substrates of other ADAMs or from juxtamembraneous regions of supposed metalloprotease substrates. With the main focus on inflammatory diseases, most of the peptides were derived from cytokines or from molecules involved in inflammatory processes. This *in vitro* peptide assay was supposed to indicate new, potential ADAM8 substrates. In addition, this approach provided comprehensive information on preferred amino acids of ADAM8 peptide substrates to define the minimal requirements for cleavage as so far no consensus sequence is known for ADAM8 substrates.

In previous work, the cell adhesion molecule CHL1 was identified as an *in vitro* substrate of ADAM8. In further biochemical studies, CHL1 turned out to be a physiological substrate of ADAM8, with the released CHL1 molecule promoting neurite outgrowth and suppressing neuronal cell death. Based on the peptide cleavage assays, corresponding proteins are further examined *in vivo* concerning the physiological relevance as ADAM8 substrates. In biochemical studies, the amyloid precursor protein (APP) was also cleaved in co-transfection experiments in cell culture, and shedding of the tumour necrosis factor receptor I (TNF-R1) was decreased in ADAM8 knockout tissues in comparison to corresponding wild type tissues. A challenging task in the analysis of ADAM proteins is the specific definition of their proteolytic activities. In a parallel project I have worked out a method for the detection of ADAM activities in cell culture and in protein extracts from primary human brain tumours. By using specific peptides for ADAM8 and ADAM19, respectively, it was demonstrated that the proteases exerted enhanced proteolytic activity in certain brain tumour specimens.

1. Introduction

The physiology of cells is regulated by intracellular as well as by extracellular signals. The extracellular matrix is a repository of molecules involved in cell differentiation, cell proliferation and cell migration – important processes in development and regeneration but also in inflammation and disease.

Among other molecules, extracellular matrix proteases play important roles in these processes by enabling cell migration via degradation of matrix components or cell communication via release of signal molecules. The ADAMs form a group of membrane-type metalloproteases which have, in addition to the extracellular protease function, an extracellular binding site for integrins and intracellular binding sites for molecules involved in cell signalling. ADAMs are therefore able to mediate cell adhesion by heterophilic or homophilic cell-cell or cell-matrix interactions and to disrupt cell adhesion or to activate signal transduction pathways by the specific release of cell adhesion molecules, cytokines, receptors or other molecules.

Specific protease-substrate relationships are important for development and maintenance of cell systems, but do also play a role in their pathology. The cell adhesion molecule L1 is released from the cell surface by ADAM10 (Mechtersheimer et al., 2001) and the soluble form of L1 is able to suppress neuronal cell death (Chen et al., 1999). Defects in proteolysis of L1 may have pathological consequences in the nervous system. Tumour necrosis factor alpha (TNF- α) is released from the membrane by ADAM17 (Black et al., 1997; Moss et al., 1997). The soluble form of TNF- α acts as a proinflammatory cytokine with a dual role in the nervous system: on the one hand it is implicated to trigger neuronal cell death (Knobloch et al., 1999; Venters et al., 1999), and on the other hand it can have neuroprotective effects (Bruce et al., 1996; Tarkowski et al., 1999).

In several diseases such as allergic asthma (King et al., 2004), the involvement of ADAMs was demonstrated. Concerning the expanding physiological roles for ADAMs and their substrates, the interest in identification and functional characterisation of ADAM-substrate relationships is increasing. Further knowledge about ADAM expression patterns under specific pathological conditions as well as analysis of new ADAM substrates may help to understand the complex regulation of cell systems and the cause of disease.

1.1 A Disintegrin And Metalloprotease (ADAM)

Numerous zinc metalloproteases contain a consensus motif “HEXXHXXGXXH” in their catalytic centre and a conserved methionine residue close to the zinc-binding active site. These enzymes referred to as metzincins can be grouped into four families, the astacins, the adamalysins, the serralysins and matrix metalloproteases (Stocker and Bode, 1995). The metzincins share a similar catalytic reaction mechanism in which the three conserved histidines coordinate the zinc ion, whereas the glutamic acid polarises a zinc-bound water molecule for nucleophilic attack of the scissile peptide bond of a bound substrate (Stocker and Bode, 1995).

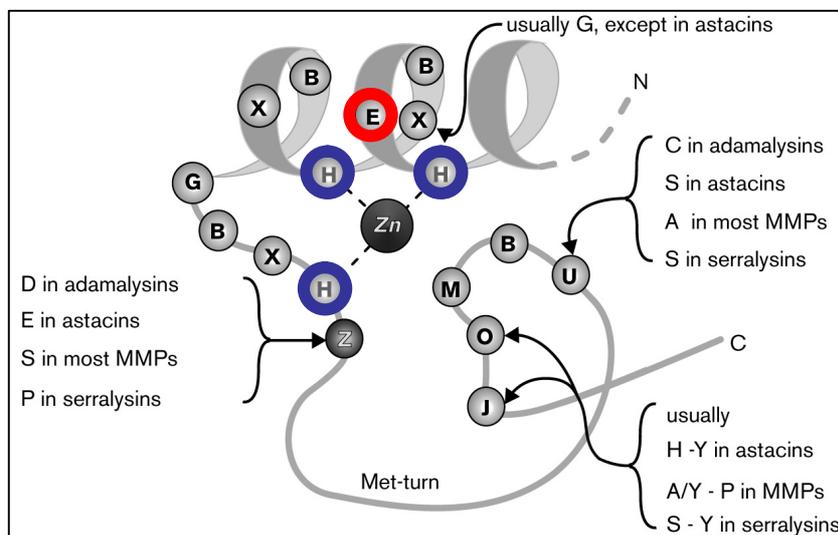


Figure 1.1-1: The catalytic centre of the Metzincins.

A zinc ion (black) is complexed by 3 histidine residues (blue) of the consensus sequence in the catalytic centre. The glutamic acid (red) in this sequence is important for electron transfer during the catalytic cleavage reaction.

(Bergers and Coussens, 2000; modified in Stocker and Bode, 1995)

ADAM proteins form a large and widely expressed subfamily of the adamalysins with high homology to the snake venome metalloproteases (SVMP). Chantry et al. (1989) discovered a metalloprotease in brain myelin membrane preparations degrading myelin basic protein (MBP) which later turned out to be ADAM10. ADAM1 and ADAM2 were originally identified as alpha and beta subunits of the sperm surface protein PH-30 which formed a complex involved in sperm-egg fusion. In this complex, the alpha domain contained a fusion peptide typical for viral fusion proteins, and the beta subunit contained a domain related to soluble integrin ligands found in snake venoms (Blobel et al., 1992; Wolfsberg et al., 1993). Finally, the ADAMs were identified as one large gene family encoding proteins with a disintegrin and metalloprotease domain (Wolfsberg et al., 1995). At present, 39 ADAMs in various species are known (http://www.people.virginia.edu/~7Ejw7g/Table_of_the_ADAMs.html), about half of them are expected to be catalytically active based on their conserved consensus sequence. ADAMs are type I membrane proteins with a multidomain

structure containing a pro, metalloprotease, disintegrin-like, cystein-rich, epidermal growth factor (EGF) like, transmembrane and cytoplasmic domain (Figure 1.1-2). According to different functional domains, ADAMs have multiple functions: catalytic activity via the metalloprotease domain, homophilic and heterophilic adhesion via the disintegrin and cysteine-rich domains and cell signalling via binding motifs in the cytoplasmic domain.

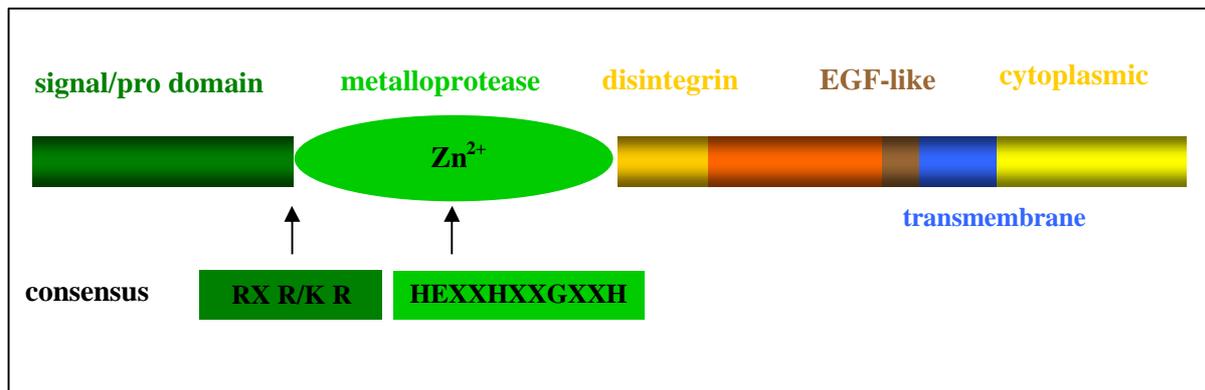


Figure 1.1-2: Overview of the conserved domain structure of ADAMs

1.1.1 The prodomain

ADAMs are expressed as inactive zymogens. Many ADAM proteases like ADAM9, ADAM10, ADAM17, and ADAM19 undergo proteolytic prodomain removal by furines or prohormone convertases. The respective recognition motifs RXR/KR are located in the hinge region located between the pro and the metalloprotease domain (Anders et al., 2001; Clarke et al., 1998; Kang et al., 2002; Roghani et al., 1999). Sequence alignment of this processing region reveals that ADAM8 as well as ADAM28 do not contain these consensus motifs. As both ADAMs show high homology to hemorrhagic snake venom proteases which are activated by autocatalysis, it was analysed whether ADAM8 and ADAM28 are processed in the same manner. In experiments in which Glutamate₃₃₀ in the HEXXH consensus motif was exchanged by a glutamine (EQ-ADAM8), prodomain removal was completely abolished (Schlomann et al., 2002). A similar experiment performed with ADAM28 with the glutamate exchanged by alanine gave an identical result (Howard et al., 2000). In further experiments, EQ-ADAM8 co-transfected with a construct encoding the ectodomain of ADAM8 was activated by the ectodomain *in cis* during transport through the Trans-Golgi network. Co-transfection of EQ-ADAM8 with a construct encoding the metalloprotease domain did not result in prodomain removal, allowing for the conclusion that the presence of the disintegrin domain influenced autocatalytic prodomain removal, e.g. by mediating the interaction of at least two ADAM8 molecules (Schlomann et al., 2002).

1.1.2 The metalloprotease domain

ADAMs can act as proteolytic enzymes releasing membrane proteins from the cell surface – therefore referred to as “shedases” – or cleaving proteins of the extracellular matrix. Proteolytic activity of ADAMs was first shown for ADAM10 cleaving MBP (Chantry et al., 1989). Thereafter, ADAM17 was purified and characterised on the basis of its catalytic activity as TNF- α converting enzyme (TACE) able to cleave TNF- α to its mature form (Black et al., 1997; Moss et al., 1997). Meanwhile, ADAM8 (Choi et al., 2001; Schlomann et al., 2002), ADAM9 (Roghani et al., 1999), ADAM12 (Loechel et al., 1998), ADAM15 (Martin et al., 2002), ADAM19 (Chesneau et al., 2003; Shirakabe et al., 2001), ADAM28 (Howard et al., 2001) and ADAM 33 (Zou et al., 2004) have been shown to be catalytically active.

ADAM substrates form a heterogeneous group of proteins with diverse function in the cell. Among them are receptors, growth factors, cytokines and cell adhesion molecules. Table 1.1.2 shows an overview of a number of ADAM substrates, synonyms of protein names are listed in 6.5. For some substrates, a modified shedding was observed in cells lacking proteolytic ADAM activity. Accordingly, Peschon et al. (1998) proposed an influence of ADAM17 in shedding of pro-transforming growth factor receptor (TGF- α) and L-Selectin. As many of the substrates listed in table 1.1.2 were identified either on the basis of *in vitro* cleavage experiments with recombinant proteins or with peptides representing the juxtmembraneous regions of membrane proteins, it is not definite that they are all of physiological relevance (e.g. Amour et al., 2002; Chesneau et al., 2003; Fourie et al., 2003; Roghani et al., 1999; Zou et al., 2004). For some proteins, additional transfection experiments *in vivo* in cell culture were performed; e.g. shedding of CD23 and TRANCE by ADAM8 and ADAM19, respectively, was confirmed in co-transfection experiments, whereas ADAM19 seemed to function as a negative regulator of kit ligand precursor (KL) shedding and ADAM33 of APP shedding (Chesneau et al., 2003; Fourie et al., 2003; Zou et al., 2004).

In other studies, mouse models were used to examine physiological relevance of ADAM substrates. Sahin et al. (2004) and Weskamp et al. (2004) used cells isolated from ADAM knockout mice lacking different candidate-releasing enzymes to study shedding of different proteins. On the basis of these experiments, ADAM10 was identified as the main shedase of pro-epidermal growth factor precursor (EGF) and betacellulin (BTC) and ADAM17 of epiregulin precursor, TGF- α , amphiregulin (AR), heparin-binding EGF-like growth factor precursor (HB-EGF) and low affinity neurotrophin receptor (p75NTR)(Sahin et al., 2004; Weskamp et al., 2004).

ADAM	Substrate	Reference
ADAM8 CD156a MS2	APP, amyloid beta A4 protein precursor	(Amour et al., 2002)
	CD23, low affinity immunoglobulin epsilon Fc receptor	(Fourie et al., 2003)
	CD27-L, CD27 ligand	(Fourie et al., 2003)
	CD30-L, CD30 ligand	(Fourie et al., 2003)
	CHL1, close homologue of L1	(Naus et al., 2004)
	IL-1R, interleukin-1 receptor	(Amour et al., 2002)
	KL, kit ligand precursor	(Amour et al., 2002)
	MBP, myelin basic protein	(Schlomann et al., 2002)
	TNF- α , tumour necrosis factor alpha	(Amour et al., 2002)
ADAM9 MDC9 Meltrin γ	APP, amyloid beta A4 protein precursor	(Koike et al., 1999; Roghani et al., 1999)
	Beta Casein	(Schwettmann and Tschesche, 2001)
	CD23, low affinity immunoglobulin epsilon Fc receptor	(Fourie et al., 2003)
	Collagen XVII	(Franzke et al., 2002)
	Fibronectin	(Schwettmann and Tschesche, 2001)
	Gelatine	(Schwettmann and Tschesche, 2001)
	Insulin β chain	(Roghani et al., 1999)
	HB-EGF, heparin-binding EGF-like growth factor precursor	(Izumi et al., 1998)
	IGFBP-3, insulin like growth factor binding protein 5 precursor	(Mohan et al., 2002)
	KL, kit ligand precursor	(Roghani et al., 1999)
p75NTR, low affinity neurotrophin receptor	(Roghani et al., 1999)	
TNF- α , tumour necrosis factor alpha	(Roghani et al., 1999)	
ADAM10 MADM Kuzbanian	APP, amyloid beta A4 protein precursor	(Lammich et al., 1999)
	BTC, betacellulin precursor	(Sahin et al., 2004)
	CD40-L, CD40 ligand	(Amour et al., 2002)
	Collagen VI	(Millichip et al., 1998)
	Collagen XVII	(Franzke et al., 2002)
	CX3CL1, fractalkine precursor	(Hundhausen et al., 2003)
	CXCL16, small inducible cytokine B16 precursor	(Abel et al., 2004; Gough et al., 2004)
	Delta, notch ligand delta	(Qi et al., 1999)
	EGF, pro-epidermal growth factor precursor	(Sahin et al., 2004)
	Ephrin-A2, ephrin-A2 precursor	(Hattori et al., 2000)
	HB-EGF, heparin-binding EGF-like growth factor precursor	(Lemjabbar and Basbaum, 2002)
	IL-1R, interleukin-1 receptor	(Amour et al., 2000)
	IL-6R-1, interleukin-6 receptor precursor	(Matthews et al., 2003)
	L1, neuronal cell adhesion molecule L1 precursor	(Mechtersheimer et al., 2001)
	MBP, myelin basic protein	(Chantry et al., 1989)
	N-Cadherin, neuronal-cadherin precursor	(Reiss et al., 2005)
	Notch, neurogenic locus notch protein precursor	(Lieber et al., 2002)
	PrP, major prion protein precursor	(Vincent et al., 2001)
	TNF- α , tumour necrosis factor alpha	(Lunn et al., 1997)
	ADAM12 Meltrin α	Casein
Collagen IV		(Roy et al., 2004)
Gelatine		(Roy et al., 2004)
HB-EGF, heparin-binding EGF-like growth factor precursor		(Asakura et al., 2002)
IGFBP-3, insulin like growth factor binding protein 3 precursor		(Loechel et al., 2000)
IGFBP-5, insulin like growth factor binding protein 5 precursor		(Loechel et al., 2000)
P-LAP, placental leucine amino peptidase		(Ito et al., 2004)
ADAM15 MDC15 Metargidin	CD23, low affinity immunoglobulin epsilon Fc receptor	(Fourie et al., 2003)
	Collagen IV	(Martin et al., 2002)
	Gelatine	(Martin et al., 2002)
ADAM17 CD156b TACE	APP, amyloid beta A4 protein precursor	(Buxbaum et al., 1998)
	AR, amphiregulin precursor	(Sunnarborg et al., 2002)
	CD30-L, CD30 ligand	(Hansen et al., 2000)
	CD40-L, CD40-ligand	(Contin et al., 2003)
	Collagen XVII	(Franzke et al., 2002)
CSF-1-R, macrophage colony-stimulating factor receptor	(Rovida et al., 2001)	

ADAM	Substrate	Reference
ADAM17 CD156b TACE	CX3CL1, fractalkine precursor	(Garton et al., 2001)
	EBOV GP, ebola virus surface glycoprotein GP	(Dolnik et al., 2004)
	Epiregulin, epiregulin precursor	(Sahin et al., 2004)
	GPIb- α , platelet glycoprotein Ib alpha chain	(Bergmeier et al., 2004)
	GHS-R, growth hormone secretagogue receptor type I	(Zhang et al., 2000)
	HB-EGF, heparin-binding EGF-like growth factor precursor	(Merlos-Suarez et al., 2001)
	HER-4, receptor protein-tyrosine kinase ErbB-4 precursor	(Rio et al., 2000)
	IL-1R-2, interleukin-1 receptor type II precursor	(Reddy et al., 2000)
	IL-6R-1, interleukin-6 receptor precursor	(Althoff et al., 2000)
	IL-15R- α , interleukin-15 receptor alpha chain precursor	(Budagian et al., 2004)
	Insulin β chain	(Roghani et al., 1999)
	Jagged1, jagged1 precursor	(LaVoie and Selkoe, 2003)
	L-Selectin	(Peschon et al., 1998)
	Mucin 1, mucin 1 precursor	(Thathiah and Carson, 2004)
	Notch1, neurogenic locus notch homolog protein 1 precursor	(Brou et al., 2000)
	NRG, pro-neuregulin precursor	(Montero et al., 2000)
	p75NTR, low affinity neurotrophin receptor	(Weskamp et al., 2004)
	PAR-1, proteinase activated receptor 1	(Ludeman et al., 2004)
	GPV, platelet glycoprotein V	(Rabie et al., 2005)
	PrP, major prion protein precursor	(Vincent et al., 2001)
TGF- α , transforming growth factor alpha	(Peschon et al., 1998)	
TNF- α , tumour necrosis factor alpha	(Black et al., 1997; Moss et al., 1997)	
TNF-R1, tumour necrosis factor alpha receptor 1	(Peschon et al., 1998)	
TNF-R2, tumour necrosis factor alpha receptor 2	(Reddy et al., 2000)	
TRANCE, TNF-related activation-induced cytokine	(Lum et al., 1999)	
V-CAM1, vascular cell adhesion molecule 1 precursor	(Garton et al., 2003)	
ADAM19 Meltrin β	α -2-M, alpha-2-macroglobulin	(Wei et al., 2001)
	Insulin β chain	(Chesneau et al., 2003)
	KL, kit ligand precursor	(Chesneau et al., 2003)
	NRG-1, pro-neuregulin-1 precursor	(Shirakabe et al., 2001)
	TNF- α , tumour necrosis factor alpha	(Chesneau et al., 2003)
TRANCE, TNF-related activation-induced cytokine	(Chesneau et al., 2003)	
ADAM28	CD23, low affinity immunoglobulin epsilon Fc receptor	(Fourie et al., 2003)
	IGFBP-3, insulin like growth factor binding protein 3 precursor	(Mochizuki et al., 2004)
	MBP, myelin basic protein	(Howard et al., 2001)
ADAM33	α -2-M, alpha-2-macroglobulin	(Garlisi et al., 2003)
	APP, amyloid beta A4 protein precursor	(Zou et al., 2004)
	Insulin β chain	(Zou et al., 2004)
	KL, kit ligand precursor	(Zou et al., 2004)
	TRANCE, TNF-related activation-induced cytokine	(Zou et al., 2004)

Table 1.1.2: Overview of known ADAM substrates

Little is known about the substrate specificity of ADAMs. Many membrane-type substrates are released from cells near the cell surface, but it is not clear if structural characteristics or primary amino acid sequences are important for protease-substrate recognition. Structural characteristics might be important for interaction, e.g. with the disintegrin-domain, bringing protease and substrate into close proximity. Furthermore, ADAM specific motifs around the cleavage sites probably exist, as *in vitro* cleavage assays with oligo peptides derived from proteolytically sensitive sequences of shed proteins resulted in the same cleavage sites than assays with the complete proteins (Amour et al., 2002; Schlomann et al., 2002).

Some work was done to define consensus sequences of ADAM cleavage sites. The low affinity immunoglobulin epsilon Fc receptor (CD23) is released from the cell surface by a metalloprotease to result in fragments of 37, 33 and 29 kDa (Mayer et al., 2002) and ADAM8 is probably involved in the cleavage (Fourie et al., 2003). A hypothetical consensus sequence was constructed from the cleavage sites of these three fragments suggesting basic amino residues preferred at positions P1 and P2' (Mayer et al., 2002). From computer models of enzyme-substrate complexes, a cleavage site with a serine or threonine residue in P1 position and a hydrophobic residue in P1' position for ADAM10 was proposed (Manzetti et al., 2003). As metalloproteases are zinc dependent, chelators like EDTA or EGTA can be used for inhibition of ADAM catalytic activity. For many metalloproteases including ADAMs, peptide-like inhibitors designed on the basis of hydroxamates are used. The four known tissue inhibitors of metalloproteases (TIMP) are known to be physiological inhibitors of matrix metalloproteases (MMP). Whereas they inhibit MMPs effectively, they are not necessarily inhibitors of ADAM proteases. ADAM8 and 9 are not inhibited by any of the TIMPs (Amour et al., 2002; Schlomann et al., 2002). ADAM10, 12, 17 and 28 are inhibited by TIMP3 (Amour et al., 2000; Amour et al., 1998; Loechel et al., 2000; Mochizuki et al., 2004). Furthermore, ADAM10 is inhibited by TIMP1 and ADAM28 by TIMP4 (Amour et al., 2000; Mochizuki et al., 2004). ADAM19 is known not to be inhibited by TIMP1 and 2 (Wei et al., 2001).

1.1.3 The disintegrin domain

The ADAM disintegrin-like domain was proposed to function as integrin ligand, as it is highly homologous to small non-enzymatic peptides isolated from the venom of snakes that function as antagonists of integrins (Gould et al., 1990; McLane et al., 1998). These peptides, referred to as disintegrins, ideally present an RGD integrin-binding motif at the end of an extended loop structure which is called the disintegrin loop. However, this condition is only found in human ADAM15, whereas other disintegrin domains contain variant sequences in the disintegrin loop such as MLD or VGD (McLane et al., 1998).

Indeed, several ADAMs have been reported to interact with integrins. ADAM2, 9, 12, 15 and 23 are known to interact with different integrins, mainly integrins $\alpha_6\beta_1$, $\alpha_v\beta_3$, $\alpha_9\beta_1$, $\alpha_v\beta_5$, $\alpha_5\beta_1$ (Evans, 2001). ADAM15 is able to interact in an RGD-dependent manner with integrin $\alpha_v\beta_3$ (Zhang et al., 1998) as well as in an RGD-independent manner with integrin $\alpha_9\beta_1$ (Eto et al. 2000). Eto et al. (2002) determined how these two integrins recognise the ADAM15 disintegrin domain by mutational analysis. They found, that the RGD motif (residues 484-

486) was critical for integrin $\alpha_v\beta_3$ binding, but other flanking regions were not. In contrast, the conserved motif RX(6)DLPEF flanking the RGD motif (residues 481-492) was critical for integrin $\alpha_9\beta_1$ binding. This flanking motif is conserved among many ADAMs, but not in ADAM10 and 17. In contrast to ADAM1, 2, 3 and 9, integrin $\alpha_9\beta_1$ did not bind to ADAM10 and 17 disintegrin domains (Eto et al., 2002). Other data indicate that an ECD tripeptide is an adhesion mediating motif for ADAM2 and ADAM23 suggesting that an acetic residue is important for interaction with specific integrins as an alanine or a lysine substitution of the terminal aspartic residue resulted in a loss of function (Evans, 2001). ADAM28 was found to interact with integrin $\alpha_4\beta_1$. As ADAM28 is expressed on lymphocytes and integrin $\alpha_4\beta_1$ on leukocytes, this interaction has particular importance for the migration of lymphocytes (Bridges et al., 2002). Experiments with substitution mutants indicated that residues located outside of the ADAM28 disintegrin loop were important for integrin $\alpha_4\beta_1$ recognition (Bridges et al., 2003). Recently it was demonstrated that functional interaction between ADAM17 and integrin $\alpha_5\beta_1$ in *trans* orientation was inhibited by RGD peptides (Bax et al., 2004).

Interaction between integrins and disintegrin domains of ADAMs have mainly been characterised concerning sperm-egg binding (Evans, 2001). But these interaction are also important for muscle development, migration of lymphocytes, cell motility and modulation of proteolytic activity (Bax et al., 2004; Bridges et al., 2002; Nath et al., 2000; Zhao et al., 2004).

1.1.4 The cytoplasmic domain

The cytoplasmic domains of ADAMs are thought to play a role in controlling functions of ADAMs like intracellular maturation, trafficking to the cell membrane and catalytic activity. They are less conserved than the ADAM extracellular domains, their sizes vary from 11 residues for ADAM11 to 197 residues for ADAM13 (Poghosyan et al., 2002). Nevertheless, many ADAM cytoplasmic domains contain binding domains for Src homology 3 (SH3) domains. Furthermore, tyrosine residues in ADAM cytoplasmic domains can be substrates for tyrosine kinases or, when phosphorylated, binding domains for Src homology 2 (SH2) or phosphotyrosine-binding domains. Several proteins interacting with ADAM cytoplasmic domains have been identified by co-immunoprecipitation, yeast-two hybrid and other methods.

The cytoplasmic domains of ADAM9 and 15 interact directly with the SH3 domain-containing proteins endophilin I and SH3PX1, a protein containing a phox homology domain

in addition to a SH3 domain (Howard et al., 1999). Mitotic Arrest Deficient 2 (MAD2) was identified as a binding protein for ADAM17 and a MAD2-related protein, MAD2 β , as a binding protein for ADAM9 (Nelson et al., 1999). PKC δ binds to the cytoplasmic domain of ADAM9. Constitutively active PKC δ or ADAM9 resulted in shedding of proHB-EGF, whereas lack either of ADAM9 metalloprotease domain or PKC δ , suppressed TPA-induced shedding of the ectodomain (Izumi et al., 1998). Poghosyan et al. (2002) demonstrated specific interaction between cytoplasmic domain of ADAM15 and several Src family protein-tyrosine kinases (PTKs) including Lck, Fyn, Abl, and Src and the adaptor protein Grb2 *in vitro*. As interaction was decreased by dephosphorylation of cell extracts, it is likely that the state of phosphorylation influences the interaction of ADAM15 cytoplasmic domain with its binding partners (Poghosyan et al., 2002).

ADAM12 is an adhesion protein implicated in differentiation and fusion of myoblasts. The interactions of the cytoplasmic domain of ADAM12 with α -actinin 1 and 2, two actin-binding and cross-linking proteins, were discussed to play a role in regulation of myogenesis mediated by ADAM12 (Cao et al., 2001; Galliano et al., 2000). In further studies, ADAM12 cytoplasmic domains were reported to interact with SH3 domains of Src and Grb2 (Kang et al., 2000; Suzuki et al., 2000) and to be phosphorylated by Src (Suzuki et al., 2000). Co-expression of Src and ADAM12 in C2C12 muscle cells led to activation of recombinant Src (Kang et al., 2000). In the same cells, phosphatidylinositol (PI) 3-kinase was activated by ADAM12 cytoplasmic domain interacting with the SH3 domain of p85 α regulatory subunit of PI 3-kinase (Kang et al., 2001). These results indicated that ADAM12 may mediate adhesion-induced signalling during myoblast differentiation.

ADAMs are implicated in signalling of epidermal growth factor receptor (EGF) by ectodomain shedding of EGF-R ligands. The corresponding activation mechanism of ADAMs remains elusive. Tanaka et al. (2004) identified a protein designated as Eve-1 as an ADAM12 binding protein. As shedding of HB-EGF, TGF- α , AR and epiregulin was decreased in case of Eve-1 knockdown by small interfering RNA, it was proposed that Eve-1 plays a role in positively regulating ADAM activity (Tanaka et al., 2004).

1.2 ADAM8

ADAM8 was originally cloned as MS2 or CD156a from macrophages (Yoshida et al., 1990). Later on, ADAM8 was identified as a catalytically active protease (Choi et al., 2001; Schlomann et al., 2002) with adhesive function of the disintegrin domain (Schlomann et al., 2002; Schluesener, 1998). Analysis of ADAM8 knockout mice showed that ADAM8 is widely expressed during development, and also in the adult mouse (Kelly et al., 2005). ADAM8 is supposed to have various physiological functions such as involvement in inflammation in the nervous system (Schlomann et al., 2000), in immune response in allergic asthma (King et al., 2004) as well as in osteoclast fusion (Choi et al., 2001).

1.2.1 Catalytic activity of ADAM8

Due to the metzincin consensus sequence in the catalytic centre, ADAM8 was expected to be catalytically active. Experiments with soluble forms of ADAM8 transfected into HEK293 cells or in COS7 cells clearly indicated protease activity (Choi et al., 2001; Schlomann et al., 2002). This proteolytic activity was dependent on the presence of the metalloprotease domain and did not require further domains. Protease activity was examined by cleavage of MBP. Inhibition of proteolytic activity was obtained by using the metalloprotease inhibitors 1,10 *ortho*-phenanthroline (OPT) and batimastat (BB-94) (Schlomann et al., 2002), whereas none of the four known TIMPs inhibited cleavage even when high concentrations up to 500 nM were used (Amour et al., 2002; Schlomann et al., 2002). The ADAM8 cleavage site in MBP within the sequence TTHYGSLP↓QKAQGQ was similar to those found for ADAM10 and ADAM28 (Howard et al., 2001). Further substrates of ADAM8 were detected on the basis of *in vitro* cleavage experiments with recombinant proteins or on the basis of peptides representing the juxtamembraneous regions of membrane proteins (Amour et al., 2002; Fourie et al., 2003).

1.2.2 Adhesive function of ADAM8

ADAM8 was detected in neurons and oligodendrocytes in the central nervous system (CNS) (Schlomann et al., 2000). As ADAM8 was up-regulated in the CNS upon neurodegeneration followed by activation of glia cells, astrocytes and microglia, Schlomann et al. (2000) suggested involvement of ADAM8 in neuron-glia interaction. ADAM8 had further been implicated in osteoclast differentiation (Choi et al., 2001). These data indicated that ADAM8 is involved in cell adhesion and cell fusion.

Evidence for the adhesive function of the ADAM8 disintegrin domain in the course of disease came from an autoimmunity model in the rat (Schluesener, 1998). In this model, a MBP peptide was used to induce severe generalised autoimmune symptoms in the brain (GANS). When rats were pre-treated with the recombinant disintegrin domain of ADAM8, the autoimmune symptoms were dramatically reduced, indicating that either the disintegrin domain competed with endogenous ADAM8 for cell-cell interactions or that the effect was indirect e.g. by influencing ADAM8 protease activity. Experiments with a recombinant ADAM8 disintegrin/cystein-rich domain argued for cell adhesion in a homophilic manner. The recombinant disintegrin protein coated as a substrate on cell culture dishes was able to mediate adhesion specifically to cells expressing ADAM8 (Schlomann et al., 2002).

1.2.3 Regulation of ADAM8

An important function for ADAM8 in immune responses was postulated on the basis of its inducibility by inflammatory stimuli. Katoaka et al. (1997) reported the induction of ADAM8 expression by lipopolysaccharide (LPS) from *E.coli* and interferon-gamma (IFN- γ) in the murine macrophage cell line aHINSB3. In another murine macrophage cell line RAW264.7, target genes for peroxisome proliferator-activated receptor gamma (PPAR γ) were identified by stimulation with a natural prostaglandin ligand of PPAR γ . ADAM8 mRNA expression as well as expression of CD36 and ATP-binding cassette transporter A1 (ABCA1), which mediated cholesterol efflux, were induced (Hodgkinson and Ye, 2003). In the same macrophage cell line, shedding of the tumour necrosis factor receptor 1 (TNF-R1) was dependent on treatment with LPS inducing ADAM8 in these cells (Wildeboer et al., unpublished data).

In an inherited model for neurodegeneration, the wobbler (WR) mouse, a model for human amyotrophic lateral sclerosis (ALS), ADAM8 mRNA was found to be prominently upregulated in degenerating neurons and in glia cells. The inducer for enhanced transcription was the cytokine TNF- α (Schlomann et al., 2000). In lungs of mice undergoing asthma induction with different allergens like ovalbumin and *Aspergillus fumigatus*, ADAM8 was strongly induced (King et al., 2004) suggesting that ADAM8 plays a role in pathogenesis of allergic asthma. Under these conditions, the induction of ADAM8 was increased by interleukins 4 and 13 (IL-4, IL-13). The pathways leading to increased expression of ADAM8 in allergic asthma were critically dependent on the IL-4 receptor α -chain and on a signal transducer and activator of transcription (STAT) protein family member, STAT6.

The cytoplasmic region of ADAM8 contains putative proline rich SH3 binding sequences, suggesting the capability of intracellular signalling (Yoshida et al., 1990; Yoshiyama et al., 1997). So far, no evidence for an intracellular regulation of ADAM8 e.g. by phorbol ester TPA has been given. This is in contrast to ADAM10 and TACE which can be activated by TPA leading to enhanced shedding of membrane proteins.

1.2.4 Expression of ADAM8

ADAM8 was first detected in macrophages (Yoshida et al., 1990), later in the nervous system and in osteoclasts (Choi et al., 2001; Schlomann et al., 2000). A systematic analysis of ADAM8 expression in the mouse came from ADAM8 deficient mice. In early embryonal development, ADAM8 was expressed by maternal cells in the decidua and by trophoblast derivatives of the embryo. At later stages of embryonal development, ADAM8 was expressed in the gonadal ridge, thymus, developing cartilage or bone, in mesenchymal tissue in close proximity to developing blood vessels and lymphatic vessels as well as in the developing brain and spinal cord. Despite this prominent expression in embryonal development, no major defects or abnormalities were evident (Kelly et al., 2005).

In adult tissues, ADAM8 expression occurred in the lymphatic organs thymus and spleen as well as in lung, epithelia of salivary glands, and in tubular epithelial cells of the kidney. In lungs, expression was restricted to epithelial cells of distinct respiratory bronchioles. Under experimental induction of allergic asthma, ADAM8 expression was upregulated in these cells (Kelly et al., 2005), whereas no expression was observed in smooth muscle layers underlying the bronchioles. In the brain, ADAM8 was expressed weakly in neurons and oligodendrocytes under normal conditions. Under inflammatory stimuli like exogenous LPS injected subcutaneously or TNF- α , expression levels enhanced, caused by additional expression in astrocytes and microglia, and also in degenerating neurons.

1.3 ADAMs in inflammation and disease

ADAM proteins form a large group with widespread cell distribution. Initially, ADAMs were classified into five subgroups according to their so far known functions (Kataoka et al., 1997). A group containing ADAM8, 10 and 17 was supposed to play a role in protein degradation. One group (ADAM1 and 12) with hydrophobic regions similar to viral fusion peptides was assumed to contribute to cell fusion, another one (ADAM2) to cell adhesion. Group 4 implied ADAM8, 9, 10, 12 and 15 mediating cell signalling or cytoskeletal attachment via SH3 domain containing proteins. The last group including ADAM7, 10 and 12 was supposed to facilitate differentiation and maturation of cells. It was expected that other ADAM family proteins would be subdivided into these groups (Kataoka et al., 1997). With the knowledge about ADAMs gained in recent years it emerged that specific functions cannot be assigned to individual ADAMs, but that ADAMs can have multiple functions. Furthermore, it turned out that ADAM function is involved in inflammation and several diseases.

1.3.1 ADAMs in inflammation

ADAM17 was identified as the TNF- α converting enzyme (TACE) able to cleave the cytokine TNF- α to its mature form (Black et al., 1997; Moss et al., 1997). Meanwhile, several ADAMs were identified as proteases of a number of cytokines (Table 1.1.2). Cytokines together with hormones and neurotransmitters are 'messenger proteins' enabling communication between cells and controlling development and homeostasis. Moreover, cytokines coordinate the activity of the immune system. As cytokines play a role in many diseases and inflammatory processes, ADAMs are thought to be involved as cytokine sheddases. Further indications for the involvement of ADAMs in inflammation came from experiments in which ADAM expression was upregulated under inflammatory conditions. Numerous publications report that several ADAM shedding activities can be induced by phorbol esters presumably by activating protein kinase C. ADAM8 was reported to be induced by stimulation with LPS and IFN- γ (Kataoka et al., 1997). ADAM8 mRNA was found to be upregulated in degenerating neurons and glia cells under inflammatory conditions in an experimental mouse model for neurodegeneration with the expression induced by the cytokine TNF- α (Schlomann et al., 2000).

ADAMs might be involved in inflammatory processes either directly by shedding of cytokines or cytokine receptors or indirectly by their activation induced by cytokines. A feedback regulation in inflammation was discussed for ADAM8. TNF- α is constitutively released from the cell surface by ADAM17. Soluble TNF- α bound to its receptor TNF-R1

induces ADAM8 transcription by intracellular signalling. Expressed ADAM8 protein on the cell surface is able to cleave TNF-R1 (unpublished data). Thereby, target cells are desensitised to high TNF- α concentration under inflammatory conditions and a dose-dependent cellular response is maintained (Moss and Bartsch, 2004; see also figure 4.4.3).

Asthma is a complex chronic pulmonary disease associated with production of immunoglobulin E (IgE), mucus hypersecretion, airway obstruction, eosinophilic inflammation and enhanced bronchial reactivity to spasmogens (Broide, 2001). The severity of asthma correlates with the presence of CD4⁺ T helper 2 lymphocytes (Th2) (Hogan et al., 1998; Robinson et al., 1992). These cells are thought to induce asthma by secretion of several cytokines, mainly IL-4 and IL-13. King et al. (2004) defined a set of “asthma signature” genes with a gene chip and detected increased ADAM8 expression levels in murine asthmatic lungs which were induced by IL-4 and IL-13. As increase of serum IgE of individuals with allergic asthma correlated with increased concentrations of CD23, CD23 was supposed to be involved in allergic asthma (Mayer et al., 2002). CD23 first detected on activated B cells is also located to other hematopoietic cells. The membrane bound form of CD23 has a modulatory role in antigen presentation and a regulatory role in IgE synthesis through a negative feedback mechanism (Mayer et al., 2002), whereas shed fragments were supposed to perform cytokine-like activities (Armant et al., 1994; Armant et al., 1995; Mossalayi et al., 1992). The fact that ADAM8 was shown to cleave CD23 (Fourie et al., 2003), further indicated involvement of ADAM8 in allergic asthma: surface bound CD23 is probably released from the cell surface by ADAM8 as a result of induction of ADAM8 expression by IL-4 and IL-13.

In a genome-wide study, the ADAM33 gene was significantly associated with asthma (Van Eerdewegh et al., 2002). Consistent with a role in the pathophysiology in asthma, ADAM33 was expressed in bronchial tissues and smooth muscle cells (Garlisi et al., 2003).

1.3.2 ADAMs in the CNS

Several catalytically active ADAMs were reported to be expressed in the normal adult rodent brain or during brain development (e.g. Bernstein et al., 2004; Bosse et al., 2000; Goddard et al., 2001; Gunn et al., 2002; Schlomann et al., 2000) suggesting a role of ADAMs in normal brain function and brain maturation. However, most studies examined differential ADAM expression in the brain mainly under inflammatory conditions or in brain tumour development (see also 1.3.3).

Amyotrophic lateral sclerosis (ALS) is a heterogeneous neurodegenerative syndrome belonging to a group of disorders known as motor neuron diseases. ALS is characterised by

rapidly progressive degeneration of motor neurons in brain and spinal cord. In the WR mouse, a model for ALS, ADAM8 mRNA levels were increased in brain stem and spinal cord in regions in which neurodegeneration occurred. Low expression levels were detected in oligodendrocytes and neurons throughout the normal mouse brain, whereas levels were highly increased in neurons, reactive astrocytes and activated microglia in the WR brainstem and spinal cord. Expression levels of TNF- α were increased with the same cellular distribution. As ADAM8 expression was induced by this cytokine and as this induction was suppressed by an interferon-regulating factor 1 (IRF-1) antisense oligonucleotide, it was supposed that IRF-1-mediated induction of ADAM8 by TNF- α is a signalling pathway relevant for neurodegenerative disorders with glia activation (Schlomann et al., 2000).

Multiple sclerosis (MS) is an inflammatory demyelinating disorder in the CNS mediated by several cytokines and proteases. This disease is characterised by demyelinated plaques which are supposed to disrupt the blood brain barrier and which contain inflammatory cells – T lymphocytes and monocytes – and activated glial cells which are thought to enhance generation of plaques (Calder et al., 1989). At least partly, the cytokine TNF- α is involved in MS pathology. As ADAM17 is able to cleave TNF- α and as ADAM10 is involved demyelination processes, both proteases are thought to play a role in MS pathology. In brain sections of post mortem CNS tissue samples from patients with MS, ADAM10 was detected in astrocytes in all MS as well as in control sections of healthy patients. However, it was exclusively detected in perivascular macrophages in the MS sections suggesting that it is constitutively expressed in astrocytes and only under inflammatory conditions in perivascular macrophages. ADAM17 was expressed in active MS plaques in invading T lymphocytes. ADAM10 co-localised with TNF- α expression in perivascular macrophages, whereas ADAM17 and TNF-R2 were expressed in MS plaques. In cerebrospinal fluid samples from MS patients, increased levels of ADAM17 and of soluble TNF-R2 were measured suggesting shedding of TNF-R2 by ADAM17. These results indicated that in MS pathogenesis, ADAM10 upregulated in perivascular macrophages and ADAM17 expressed by invading T lymphocytes are actively involved in the inflammatory disorder (Kieseier et al., 2003).

Alzheimer's disease and Creutzfeldt-Jacob's disease are spongiform encephalopathies caused by accumulation of cerebral plaques of infectious forms of proteins. ADAMs are thought to play a role in these neurodegenerative diseases by cleaving either the amyloid beta A4 protein precursor (APP) or the prion precursor protein (PrPc) into non-infectious forms preventing accumulation of infectious forms to cerebral plaques and subsequent neurodegeneration.

The Alzheimer's APP is cleaved proteolytically in two different pathways. It is cleaved in the amyloidogenic pathway by β - and γ -secretases to release a toxic amyloid β (A β) peptide forming toxic non-soluble plaques. In a non-amyloidogenic pathway, it is cleaved within the amyloidogenic A β domain by an α -secretase to release a non-amyloidogenic p3 peptide and, furthermore, a large ectodomain of APP (sAPP α) with neuroprotective and memory-enhancing function (Furukawa et al., 1996; Mattson et al., 1999; Meziane et al., 1998, see also figure 4.4.2). ADAM10 was the first ADAM described to have α -secretase activity. In ADAM10 overexpressing cells, basal and protein kinase C stimulated α -secretase activity was increased and inhibited by using a catalytically inactive form of ADAM10. Purified non-amyloidogenic p3 fragments exhibited the correct α -secretase cleavage sites. Increase of ADAM10 expression was suggested as treatment of Alzheimer's disease (Lammich et al., 1999). *In vivo* studies with mice overexpressing either ADAM10 or a catalytically inactive ADAM10 mutant supported this idea, as secretion of neurotrophic sAPP α was enhanced and the formation of A β peptides into plaques was reduced in neurons of mice overexpressing wild type ADAM10 (Postina et al., 2004). Furthermore, ADAM9 and ADAM17 were suggested to act as α -secretases (Buxbaum et al., 1998; Koike et al., 1999). In a comparative study, the physiological roles of ADAM9, ADAM10 and ADAM17 were examined. In human glioblastoma cells, the expression of these ADAMs was suppressed by lipofection of double-stranded RNA encoding these ADAMs. As ADAM9, ADAM10 and ADAM17 were active as α -secretases, it was suggested that the endogenous α -secretase was composed of several ADAM enzymes (Asai et al., 2003).

Usually, prion precursor protein PrP^c is cleaved in the 106-126 toxic domain of the protein producing a non-toxic protein N1. The pathological cleavage more N-terminally at the 90-91 site leads to a toxic isoform which accumulates and which is resistant to proteases (Chen et al., 1995). In stable HEK cells overexpressing ADAM10 and 17, N1 formation was drastically reduced by inhibitors against these two ADAMs. Furthermore, in ADAM10 and ADAM17 deficient cells N1 formation was reduced. These data indicated that ADAM10 and 17 might be responsible for normal cleavage of PrP^c and therefore for avoiding formation of toxic prion protein. These ADAMs were therefore discussed as putative cellular targets for therapeutic strategies (Vincent et al., 2001). Recently, dual mechanisms for shedding of PrP^c were detected. Apart from cleavage by ADAM proteases, another cleavage mechanism most likely to occur via phospholipase cleavage of the glycosylphosphatidylinositol (GPI) anchor of PrP was observed (Parkin et al., 2004). Both cleavage mechanisms reduced the amount of

membrane bound prion precursor for subsequent conversion into the toxic form and might therefore both be important in the pathogenesis of Creutzfeldt-Jacob's disease.

Neuronal cell adhesion molecules form a group of further potential target proteins for ADAMs in the brain. They form a subfamily of the immunoglobulin superfamily which is important for mediating interactions between cells in the nervous system. These molecules are widely expressed during brain development and in the adult brain and have multiple functions. They are involved in neurite outgrowth, regeneration and cell migration (e.g. Brummendorf et al., 1998; Chaisuksunt et al., 2000; Kamiguchi and Lemmon, 1997). In chicks, the neuronal cell adhesion molecule L1 was involved in consolidation of memory (Scholey et al., 1995). Defects in the genes for L1 or the close homologue of L1 (CHL1) resulted in neuropsychiatric disorders such as schizophrenia (Irintchev et al., 2004). As neuronal cell adhesion molecules promoted cell migration, they might also contribute to migration and proliferation of tumour cells (Chen et al., 2004; Tsuzuki et al., 1998). Soluble forms of CHL1 as well as of L1 prevented neuronal cell death (Chen et al., 1999). L1 is known to be released from the cell surface by ADAM10 (Mechtersheimer et al., 2001). Assuming that other members of this family are also cleaved by ADAMs, research concerning the physiological role of ADAMs in nervous system would expand.

1.3.3 ADAMs in cancer

ADAMs have potential implication for metastasis of cancer cells via cell adhesion and protease activity. In several studies, either chip technology, immunohistochemistry, PCR or immunoblotting methods were used to identify molecular markers for diagnosis and prognosis of cancer. Indeed, increased levels of ADAMs – mainly of ADAM8, 9, 10, 12, 15, 17 and 19 – were detected in cancer cells compared to normal cells indicating involvement of ADAMs in cancer.

Increased levels of ADAMs were detected in various kinds of cancers: in cancer cells derived from haematological malignancies (Wu et al., 1997), in breast cancer cells (Lendeckel et al., 2005; O'Shea et al., 2003), in lung cancer cells (Ishikawa et al., 2004; Shintani et al., 2004), in prostatic tumour cell lines (Karan et al., 2003), in gastric cancer cell lines (Carl-McGrath et al., 2005), in pancreatic ductal adenocarcinomas (Grutzmann et al., 2003; Grutzmann et al., 2004), in hepatocellular carcinomas (Ding et al., 2004; Tannapfel et al., 2003), in renal cell carcinomas (Roemer et al., 2004), in glioblastomas (Kodama et al., 2004) and primary human brain tumours (Wildeboer et al., submitted). In many studies, increased ADAM expression levels correlated with cancer progression. In human renal cell carcinoma, ADAM8 expression

correlated with a shorter survival of patients (Roemer et al., 2004), and high levels of ADAM8 were significantly more common in advanced-stage lung cancer adenocarcinomas than in those at earlier stages (Ishikawa et al., 2004). ADAM12 expression in glioblastomas was associated with glioblastoma cell proliferation (Kodama et al., 2004).

The ability of invasive tumour cells to degrade connective tissue stroma and basement membrane is one important step for the metastatic process (Liotta and Stetler-Stevenson, 1991; Stetler-Stevenson et al., 1993). ADAM15 degrades collagen IV and gelatine (Martin et al., 2002) and only recently, it was demonstrated by zymography that ADAM12 degrades collagen IV (Roy et al., 2004). ADAMs are therefore potentially able to degrade the basement membrane and to provide metastasis directly by matrix degradation. In addition – at least partially – ADAMs are involved in cancer progression indirectly by modification of signalling pathways regulating cell proliferation and differentiation; e.g. via the epidermal growth factor receptor (EGF-R).

The EGF-R signalling pathway has a critical function for cell proliferation. EGF-R ligands are integral membrane proteins that are proteolytically released from the cell surface and that stimulate intracellular signalling pathways by binding to their receptor as soluble proteins. Several EGF-R ligands have been identified as ADAM substrates and indeed, ADAMs are discussed to be involved in cancer progression by shedding of these ligands, e.g. when ADAM17 expression was prevented, TGF- α interacted with EGF-R but did not activate the receptor (Borrell-Pages et al., 2003).

1.4 Proteomic approaches for the analysis of proteases

Proteases form a part of physiological systems operating with other proteases, substrates, inhibitors, receptors and binding molecules. Often, one protease can cleave several substrates and conversely, several proteases can cleave the same substrate. Proteolytic processing is possible in *trans* as well as in *cis* and can lead to activation of other proteases, release of signal proteins thereby activating signal cascades or release of other proteins of the physiological system. As the involvement of proteases in many diseases is evident, proteases have become more relevant as drug targets. Therefore, the characterisation of the hierarchical status of proteases within a system, their physiological functions as well as their interaction partners is of importance, in addition to the detailed characterisation of individual proteases and their substrates.

A substrate identified *in vitro* is necessarily of physiological relevance *in vivo*. Several proteases may cleave a substrate *in vitro*, but *in vivo* only one protease might be of importance. Tools for examination of all expressed proteases of a physiological systems as well as for screening for new physiologically relevant protease substrates are eligible. All genomic and proteomic applications to identify protease and protease-substrate in their entirety in an organism can be summarised as “Degradomics”. Different degradomic approaches have been developed in order to determine proteases expressed in cells or tissues and to identify new substrates (Lopez-Otin and Overall, 2002).

1.4.1 Identification of protease profiles

With the examination of proteases under distinct physiological conditions it can be ascertained if specific sets of proteases are switched on under specific pathological conditions, in specific cell types or tissues. Conventional genomic methods such as reverse transcriptase PCR, real-time PCR as well as microarray chip technique are used to examine expression of proteases under specific physiological conditions. A DNA microarray with oligonucleotides for identification of all 715 human proteases, their inactive homologs and inhibitors was used to compare protease expression levels of cancerous breast tissues in comparison to normal ones. In invasive ductal cell carcinoma, expression of ADAMTS17, carboxypeptidases A5 and M, tryptase-gamma, matriptase-2 and MMP-28 was increased (Overall et al., 2004). Nevertheless, the examination of protease expression levels do not necessarily correspond to the activity of corresponding proteins, as other mechanisms such as post-translational regulation, prodomain removal or regulation by inhibitors can be of importance for proteolytic activity. The same disadvantages arise with identification of

proteases using conventional proteomic methods involving denaturation, two dimensional electrophoreses, tryptic digestion and subsequent mass spectrometry. For examination of active proteases in complex samples, approaches distinguishing between active proteases, inactive precursor or inhibitor-bound forms have to be used. These can be based on capturing of proteolytically active proteases by immobilised inhibitors, substrates or chemicals (Lopez-Otin and Overall, 2002).

1.4.2 Identification of protease substrates

Several protease substrates were identified in knockout studies because of accumulation of non-processed proteins; e.g. in such studies, L-Selectin, TNF-R1 and 2, TGF- α , APP and IL-1R-2 were identified as ADAM17 substrates (Buxbaum et al., 1998; Peschon et al., 1998; Reddy et al., 2000). Mostly, only one or a few substrates were identified for particular proteases.

As more systematic approaches for the identification of substrate sets, two different states in cells or tissues with different protease expression were analysed in 2D gel electrophoreses with subsequent mass spectrometry. Using specific labelling of cell surface proteins in cell culture, released proteins could be analysed directly by mass spectrometry without performing 2D gel electrophoresis and corresponding tryptic digestion. Supernatants from isotope-coded affinity tag (ICAT) labelled breast carcinoma cells either in the presence or absence of membrane type 1 matrix metalloprotease (MT1-MMP) were analysed by mass spectrometry. Among others, the cytokines interleukin-8 (IL-8) and TNF- α were determined as new substrates of MT1-MMP (Tam et al., 2004). As further systematic approaches, phage display peptide libraries as well as combinatorial peptide libraries with either quenched or fluorophore-coupled peptides were used to express diverse peptide sequences as potential protease substrates. For some proteases, conserved cleavage sites could be derived with this method by sequencing of peptide substrates. Furthermore, new substrates could be determined using protein databases. For ADAMs, only several new substrates were determined either on the basis of peptides known to be metalloprotease substrates (e.g. Amour et al., 2002) or on the basis of combinatorial peptide libraries (e.g. Fourie et al., 2003). Cleavage in these assays did not necessarily indicate substrates *in vivo*. For that reason, e.g. CD23 cleavage by ADAM8 was confirmed in transfection experiments (Fourie et al., 2003).

In a yeast-two hybrid approach, a catalytically inactive protease domain lacking the prodomain was used as bait which bound to but did not cleave substrates. Using catalytically inactive MT1-MMP lacking the prodomain, a human fibroblast cDNA library was screened

for interacting proteins in order to find new MT1-MMP substrates. *Wnt*-induced signalling protein-2 (WISP-2) was identified by this approach and biochemically confirmed as a new substrates by further experiments (Ortiz et al., 2004). Another yeast-two hybrid approach took into account that binding sites outside the catalytic domain might be necessary for cleavage specificity. The characteristic C-terminal hemopexin-like domains in MMPs have specific roles in individual MMPs like activation of proenzymes, substrate specificity and TIMP binding. For the detection of MMP substrates in the yeast two-hybrid approach, the hemopexin domain was used as a bait in order to find binding partners which were potential new substrates (McQuibban et al., 2000). Transferred to ADAMs, the disintegrin and cysteine-rich domains could be used in such an approach, as they are supposed to be of importance for substrate recognition and cleavage. For ADAM8, it was suggested that the disintegrin domain influences autocatalytic prodomain removal by mediating the interaction of at least two ADAM8 molecules (Schlomann et al., 2000).

1.5 Objectives

The ADAMs form a family of metalloproteases which is known for about 10 years. A number of physiologically relevant ADAM substrates have been determined since, but only little work was done on systematic substrate search and on determination of ADAM expression patterns. The objective of this work focused on search for physiologically relevant protease substrates of the family member ADAM8.

In order to find new ADAM8 substrates, an *in vitro* approach with fluorogenic peptides was established. Therefore, a collection of peptides was designed. Sequences were either derived from known peptide substrates of other ADAMs or from juxtamembraneous regions of supposed metalloprotease substrates. To get soluble, catalytically active forms of ADAM8 for the *in vitro* peptide assays, new ADAM8 cDNA constructs encoding the pro and metalloprotease domain were cloned and expressed in cell culture as well as in *E.coli*.

Cleaved peptides were examined in order to find a consensus sequence for ADAM8 substrates. The corresponding proteins of positively tested peptide substrates were supposed to be tested for their physiological relevance *in vivo* as ADAM8 substrates. In addition to these proteins, the physiological relevance of the ADAM8 substrate close homologue of L1 (CHL1) in the nervous system was determined.

A test method for the determination of ADAM activities in tumour tissue extractions was established using specific peptides for ADAM8 and 19, respectively, in order to determine whether expression levels of ADAM8 and 19 correlated with proteolytic activity.

2. Materials and methods

2.1 Materials

2.1.1 Materials and chemicals

Chemicals and molecular biology reagents were mainly obtained from the companies Applichem (Darmstadt, Germany), Sigma (Taufkirchen, Germany), Merck (Darmstadt, Germany) and Roth (Karlsruhe, Germany), cell culture materials from Invitrogen (Groningen, Netherlands), Seromed Biochrom AG (Berlin, Germany) and PAA Laboratories GmbH (Linz, Austria), plastic articles from Greiner Labortechnik GmbH (Solingen, Germany), Eppendorf (Hamburg, Germany) and Nunc (Wiesbaden, Germany). The tissue inhibitors of metalloproteases 1 (TIMP1) as well as the broad range matrix metalloprotease protease inhibitor batimastat (BB-94) was kindly provided by Prof. Tschesche (Biochemie, Chemische Fakultät, Universität Bielefeld). TIMP3 was obtained from Chemicon (Heidelberg, Germany). The metalloprotease inhibitor 1,10-*ortho*-phenanthroline (OPT) was obtained from Sigma (Taufkirchen, Germany) and the protease inhibitor cocktail, CompleteTM EDTA-free from Roche (Mannheim, Germany).

2.1.2 Cell lines

COS1 cells (ATTC No. CRL-1650) and COS7 cells (ATTC No CRL-1651) were derived from the fibroblast-like kidney cell line CV-1 (ATTC No. CCL-70) from African green monkey (*Cercopithecus aethiops*). They were transformed with an origin defective mutant of SV40 coding for the wild type T antigen. The cells contain a single integrated copy of the complete early region of the SV40 genome. COS7 cells were allocated from Prof. Lehmann (Zellkulturtechnik, Technische Fakultät, Universität Bielefeld) and COS1 cells from Prof. Frey (Biochemie, Fakultät für Chemie, Universität Bielefeld).

2.1.3 Animals

Breeding and experimental use of mice were performed in agreement with the German law on the protection of animals, with a permit by the local authorities. The wobbler mutation (Duchen and Strich, 1968) as well as the ADAM8 knockout mouse was maintained on a C57BL/6 background. The mouse deficient in ADAM8 was obtained from Dr. A.J.P Docherty (Celltech, Slough, UK).

2.1.4 Recombinant proteins

The three recombinant cell adhesion molecules L1-Fc, CHL1-Fc, NCAM-Fc were allocated by Prof. Melitta Schachner (ZMN Hamburg, Germany) as recombinant fusion proteins containing the extracellular portions of the proteins fused to a human immunoglobulin Fc-tag and were used for *in vitro* cleavage experiments. Recombinant myelin basic protein (MBP) was obtained from Sigma (Taufkirchen, Germany).

2.1.5 Antibiotics

Penicillin (Seromed Biochrom AG, Berlin, Germany) und streptomycin (Sigma, Taufkirchen, Germany) were used in cell culture media for COS1 and COS7 cells to prevent contaminations. Ampicillin, chloramphenicol and streptomycin (Roth, Karlsruhe, Germany), were used for selection of transformed bacteria and were added to culture media. Anhydrotetracyclin (IBA, Göttingen, Germany) was used for induction of ADAM8 expression in *E.coli*.

2.1.6 Antibodies

Antibody dilutions are quoted either for immunoblotting (*) or for immunohistochemistry (§).

Primary antibody	Host animal	Characterisation	Dilution
anti-BiPro antibody	mouse	monoclonal antibody against a birch pollen profilin tag (Rudiger et al., 1997), allocated by Prof. Jockusch (Universität Braunschweig)	1:25*
anti-ADAM8 antibody	rabbit	polyclonal antibody against cytoplasmic domain of mouse ADAM8 (AA 766-780) (Schlomann et al., 2000)	1:1000*
anti-ADAM10 antibody	rabbit	polyclonal antibody against the cytoplasmic domain of mouse ADAM10 (AA 727-741)	1:1000*
anti human IgG antibody	rabbit	polyclonal, non conjugated antibody against human IgG, allocated by Prof. Melitta Schachner (ZMN Hamburg)	1:10000*
anti-CHL1 antibody	rabbit	polyclonal antibody against the extracellular domain of mouse CHL1, allocated by Prof. Melitta Schachner (ZMN Hamburg)	1:10000*
anti-GAPDH antibody	mouse	monoclonal antibody against glyceraldehyde-3-phosphate dehydrogenase (Chemicon, Darmstadt, Germany)	1:500*
anti-NF200 antibody	rabbit	polyclonal antibody against neurofilament NF200, heavy chain (Sigma, Taufkirchen, Germany)	1:100§

Secondary antibody	Characterisation	Dilution
peroxidase-conjugated affine pure goat anti mouse	horse radish peroxidase coupled polyclonal anti mouse antibody (Sigma, Taufkirchen, Germany)	1:7500*
anti rabbit IgG antibody	horse radish peroxidase coupled polyclonal antibody against rabbit IgG (Sigma, Taufkirchen, Germany)	1: 5000*
anti rabbit IgG-cy3 antibody	cy3 coupled polyclonal antibody against rabbit IgG (Sigma, Taufkirchen, Germany)	1:250 [§]

2.1.7 Oligonucleotide primer

Oligonucleotide primer were obtained from MWG-Biotech (Ebersberg, Germany) und TIB-Molbiol (Berlin, Germany).

Primer	Primer sequence	Annealing T[°C]	Cycles	DNA product
mADAM8.a	5'-ctc tgg ctg ctc agc gtc tta-3'	64	30	459 bp
mADAM8.b	5'-gat gct ttg cct gat aca tcg-3'	64	30	459 bp
mA8MPSec.s	5'-gaa ttc act tgg cct ctg gct gct cag-3'	67	see 2.2.6	
mA8MPSec.as	5'-ctc gag cga tct cct gag gct taa act gag gga agg aca cga acc ggt tga cat ctg g-3'	67		

2.1.8 Restriction endonucleases

Endonucleases were obtained from MBI Fermentas (St.Leon-Rot, Germany). The webcutter 2.0 (<http://rna.lundberg.gu.se/cutter2/>) was used for the identification of enzymes cutting a given DNA sequence.

Restriktion endonuclease	Cleaved sequence
EcoRI	5'- G / AATC -3'
XhoI	5'- C / TCGAG -3'
HindIII	5'- A / AGCTT-3'

2.1.9 Bacterial strains

For the cloning vector pCR[®] II-TOPO, provided chemical-competent *E.coli* TOP10F' cells from the TOPO TA Cloning[®] Kit (Invitrogen, Groningen, NL) were used. For expression of cDNA constructs in pTarget[™] or pSecTag2 B expression vectors, either chemical-competent *E.coli* JM109 cells or electro-competent *E.coli* XL1-Blue cells were used. For expression of the pro and metalloprotease domain of ADAM8 in bacteria the chemical-competent *E.coli* BL21(DE3)pLysS cells obtained from Invitrogen (Groningen, NL) were used.

2.1.10 Vectors

pASK-IBA 3+

The expression vector pASK-IBA 3+ (IBA, Göttingen, Germany) was used for expression of the pro and metalloprotease domain of ADAM8 in *E.coli*. The protein was expressed as a fusion protein with a Strep-tag at the C-terminal end. The expression of ADAM8 was induced via the tetA promotor with anhydrotetracyclin.

pcDNATM 3.1(+)

The expression vector pcDNATM 3.1(+) (Invitrogen, Groningen, NL) was used for the expression of full length CHL1 in cell culture and was kindly provided by Prof. Melitta Schachner (ZMN Hamburg).

peGFP-N3

The expression vector peGFP-N3 (BD Biosciences Clontech, Heidelberg, Germany) was transfected into cell culture cells as a control for transfection efficiency. According to the portion of GFP expressing cells, the efficiencies of transfections were evaluated.

pTargetTM

The expression vector pTargetTM (Promega, Heidelberg, Germany) was used by Uwe Schlomann for cloning of cDNA constructs encoding full length mouse ADAM8, EQ-ADAM8, ADAM10 and ADAM17 and for expression of these proteins in cell culture.

pSecTag2 B

The expression vector pSecTag2 B (Invitrogen, Groningen, NL) was used for cloning of the cDNA construct expressing the pro and metalloprotease domain of ADAM8 as a fusion protein with a SecTag at the C-terminal end encoding for a secretion tag for additional secretion of expressed proteins.

pCR[®] II-TOPO

pCR[®] II-TOPO (Invitrogen, Groningen, NL) was used as cloning vector.

2.2 Molecular biology methods

2.2.1 Culture media for bacteria

For bacterial cultures, autoclaved LB-Medium (Luria-Bertani) containing 10 g NaCl, 5 g yeast extract and 10 g trypton or pepton per litre medium (pH 7.0) was used. Appropriate antibiotics were added after media had cooled down. For the preparation of LB-agar plates which were used to obtain monocultures, 15 g/l agar-agar were added to 1 l medium before autoclaving. After cooling down to ~60 °C, respective antibiotics were added, and 20 ml were filled in each petri dish with 100 mm diameter. Media and agar plates were stored at 4 °C.

2.2.2 Glycerol cultures

For long-time storage of recombinant bacterial colonies, glycerol cultures were prepared by mixing 800 µl of a bacterial culture in stationary growth phase and 200 µl of glycerol and were stored at -80 °C.

2.2.3 Isolation of plasmid DNA

For preparation of plasmid DNA preparation, either the QIAprep Spin Miniprep Kit for isolation of up to 20 µg high-copy plasmid DNA or the Qiagen Plasmid Midi Kit for purification up to 100 µg plasmid DNA was used (Qiagen, Hilden, Germany). These kits were based on alkaline lysis without using phenol/chloroform and subsequent DNA purification with ion exchanger columns. Either 3 ml or 40 ml bacterial cultures were prepared according to the manufacturers instructions.

2.2.4 Determination of DNA concentrations

DNA concentrations were determined with a Bio Photometer (Eppendorf, Hamburg, Germany) by measuring the absorption at 260 nm of 2 µl DNA solution mixed with 98 µl 20 mM Tris, pH 7.4 and calculating the corresponding DNA concentration. Additionally, the photometer measured the absorptions at 280 nm and 230 nm. A value of 1.8 for the ratio A_{260}/A_{280} indicated high purity of the DNA sample, whereas a small value for A_{230} indicated little stray light.

2.2.5 Precipitation of DNA

For removal of buffer components as well as for increasing DNA concentrations, DNA solutions were precipitated. The smallest volume of DNA solution used for precipitation was

100 μ l. DNA solutions were mixed with 1/10 volume 3 M sodium acetate, pH 5.2 and 2.5 volumes 100 % ethanol. Batches were either precipitated for 15 minutes at -80 °C or overnight at -20 °C. After centrifugation for 30 minutes at 13000 x g, supernatants were discarded. Pellets were washed with 500 μ l ice cold 70 % ethanol. After centrifugation for 15 minutes at 13000 x g, supernatants were discarded. Pellets were dried and resuspended in distilled water or adequate buffer. 20 μ l ligation sample (2.2.11) was precipitated with 2 μ l yeast tRNA and 78 μ l distilled water and washed as described above. Yeast t-RNA served as carrier improving precipitation.

2.2.6 Polymerase chain reaction (PCR)

PCR is a technique for amplifying specific DNA sequences in complex mixtures. A DNA sequence located between two oligonucleotide primers present in excess concentration is amplified in an exponential fashion by repeated cycles of melting DNA double strands to single strands, binding of primers to complementary DNA sequences on the single strands, and synthesis of new DNA fragments. Criteria for oligonucleotide primers are GC contents of 40-60 %, lengths of 18-30 nucleotides, no complementary sequences to each other and annealing temperatures of 50-65 °C.

Usually, the 2 x Taq PCR-Mastermix (Quiagen, Hilden, Germany) containing Taq-Polymerase, dNTPs and corresponding salts was used for PCR. For 10 μ l reaction batches, 5 μ l Mastermix were mixed with 0.2 μ l 50 μ M sense and anti-sense oligonucleotide primer, 50 ng DNA and water. For the control of bacterial clones on agar plates, as template DNA a little of the whole colony was picked and mixed with the batch. Furthermore, this colony was seeded on a new agar plate for isolation. For DNA amplification, either a Trio Thermoblock (Biometra, Göttingen, Germany) or a Mastercycler Gradient (Eppendorf, Hamburg, Germany) was used. A standard protocol for amplification with Taq-Polymerase was as follows. Used number of cycles and annealing temperatures are listed in table 2.1.7. Depending on the length of amplified DNA fragments, 45 to 60 seconds were used for denaturation and annealing.

Denaturation	4 min	94 °C	
Denaturation	45-60 sec	94 °C	18-35 cycles
Annealing	45-60 sec	X °C	
Polymerisation	1 min	72 °C	
A-Tailing	10 minutes	72 °C	

For amplification of DNA fragments used for further cloning, proofreading DNA polymerases that efficiently add 3'-deoxyadenoside overhangs to PCR amplicons for subsequent TA-cloning were used. For amplification of the ADAM8 pro and metalloprotease domain cloned into the pSecTag2 B vector (Invitrogen, Groningen, NL), the Expand Long Template PCR System (Roche, Mannheim, Germany) was used. 25 µl buffer III containing 5 µl 10 x buffer III, 0.75 µl enzyme and distilled water were mixed with a 25 µl reaction batch containing 10 µl 2.5 mM dNTPs, 0.6 µl of each primer (50 mM), 2 µl template cDNA and distilled water. A Mastercycler Gradient (Eppendorf, Hamburg, Germany) was used for amplification according to the following protocol.

cDNA was generated from total RNA extracted from cells of the macrophage cell line J774A.1 with the Nucleospin® RNAII Kit (Macherey-Nagel, Düren, Germany). For reverse transcription, SuperscriptII Reverse Transcriptase (Roche, Mannheim, Germany) was used.

Denaturation	2 min	94 °C	
Denaturation	10 sec	94 °C	10 cycles
Annealing	30 sec	67 °C	
Polymerisation	2 min	68 °C	
Denaturation	10 sec	94 °C	25 cycles
Annealing	30 sec	67 °C	
Polymerisation	2 min (+20sec)	68 °C	
A-Tailing	10 minutes	72 °C	

2.2.7 Agarose gel electrophoresis

DNA fragments were separated according to their molecular mass by agarose gel electrophoresis. For DNA fragments larger than 1000 bp, 1 % gels were used and for smaller fragments 2 % gels (SeaKem LE Agarose, Cambrex, USA). Agarose was mixed and melted in TAE buffer. Samples were mixed with 6 x sample buffer, and either DNA-Marker 100 bp-Leiter or DNA-Marker Lambda Mix II (MBBL, Bielefeld, Germany) was used as DNA molecular mass marker. Gels were run with constant voltage of 5 V per cm electrode distance. The DNA in the gels was stained with ethidium bromide by incubation in TAE buffer containing 1 µg/ml ethidium bromide for 15 minutes. The DNA with the intercalated dye was detected on an illuminator (excitation: 312 nm, emission: 590 nm).

TAE buffer

0.04 M Tris, HAC

1 mM EDTA, pH 8.0

6 × sample buffer

0.25 % Bromphenol blue

0.25 % Xylencyanol FF

30 % Glycerol in distilled water.

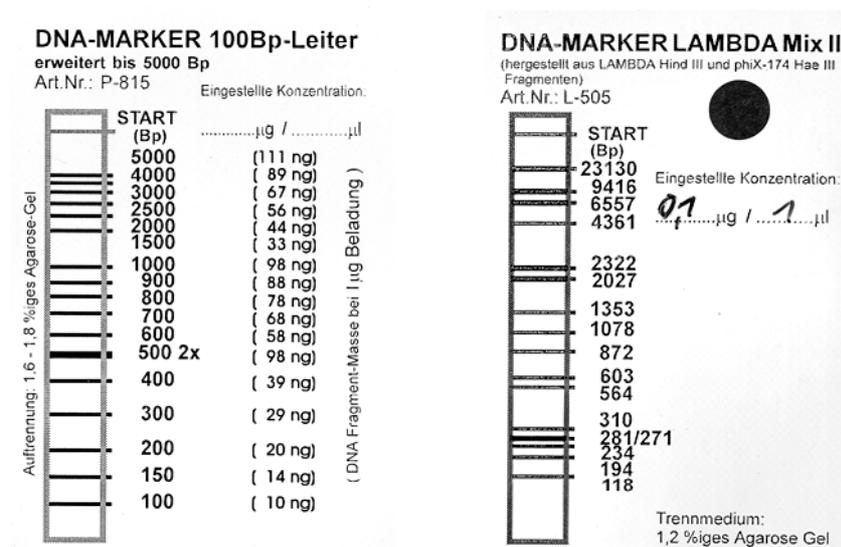


Figure 2.2.7: DNA markers

2.2.8 DNA isolation from agarose gels

DNA isolation from agarose gels was performed with the MinElute™ Gel Extraction Kit (Qiagen, Hilden, Germany) according to the manufacturers instructions in order to purify DNA fragments needed for subsequent cloning steps.

2.2.9 TA-cloning

Before cloning into the target expression vector, PCR fragments were inserted into the cloning vector pCR® II-TOPO by TA-cloning with the TOPO TA Cloning® Kit (Invitrogen, Groningen, NL). Topoisomerase I bound to a terminal thymine of the linear pCR® II-TOPO cloning vector combined the thymine with the 3'-deoxyadenoside overhangs in the PCR amplicons.

The ligation reaction was performed with 2 µl PCR product, 1 µl provided salt solution and 1 µl vector for 5 minutes at room temperature. For transformation, 2 µl of this reaction batch were mixed with an aliquot of provided chemical-competent TOP10F' One Shot® cells and

were incubated on ice for 20 minutes. After a heat-shock at 42 °C for 30 seconds and quick cooling down on ice, 250 µl SOC medium were added. The transformed cells were incubated for 1 hour at 37 °C in a shaker. Subsequently, 50 µl and 100 µl were plated on LB agar plates which were then incubated overnight at 37 °C. In addition to 50 µg/ml ampicillin, the agar plates contained isopropyl-β-thiogalactopyranosid (IPTG, 40 µl of a 100 mM stock solution) and the synthetic β-galactosidase substrate 5-brom-4-chloro-3-indigo-β-D-galactoside (X-Gal, 40 µl of a 40 mg/ml stock solution) for the selection of transformed colonies.

In the untransformed *E.coli* strain TOP10F', the multiple cloning site was located in the LacZ gene and the lac repressor was overexpressed. Induction with IPTG resulted in transcription of the LacZ gene and expression of β-galactosidase which turned over X-Gal to the dye 5-brom-4-chlor-indigo. Corresponding colonies were blue coloured. In case of integration of a PCR fragment into the multiple cloning site, lacZ promoter and lacZ gene were separated and β-galactosidase was not expressed. Corresponding colonies remained colourless. Some of these colourless colonies were further tested for the integrated DNA in colony PCR reactions (2.2.6).

2.2.10 Restriction of DNA

Restriction reactions were performed either to provide PCR fragments and vectors with complementary ends for ligation reaction or to examine insertion and orientation of inserted DNA fragments in cloned constructs. The used restriction enzymes (MBI Fermentas, St. Leon-Rot, Germany) were type II endonucleases. For reactions with two or more endonucleases, MBI Fermentas provided information for the best reaction conditions (www.fermentas.com/doubledigest/double.php).

For 20 µl restriction reaction samples, 2 µg DNA, 0.6 µl of each enzyme (10 U/µl), 2 µl 10 x buffer (MBI Fermentas, St. Leon-Rot, Germany) and distilled water were mixed and incubated overnight at 37 °C. Digested fragments were analysed by agarose gel electrophoresis (2.2.7).

2.2.11 Ligation

PCR fragments and linearised target expression vectors with complementary ends were ligated with T4-Ligase (MBI Fermentas, St.Leon-Rot, Germany). 1 µl T4 Ligase and 2 µl 5 x buffer (MBI Fermentas, St.Leon-Rot, Germany), ~100 ng vector and ~300 ng insert were applied for 20 µl reaction batches. The ligation reaction was incubated overnight in a water bath at 16 °C. DNA was then precipitated (2.2.5) and transformed into bacteria.

2.2.12 Transformation of electro-competent *E.coli* cells

The *E.coli* strain XL1 blue was used for transformation by electroporation. Electro-competent cells were stored in aliquots at -80 °C. For electroporation, cells were defrosted on ice and carefully mixed with either 10 µl of a ligation batch (2.2.11), 2 µl of precipitated DNA (2.2.5) or 50 ng purified plasmid DNA (2.2.3). After incubation for 1 minute on ice, the mixture was pipetted into an electro cuvette and electroporation was performed with a GenePulserTM (Bio-Rad, München, Germany) with a voltage of 2.5 kV, an electric resistance of 400 Ω and an electric capacity of 25 µF. 900 µl SOC medium were added into the cuvette immediately. The mix was decanted into an Eppendorf tube and incubated in a shaker for 1 hour at 37 °C. 50 µl and 100 µl were plated on LB agar plates containing 50 µg/ml ampicillin, IPTG and X-Gal (see also 2.2.9) which were then incubated overnight at 37 °C.

SOB medium

- 20 g trypton
- 5 g yeast extract
- 0.5 g NaCl

Components were mixed in 950 ml distilled water and 10 mM KCl was added. The pH was adjusted to 7.0. The solution was filled up to 1 l and was autoclaved. Subsequently, 5 ml sterile 2 M MgCl₂ solution were added.

SOC medium

- SOB medium
- 20 mM sterile glucose

2.2.13 Transformation of chemical-competent *E.coli* cells

The *E.coli* strain TOP 10F' (TOPO TA Cloning[®] Kit, Invitrogen, Groningen, NL) was used for chemical transformation. Transformation was performed according to manufacturers instruction (see also 2.2.9).

2.2.14 DNA sequencing

Sequencing was performed by the IIT sequencing service (Universität Bielefeld) according to the Sanger sequencing method. Examination of sequencing data was performed with the software ClustalX 1.81 and Chromas 2.24.

2.3 Protein biochemical methods

2.3.1 Preparation of proteins from cell culture cells and supernatants

Cells were grown to 90 % confluence. Cell supernatants were collected and spun for removal of cells and other particles and were enriched by ultrafiltration (2.3.6) or subjected to affinity chromatography either with ConA sepharose or Nickel-NTA (2.3.3, 2.3.4).

Cells were washed twice with PBS and lysed with RIPA containing protease inhibitor cocktail. After incubation with lysis buffer for 15 minutes at room temperature, cells were abraded from the cell culture plates and were sonicated at medial amplitude twice for 30 seconds. After removal of insoluble particles by centrifugation, samples were used directly or were subjected to affinity chromatography with ConA sepharose (2.3.3). If not used immediately, samples were frozen on liquid nitrogen.

For native preparation of cells and subsequent analysis of protease activities in cell lysates, a HEPES lysis buffer containing protease inhibitor cocktail was used for lysis. After incubation at room temperature for 15 minutes, cells were abraded from the culture plates and spun at 13000 x g for 2 minutes. The resulting pellets were directly assayed for protease activity.

1 x PBS (pH 7.4)

136 mM NaCl

3 mM KCl

8 mM Na₂HPO₄ x 2H₂O

1.5 mM KH₂PO₄

RIPA

1 x PBS

1 % Nonidet P-40

0.5 % sodium deoxycholate

0.1 % SDS

HEPES lysis buffer

20 mM HEPES pH 7.4

1 % NP-40

0.5 % sodium deoxycholate

2.3.2 Preparation of proteins from tissues

Wobbler mice and ADAM8 knockout mice as well as wild type litter mates were killed by neck fraction. Brain tissues, spinal chord and lungs were prepared and either used immediately or frozen on liquid nitrogen. 30-day old wobbler mice with an evident wobbler phenotype were used for biochemical analysis.

Mouse tissues were lysed with either an ultraturrax or with pestles in 500 µl RIPA (2.3.1) containing protease inhibitor cocktail per 100 mg fresh weight. After incubation on ice for 30 minutes, samples were sonicated at medial amplitude twice for 30 seconds. After removal of insoluble particles by centrifugation, samples were used directly or were subjected to ConA sepharose affinity chromatography (2.3.3). If not used immediately, samples were frozen on liquid nitrogen.

Brain tumour tissues were prepared under native conditions for subsequent analysis of protease activities. Brain tumour specimens were obtained from archival material of patients who underwent neurosurgery for tumour resection. Histologically normal temporal cortex with adjacent white matter served as control. This material was removed in the course of surgical hippocampectomy for therapy of medical refractory epilepsy. The tissue specimens used for protein extractions were obtained frozen in liquid nitrogen from a collaborating group (Dr. Axel Pagenstecher, Abteilung Neuropathologie, Pathologisches Institut, Universität Freiburg). Brain tissue extracts were lysed with pestles in 10 µl HEPES lysis buffer (2.3.1) containing protease inhibitor cocktail per mg fresh weight. Samples were spun down and supernatants were purified with ConA sepharose (2.3.3). ConA sepharose with bound proteins was used in subsequent activity assays.

2.3.3 Affinity chromatography with ConA sepharose

For enrichment of glycosylated cell surface proteins from tissue extracts, cell lysates or cell supernatants, affinity chromatography with the lectin concanavalin A was performed under native conditions and in a batchwise procedure. ConA sepharose obtained from Amersham Biosciences (Uppsala, Sweden) is ConA coupled to Sepharose 4B by the cyanogens bromide method with a capacity of binding 20-45 mg glycosylated protein per ml sepharose.

ConA sepharose was washed twice with 20 mM HEPES (pH 7.4). In case of small sample volumes, about five volumes of ConA buffer were added. For large volumes of supernatants, NaCl was added to a final concentration of 300 mM NaCl and the pH was adjusted to pH 7.4. After having added ConA sepharose, samples were incubated on an orbital shaker for at least 90 minutes at 4 °C. Subsequently, supernatants were discarded and the sepharose was washed

twice with 20 mM HEPES (pH 7.4). Elution was performed with 0.2-0.4 M α -D-methylmannoside on an orbital shaker for at least 90 minutes at 4 °C or with 2 x SDS sample buffer (from 5 x SDS sample buffer, 2.3.10) for 5 minutes at 95 °C. For activity assays, protein was not eluted, but ConA sepharose pellets with bound protein were used.

ConA buffer

20 mM HEPES, pH 7.4
1 mM CaCl₂
1 mM MnCl₂
0.3 M NaCl
Protease inhibitor cocktail

2.3.4 Affinity chromatography with Nickel-NTA

For enrichment of proteins with a His-tag consisting of six histidines, immobilised metal-ion affinity chromatography (IMAC) with Nickel-NTA was performed in batchwise procedure under native conditions. The chelate nitrolotriactic acid (NTA) was bound to a matrix and charged with nickel for specific binding of histidine containing proteins. Either the Ni-NTA Purification System (Invitrogen, Groningen, NL) or HIS-Select™ Nickel Affinity Gel (Sigma, Taufkirchen, Germany) was used. The manufacturers protocols were slightly modified.

Ni-NTA Purification System (Invitrogen, Groningen, NL)

Centrifugations steps were performed at 4000 x g. Incubation and washing steps were performed at 4 °C on an orbital shaker.

1 ml Ni-NTA matrix was washed with 6 ml distilled water, and was equilibrated twice with 6 ml native wash buffer. Ni-NTA gel and samples were mixed and incubated for 90 minutes. After centrifugation, supernatants were discarded. The gel was washed 4 times with 8 ml native wash buffer. Subsequently, protein was eluted from the gel with 8 ml native elution buffer for 10 minutes. Elution steps were repeated to recover more protein. Samples were dialysed and enriched after elution by ultrafiltration (2.3.8, 2.3.6).

Native wash buffer (pH 8.0)

50 mM Na₃PO₄
500 mM NaCl
10 mM imidazole

Native elution buffer (pH 8.0)

50 mM Na₃PO₄

500 mM NaCl

250 mM imidazole

HIS-Select™ Nickel Affinity Gel (Sigma, Taufkirchen, Germany)

The gel had to be handled carefully. Centrifugations steps were performed with 4000 x g. Incubation and washing steps were performed at 4 °C on an orbital shaker.

The gel was washed three times with 10 gel volumes wash buffer. Gel and samples were mixed and incubated for 30 minutes. After centrifugation, supernatants were discarded. The gel was washed twice with 10 gel volumes wash buffer for 5 minutes. Subsequently, protein was eluted from the gel with 2 gel volumes elution buffer for 10 minutes. Elution steps were repeated to recover more protein. Samples were dialysed, and depending on the elution volume, samples were enriched after elution by ultrafiltration (2.3.8, 2.3.6).

Wash buffer (pH 8.0)

50 mM Na₃PO₄

300 mM NaCl

Elution buffer (pH 8.0)

50 mM Na₃PO₄

300 mM NaCl

250 mM imidazole

2.3.5 Affinity chromatography with Strep-tag®/Strep-Tactin®

Recombinant proteins expressed in *E.coli* containing a streptavidin-tag (NH₂-WSHPQFEK-COOH) were enriched and purified with the Strep-tag®/Strep-Tactin® system (IBA, Göttingen, Germany). Cloning of the corresponding construct A8-ProMP into the pASK-3+ vector (IBA, Göttingen, Germany) as well as improvement of expression conditions and purification methods was performed by the diploma student Simone Reipschläger.

This purification method was based on the binding of biotin to streptavidin. Recombinant ADAM8 protein containing a strep-tag II bound to the streptavidin derivate Strep-Tactin on a matrix. Sepharose® and MacroPrep® (Polymethacrylat) were used as matrices. But as MacroPrep® turned out to be more specific, this matrix was used for large scale purifications.

Elution was performed with the biotin derivate desthiobiotin, and regeneration with a HABA (2-[4-hydroxy-phenylazo] benzoic acid) containing buffer.

HPLC with a Strep-Tactin MacroPrep Cartridge (IBA, Göttingen, Germany) with a bed volume of 1 ml and a binding capacity of 50-100 nMol per 1 ml was performed. After equilibration of the column with 15 ml washing buffer, 10 ml protein sample (2.3.15) was loaded. Subsequently, the column was washed with 20-30 ml washing buffer, and elution was performed with 20 ml elution buffer collecting several fractions. Regeneration was performed with 20 ml regeneration buffer, which was removed afterwards with 40 ml wash buffer. The elution fractions were concentrated to 500 µl by ultrafiltration (2.3.6) and protein concentrations were determined (2.3.9). Samples were analysed by immunoblotting and in Coomassie-Brilliant-Blue or silver stained gels (2.3.12, 2.3.13) and were then used for activity assays. Initially, HPLC was performed in the research group of Prof. Horst Hinszen (Biochemische Zellbiologie, Fakultät für Biologie, Universität Bielefeld).

Resuspension buffer (pH 8.0)

100mM Tris
150 mM NaCl
1 mM PMSF

Wash buffer (pH 8.0)

100 mM Tris
150 mM NaCl

Elution buffer (pH 8.0)

100 mM Tris
150 mM NaCl
2.5 mM desthiobiotin

10 x regeneration buffer (pH 8.0) (IBA, Göttingen, Germany)

1 M Tris-Cl
1.5 M NaCl
10 mM EDTA
10 mM HABA

2.3.6 Enrichment of protein solutions

For enrichment of protein solutions and for reduction of the volumes of cell culture supernatants, ultrafiltration with either Amicon[®] Ultra-4 filtration units (Millipore Corporation, Bedford, UK) or Centricon[®] YM-10 filtration units (Millipore Corporation, Bedford) was performed. Both filtration units had an exclusion volume of 10 kDa. Centrifugation was performed according manufacturers instructions at 4 °C. Filtration units were refilled and used up to three times for equal samples.

2.3.7 Precipitation of proteins

Protein precipitation was performed for enrichment of protein containing solutions. Precipitation with acetone as well as with TCA denatured proteins and was only performed for samples subjected to SDS-PAGE.

For acetone precipitation, samples were incubated with 7 volumes ice cold acetone for 30 minutes at -20 °C. After centrifugation, the supernatants were discarded and the pellets were washed twice with ice cold acetone. The pellets were dried and resuspended in distilled water. For TCA precipitation, samples were incubated with one volume of cold 10 % trichloric acetic acid for 20 minutes on ice. After centrifugation, the supernatants were discarded and the pellets were washed extensively four times with 70 % ethanol. The pellets were dried and resuspended in distilled water.

2.3.8 Dialysis

Dialysis was used for the exchange of buffers of protein containing solutions. Dialysis membranes with an exclusion volume of 14 kDa were used (Medicell International Ltd, London, UK) and dialysis was performed overnight at 4 °C under constant stirring. Protein solutions with soluble ADAM8 were dialysed against a buffer containing 20 mM HEPES, pH 7.4, 5-10 mM CaCl₂, 25 µM ZnCl₂ and 100-200 mM NaCl.

2.3.9 Determination of protein concentrations with BCA

The bicinchoninic acid (BCA) Protein Assay Kit (Pierce, Rockford, USA) was used according to manufacturers instructions with BSA used as standard. The assay is based on the reduction of Cu(II) to Cu(I) by proteins in alkaline solutions. Two molecules BCA and one molecule Cu(I) form a complex which is quantifiable at 562 nm.

2.3.10 SDS polyacrylamid gelelectrophoresis (SDS-PAGE)

For separation of proteins from cell extracts, cell lysates and cell culture supernatants according to their mass, SDS-PAGE with a SDS-containing discontinuous HCl/Tris-glycine buffer system was used (Laemmli et al., 1970). Depending on the size of the analysed proteins, 5 % to 15 % mini gels with a width of 1 mm were used. Usually, 5 % gels were used for CHL1, 7.5 % gels for full length ADAM8, 10 % gels for recombinant forms of ADAM8 and 15 % gels for MBP. A dual plate vertical electrophoresis unit with corresponding supplies (Sigma, Taufkirchen, Germany) was used for preparing and running the gels.

The radical starter TEMED and the catalyst of the polymerisation reaction APS were added to gel solutions immediately before casting gels. The separating gels were covered with isopropanol during polymerisation for smoothing the upper edge of the gels. Combs with either 10 or 12 wells depending on the number of samples were inserted into the stacking gels. Samples were denatured by incubation with 5 x SDS sample buffer for 5 minutes at 95 °C. Gels were run with a constant current of 12 mA per gel. Mark 12 (Invitrogen, Groningen, NL) was used as a protein marker.

1 mini gel	Separating gel (~8 ml)				Stacking gel (~2 ml)
	5 %	7.5 %	10 %	15 %	3.75 %
Acrylamid mix, 30 % AA, 0.8 % BisAA (Rotiphorese Gel 30, Roth, Karlsruhe)	2.65 ml	3 ml	3.3 ml	4 ml	250 µl
Distilled water	4.2 ml	3.8 ml	3.5 ml	2.8 ml	960 µl
3 M Tris, pH 8.8	1 ml				-
1 M Tris, pH 6.8	-				250 µl
60 % sucrose	-				500 µl
10 % SDS	80 µl				20 µl
10 % APS	80 µl				20 µl
TEMED	8 µl				2 µl

5 x SDS sample buffer (pH 6.8)

250 mM Tris

10 % SDS

50 % glycerol

0.05 % Bromphenol blue

100 mM β -mercaptoethanol

10 x SDS running buffer (pH 8.9)

2.5 M glycine

250 mM Tris base

1 % SDS

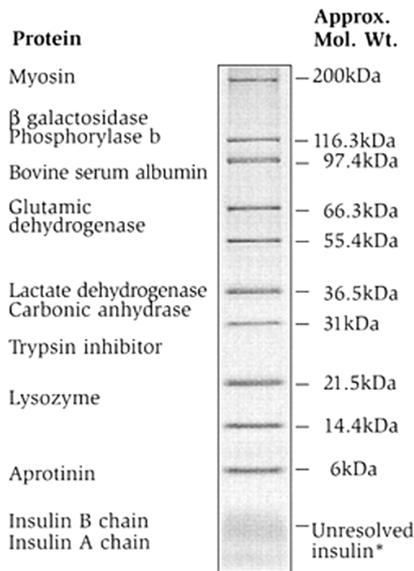


Figure 2.3.10: Mark12

2.3.11 Detection of proteins in immunoblots

Proteins separated by SDS-PAGE were electrically transferred to nitrocellulose membranes (PROTRAN[®] BA 79, Pore size 0.1 μm, Schleicher & Schuell, Dassel, Germany) by a semidry-electroblotter at a constant current of 2.5 mA per cm² gel surface. As a control for blotting efficiencies and for marking marker bands, membranes were stained with Ponceau S (Sigma, Taufkirchen, Germany) and subsequently destained with TBS.

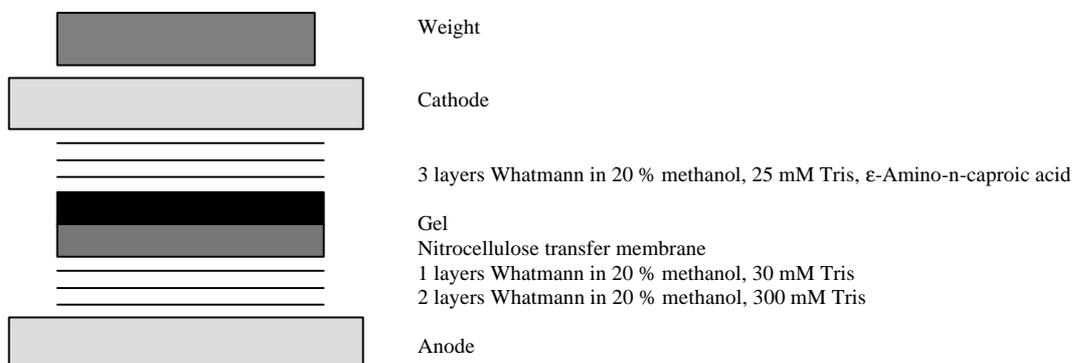


Figure 3.2.11: Semidry-electroblot

To avoid unspecific binding of antibodies, nitrocellulose membranes were incubated with 5 % non-fat dried milk powder in TBS either for 2 hours at room temperature or overnight at 4 °C. Primary antibodies were used in 5 % milk powder in TBS in concentrations listed previously (2.1.5) and were incubated with the membranes either overnight at 4 °C or for 3 hours at room temperature. Membranes were washed with TBS containing 0.1 % Tween20 three times for 10 minutes, before incubation with horse radish peroxidase coupled secondary antibodies in 5 % milk powder in TBS for 60 minutes at room temperature. Secondary antibodies were used in concentrations listed previously (2.1.6). After another three washing steps with TBS containing 0.1 % Tween20, the membranes were subjected to development with the ECL-System Lumi-Light^{PLUS} (Roche, Mannheim, Germany). Horse radish peroxidase coupled to the secondary antibodies oxidised the substrate Luminol in the presence of hydrogen peroxide. With the emitted light, Kodak X-OMAT X-ray films were exposed for 30 seconds to 24 hours depending on the intensity of the reaction. Fixing and developing of the films was performed with Kodak X-ray Developer LX 24 and Kodak X-ray Fixer AL4 (Eastman Kodak, Rochester, NY, USA).

For a new incubation with different antibodies, the membranes were stripped in PBS containing 2 % SDS for 15 minutes at 37 °C and 1 hour at room temperature. After washing with TBS containing 0.1 % Tween20, unspecific binding was blocked with 5 % milk powder.

10 x TBS

1.37 M NaCl

100 mM Tris, pH7.3

2.3.12 Detection of proteins by Coomassie-Brilliant-Blue staining

With Coomassie-Brilliant-Blue staining, proteins in concentrations of 200-400 ng per band are detectable in SDS gels.

The gels were stained for one hour in a staining and fixing solution containing 10 % acetic acid, 25 % isopropanol and 2.5 % Coomassie-Brilliant-Blue. Subsequently, the gels were destained for several hours in 10 % acetic acid and 12.5 % isopropanol until protein bands were clearly visible. All staining steps were performed at room temperature. Stained gels were either stored in water or were wrapped and dried in cellophane foil (Ostmann, Bielefeld, Germany).

2.3.13 Detection of proteins by silver staining

Silver staining is sensitive for protein concentrations of 5 ng per band. Proteins in SDS gels were fixed with 50 % methanol, 12 % acetic acid and 0.02 % formaldehyde for 15 minutes. After three washing steps in 50 % ethanol for 10 minutes each and 1 minute in 0.02 % sodium thiosulfate, the gels were incubated in the staining solution containing 0.2 % silver nitrate and 0.05 % formaldehyde for 15 minutes. During that incubation, silver nitrate accumulated to proteins. After a washing step with water, the silver nitrate was reduced to metallic silver in the following developing step in a solution containing 6 % sodium carbonate, 0.02 % formaldehyde and 0.4×10^{-3} % sodium thiosulfate. The staining reaction was stopped with 50 % methanol and 12 % acetic acid when bands were clearly visible. All staining steps were performed at room temperature. Stained gels were either stored in water or were wrapped and dried in cellophane foil (Ostmann, Bielefeld, Germany).

2.3.14 Preparative blot for protein sequencing

For sequencing, CHL1 fragments were separated by SDS-PAGE. SDS was removed from the gel by washing in blotting buffer. A Porablot-PVDF membrane (Macherey und Nagel, Düren, Germany) was activated in ethanol and equilibrated in blotting buffer for each 1 minute. By semidry-electroblotting (2.3.11), protein fragments were transferred onto the Porablot-PVDF membrane for 60 minutes with a constant current of 2 mA per cm^2 gel surface. Afterwards, the membrane was stained for 5 minutes and subsequently destained. Corresponding CHL1 fragments were cut out of the membrane and subjected to sequencing. Sequencing was kindly performed by Kerstin Bröker from the research group of Prof. Tschesche (Biochemie, Fakultät für Chemie, Universität Bielefeld) with a sequencer of the company Knauer.

Blotting buffer

50 mM H_3BO_3 , pH 9.0

20 % ethanol

1 mM DTT

Staining solution

0.02 % Coomassie-Brilliant blue

45.5 % ethanol

9 % acetic acid

1 mM DTT

Destaining solution

45.5 % ethanol

9 % acetic acid

1 mM DTT

2.3.15 Expression of recombinant ADAM8 in *E.coli*

Cloning of the corresponding construct A8-ProMP in the pASK-3+ vector (IBA, Göttingen, Germany), transformation in the *E.coli* strain BL21(DE3)pLysS (Invitrogen, Groningen, NL) as well as improvement of expression conditions and purification methods was performed by the diploma student Simone Reipschläger.

For large scale purifications of recombinant ADAM8 from the cytoplasm of *E.coli*, 1 l to 1.5 l expression cultures were used. Bacteria were grown in LB-Medium (2.2.1) containing ampicillin (100 µg/ml) and chloramphenicol (34 µg/µl). Expression was induced at OD 0.5 by 0.2 µg/ml anhydrotetracyclin for 6 hours at 30 °C with 50 µM ZnCl₂ added to the medium. Subsequently, media were spun and supernatants were discarded. Pellets were resuspended in 10 ml resuspension buffer containing 150 mM NaCl, 100 mM Tris (pH 8.0) and 1 mM phenylmethylsulfonyl fluoride (PMSF) for inhibition of serine proteases. The suspension was subjected to ultrasonic treatment at medial amplitude three times for 30 seconds. 5 µg/ml DNase I and 10 µg/ml RNaseA were added to the sample to decrease viscosity. After spinning at 50000 x g and filtration with a 0.2 µm filter unit, supernatants were subjected to HPLC in a Strep-Tactin MacroPrep Cartridge (IBA, Göttingen, Germany) (2.3.5). Samples were analysed by immunoblotting (2.3.11) and Coomassie-Brilliant-Blue or silver stained gels (2.3.12, 2.3.13) and were then used for activity assays.

2.3.16 Expression of recombinant ADAM8 in COS1 cells

COS1 cells were transiently transfected with a cDNA encoding the pro and metalloprotease domain of ADAM8 in a SecTag2 B vector according to the DEAE/Dextran method (2.4.3). Cells were maintained in DMEM with 10 % SerEx for 36-48 hours after transfection. Supernatants were collected and subjected to ultrafiltration (2.3.6), ConA (2.3.3) or Nickel-NTA affinity chromatography (2.3.4). Samples were analysed by immunoblotting (2.3.11) and silver stained gels (2.3.13) and were used for activity assays.

2.3.15 Cleavage assays with recombinant proteins

Myelin basic protein (MBP) was used for *in vitro* cleavage to confirm proteolytic activity of purified ADAM8 (Schlomann et al., 2002). 20 µl samples were prepared containing 10 µg recombinant MBP (Sigma, Taufkirchen, Germany), digestion buffer, ADAM8 solution containing 2-5 µg protein and protease inhibitor cocktail. Cleavage assays were performed likewise with 1 µg recombinant L1-Fc, CHL1-Fc and NCAM-Fc. Samples were incubated overnight at 37 °C and cleavage products of MBP were analysed by 15 % SDS-PAGE and cleavage products of L1-Fc, CHL1-Fc and NCAM-Fc were analysed by 5 % SDS-PAGE (2.3.10). Gels were subsequently silver stained (2.3.13). For inhibition experiments, ADAM8 was preincubated with inhibitors for 90 minutes at 37 °C. Metalloprotease inhibitors were used as follows: EDTA 10 mM, TIMPs 500 nM, OPT 10 mM, hydroxamates 200 nM.

20 × digestion buffer (pH 7.3)

0.4 M Tris

0.1 M CaCl₂

1 mM ZnCl₂

2 M NaCl

2.3.16 Quantitative analysis of CHL1 cleavage

After cleavage assay with CHL1, ADAM8 and inhibitors where necessary, a quantitative analysis of CHL1 cleavage was performed. The band intensities of uncleaved CHL1-Fc in a silver stained gel were determined for each cleavage condition. These intensities were compared to 1 µg of uncleaved CHL1-Fc protein which intensity was set to 100 % and were given as means. For quantification, Quantiscan software (Bio-Rad, Göttingen, Germany) was used.

2.3.17 Peptide synthesis

The three peptides NH₂-NDLGPRALEI-COOH, NH₂-PRALEIYRAQ-COOH and NH₂-RAQPRNWLIP-COOH were synthesised with an automatic peptide synthesiser by Fmoc/tBu method in a batchwise procedure. In case of peptides NH₂-NDLGPRALEI-COOH and NH₂-PRALEIYRAQ-COOH Wang resin was used, in case of peptide NH₂-RAQPRNWLIP-COOH 2-Chlorotrityl-resin.

The first amino acid – isoleucine in case of peptide 1, glutamine in case of peptide 2 and proline in case of peptide 3 – was coupled manually to the resin with 1 equivalent TBTU and

2 equivalents DIPEA corresponding to the molar amounts of used amino acid. Coupling efficiencies were controlled by decoupling a small aliquot of resin and measurement of Fmoc concentration at wavelength 292 nm. From the result, the amount of amino acids coupled on the resin was extrapolated. In the automated synthesis coupling was performed with a threefold excess of amino acids and TBTU and a sixfold excess of DIPEA based on the coupled amino acids on the resin. Cleavage of Fmoc groups was obtained by 2 % piperidine and 2 % DBU in DMF. Washing steps were performed with DMF. Permanent side chains and bonds between peptides and resin were cleaved in a solution containing 95 % TFA, 2.5 % TIPS and 2.5 % H₂O. The peptide solution was evaporated to an oily consistency and precipitated twice in Diethylether followed by lyophilisation. Correct synthesis was confirmed by MALDI-TOF MS and reverse phase HPLC.

The peptide synthesis was performed in the study group of Prof. Norbert Sewald (Bioorganische Chemie, Fakultät für Chemie, Universität Bielefeld) with kind aid by the technician Marco Wißbrock.

2.3.18 Design of peptide collection

A number of peptides was designed in order to perform peptide assays with proteolytically active forms of ADAM8 and to find new peptide substrates of ADAM8. Peptides were derived from mouse proteins and included sequences of other metalloprotease peptide substrates as well as sequences from membrane proximal regions of proteins supposed to be released from the cell surface by metalloproteases.

For literature search the database of the National Center of Biotechnology Information (NCBI) (<http://www.ncbi.nlm.nih.gov>) was used. For protein information the NCBI database as well as the Swiss-Prot/TrEMBL protein database (<http://www.expasy.org/sprot/>) was used. For known human metalloprotease peptide substrates, homologous mouse sequences were chosen using the programme ClustalX as alignment tool. If no cleavage site details were available, overlapping peptides covering either juxtamembraneous regions or other proposed cleavage site regions were designed. The overlap with three amino acids guaranteed that all possible cleavage sites were covered. Table 3.3.1 shows the list of designed peptides used in cleavage experiments.

2.3.19 MBP peptide cleavage assay

The peptide Suc-H-(Mcp)-GSLPQKSH-K-(Dpa)-R-amide derived from the MBP cleavage site (Schlomann et al. 2002) was used in *in vitro* cleavage experiments either to confirm proteolytic activity of purified ADAM8 or to analyse protease activities in cells transfected with ADAM8, EQ-ADAM8 or ADAM10. The peptide was N-terminally coupled to the fluorophor Mcp (DL-2-amino-3-(7-methoxycoumaryl)-propionic acid) and C-terminally to the quencher Dpa (L-2-amino-3-(2,4-dinitrophenyl)aminopropionic acid). Fluorescence of the fluorophor was detectable after peptide cleavage, when fluorophor and quencher were separated. The assays were performed with a sample volume of 100 μ l. 100 μ M peptide were mixed with either purified ADAM8 solution containing 2-5 μ g protein, 10-20 μ l ultrafiltrated supernatants, ConA pellets or cell pellets and assay buffer. Fluorescence (excitation: 340 nm, emission: 405 nm) was measured over a time course of 2 hours with a Perkin-Elmer LS50B Luminescence spectrometer in black-coated 96 well plates (Nunc, Wiesbaden, Germany). Fluorescence measurements were performed in the research group of Dr. Olaf Kruse (Molekulare Zellphysiologie, Fakultät für Biologie, Universität Bielefeld).

Assay buffer

20 mM HEPES, pH 7.4

0.06 % CHAPS

2.3.20 Peptide cleavage assays with peptide collection

This assay was based on peptides synthesised on continuous cellulose membranes using the SPOT-synthesis technique. The peptides were synthesised with an amino-benzoic-acid (Abz) fluorescent moiety at the N-terminal end and were punched out as disks into 96 well plates. Peptides were obtained from JPT Peptide Technologies GmbH (Berlin, Germany).

Cellulose disks were cut into equivalent pieces, activated in methanol for 5 minutes and subsequently washed 4 times with assay buffer (2.3.19). For the reactions, disks were incubated at 37 °C with 100 μ l purified ADAM8 solution containing 2-5 μ g protein, protease inhibitor cocktail and assay buffer. In negative controls, 10 mM EDTA was added. As positive controls, some peptides containing either arginine or lysine residues were incubated with trypsin. Fluorescence (excitation: 325 nm, emission: 420 nm) was measured immediately, after 2 and 6 hours and after overnight incubation with a Perkin-Elmer LS50B Luminescence spectrometer in black-coated 96 well plates (Nunc, Wiesbaden, Germany).

2.3.21 Peptide cleavage assays with fluorescamine

This assay method was based on binding of the fluorophor fluorescamine to alpha amino groups of new N-terminal ends from peptide cleavage resulting in increase of fluorescence. Fluorescamine did not specifically bind to the assayed peptide, but to all N-terminal amino groups as well as to epsilon amino groups of lysine residues in a sample. Depending on the purity of the protease sample, large variations could occur because of different background fluorescence. In the work presented here, peptide cleavage assays with A8.1 peptide (NH₂-NDLGPRALEI-COOH) A8.2 peptide (NH₂-PRALEIYRAQ-COOH), A8.3 peptide (NH₂-RAQPRNWLIP-COOH), CD23 peptide (DNP-SHHGDQMAQKSQSTQI-COOH), TNF- α peptide (DNP-SPLAQAVRSSSR-COOH) and CRDA19 peptide (Ac-RPLESNAV-COOH) were performed. The peptides A8.1, A8.2 and A8.3 were derived from the autocatalytic cleavage site of ADAM8 (Schlomann et al., 2002) and were tested as new peptide substrates of ADAM8. According to an established protocol, the CD23 peptide was determined as new substrate of ADAM8 (Diploma thesis, Simone Reipschläger). TNF- α and CRDA19 peptides were known as ADAM17 and ADAM19 substrates respectively (personal communication, M.Moss, Biozyme Inc., NC, USA; Kang et al., 2002). The assay method was established by modifying a protocol from Kang et al. (2002). As the protocol was modified and improved, the latest one is given.

In 100 μ l reaction batches, 100-200 μ M peptide were mixed with either ADAM8 solution containing 2-5 μ g protein, ConA sepharose pellets or cell pellets, protease inhibitor cocktail and HEPES assay buffer. For the brain tumour activity assays, 20 μ l ConA sepharose pellets and 500 μ M CD23 peptide and 1 mM CRDA19, respectively, were used.

Where necessary, inhibitors were added (EDTA 10 mM, TIMP1 40 nM, TIMP3 40 nM, OPT 10 mM, hydroxamates 200 nM). Peptides were added to reaction batches after preincubation of protease with inhibitors which was performed for 90 minutes at 37 °C. Immediately and after 1, 2 and 3 hours incubation time at 37 °C, 25 μ l of the reaction batches were removed and mixed with 70 μ l HEPES assay buffer and 5 μ l 1 % fluorescamine in DMSO. Fluorescence (excitation: 386 nm, emission: 477 nm) was measured with a Perkin-Elmer LS50B Luminescence spectrometer in black-coated 96 well plates (Nunc, Wiesbaden, Germany).

HEPES assay buffer

20 mM HEPES, pH 7.4

0.015 % Brij-35

2.4 Cell culture methods

2.4.1 Working with sterile material

Glass bottles, phosphate buffer and endotoxin-free Milli-Q-water were sterilised by autoclaving before use. Glass pipettes were sterilised for 4 hours at 160 °C. Culture plates, flasks, pipette tips and further plastic ware were obtained as expendable products. Dulbecco's Modified Eagle Medium (DMEM) and additives for media (fetal calf serum (FCS), glutamine, non-essential amino acids (NEAS), Trypsin-EDTA) were obtained as sterile products from PAA Laboratories (Linz, Austria). Further additives like antibiotics were dissolved in Milli-Q-water and were sterilised by filtration with syringe filter units with a pore size of 0.2 µm (Schleicher und Schüll, Dassel, Germany). Cell culture work was performed at a class II cell culture workbench (SterilGuard, Baker, USA).

2.4.2 Cultivation of cell culture cells

COS7 and COS1 cells were cultivated in growth medium containing DMEM, 10 % FCS, NEAS and 2 mM L-glutamine (PAA Laboratories, Linz, Austria) at 37 °C in 7.5 % CO₂-atmosphere. To avoid bacterial contamination, 5 µl of a penicillin-streptomycin stock solution per ml medium were added (0.4 g penicillin and 1.28 mg streptomycin in 20 ml Milli-Q-water). For splitting, seeding and freezing, cells were washed with PBS (2.3.1) and incubated with Trypsin-EDTA for 2 minutes at 37° C. When cells detached from the bottom, tryptic reaction was stopped with 10 volumes growth medium compared to the used Trypsin-EDTA and cells were resuspended by pipetting up and down. For counting cells, a Neubauer counting chamber was used.

For freezing, trypsinised cells were spun and the supernatants were discarded. About 10⁶ cells were resuspended in 1 ml freezing medium containing 50 % fetal calf serum, 45 % DMEM and 5 % DMSO and were filled into cryo tubes. Cells were frozen immediately in a Nalgene Cryo 1 °C Freezing container (Nunc, Wiesbaden, Germany) to -80 °C. Long-term storing was performed in a tank with liquid nitrogen. To avoid osmotic damage caused by cytotoxic DMSO, defrosting of frozen cells was performed quickly. Cryo tubes were incubated in a 37 °C water bath and thawed cell suspension was mixed with 9 ml warm growth medium.

2.4.3 Transient transfections

Transient transfections of COS7 cells were performed with LipofectAMINE™ Plus (Invitrogen, Groningen, NL) and transient transfections of COS1 cells according to the DEAE/Dextran transfection method (Seed and Aruffo, 1987).

For the liposome transfection with LipofectAMINE™ Plus, COS7 cells were seeded one day before transfection to achieve 70 % confluency on target day. On target day, DNA and PLUS reagent were diluted in serum-free medium according to table 2.4.3-1 and were incubated for 15 minutes at room temperature. LipofectAMINE™ diluted in medium was added to the mixture and incubated another 15 minutes. The transfection mixture was dropped onto the cells after exchange of medium by serum-free DMEM. After incubation of 3 hours, the medium was exchanged against serum-containing DMEM.

Plate size	Cells seeded	DNA (step1)	PLUS reagent (step1)	Medium for dilution (step1+2)	Lipofect AMINE reagent (step2)	Transfection medium	Transfection volume
30 mm	1 x 10 ⁵	1 µg	6 µl	100 µl	4 µl	0.8 ml	1 ml
60 mm	5 x 10 ⁵	2 µg	8 µl	250 µl	12 µl	2 ml	2.5 ml
90 mm	1 x 10 ⁶	4 µg	20 µl	750 µl	30 µl	4 ml	6.5 ml
140 mm	3 x 10 ⁶	14 µg	50 µl	1800 µl	75 µl	8 ml	12 ml

Table 2.4.3-1: LipofectAMINE™ Plus transfection

For the DEAE/Dextran transfection, COS1 cells were seeded one day before target day to achieve 80 % confluency for transfection. On target day, cells were washed with ice cold PBS and were subsequently incubated with a transfection mix containing DNA, DMEM + 10 % fetal bovine serum PAA clone (PAA Laboratories, Linz, Austria), 400 µg/ml DEAE Dextran and 100 µM chloroquine according to details in table 2.4.3-2. After 3 hours incubation, the transfection mix was removed and a DMSO-shock was performed. Cells were incubated at room temperature for 2 minutes with PBS containing 10 % DMSO. Afterwards, the PBS/DMSO mix was exchanged by growth medium.

Plate size	Cells seeded	DNA	Transfection mix / PBS/DMSO mix
30 mm	1 x 10 ⁵	1 µg	1 ml
60 mm	5 x 10 ⁵	2 µg	2 ml
90 mm	1 x 10 ⁶	5 µg	5 ml
140 mm	3 x 10 ⁶	20 µg	10 ml

Table 2.4.3-2: DEAE/Dextran transfection

The medium used after transfection depended on further treatment of cells. Usually, growth medium was used. But as fetal calf serum contains an undefined concentration of various proteins, it was not appropriate for the isolation of proteins from supernatants and for stimulation studies. For those cases, medium was either changed to serum-free conditions on the day after transfection or to DMEM with 10 % SerExTM (PAA Laboratories, Linz, Austria) – an additive for serum-free media.

In shedding experiments with CHL1, co-transfected cells were kept under different conditions by either adding activators of protein kinase C or 10 mM β -mercaptoethanol as a reducing agent to media. Media were changed to serum-free conditions the day after transfection and cells were incubated with the phorbol ester 12-O-tetradecanoylphorbol-13-acetate (TPA, 50 and 500 ng/ml) and pervanadate (77 and 770 μ M) for six hours before harvesting the cells. TPA was obtained from Sigma (Taufkirchen, Germany). Pervanadate, a phosphatase inhibitor, had to be prepared by mixing 100 μ l of 50 mM Na₃VO₄ with 30 μ l of 30 % H₂O₂ for 5 minutes at room temperature, followed by adding 520 μ l distilled water to obtain a 7.7 mM pervanadate solution.

2.4.4 Poly-L-Lysine (PLL) coating of coverslips

Coverslips were treated for 30 minutes with 3 M HCl at room temperature and were subsequently washed twice for 10 minutes with distilled water. Coverslips were then treated with acetone for 2 hours at room temperature and washed 5 times with distilled water and twice for 10 minutes with ethanol. All these steps were carried out under gentle shaking in a beaker. The coverslips were then sterilised for 2 hours at 160 °C with the beaker covered with aluminium foil. All further steps were carried out sterile under the workbench. After cooling down to room temperature, coverslips were incubated with sterile 0.01 % PLL (mol wt. 70000-150000, Sigma, Taufkirchen, Germany) for 2 hours at room temperature. Subsequently, they were washed three times with distilled water, dried and incubated under UV light for at least 30 minutes. Until use, they were stored sterile.

2.4.5 Preparation and cultivation of primary cerebellar granule cells

For cerebellum preparation, sterile scissors and forceps were used. 1-4 day old mice were held over petri dishes containing ice cold HBSS, and the head was cut off behind the ears. The head was fixed with a sharp curved forceps. Skin, flesh and ears were cut away from back and middle of the skull. A small scissor was inserted into the spinal cord opening, and the skull was cut low on either side in order to be able to pull back the skull and to expose the brain.

The cerebellum was pinched off with a fine curved forceps and placed into ice cold HBSS. Foreign tissue, blood vessels and membrane surrounding the cerebellum were carefully pinched off under the stereomicroscope. Clean cerebella were put into new petri dishes with chilled HBSS and cut into several pieces.

Cell preparation steps were carried out under sterile conditions under the workbench and centrifugation steps with 100 x g. The cerebella pieces were put into a 15 ml Greiner tube and washed with 5 ml HBSS. After centrifugation for 2 minutes, HBSS was discarded. Each three prepared cerebella were incubated with 1 ml Trypsin/DNase solution for 15 minutes at room temperature. Samples were spun down, Trypsin/DNase solutions were discarded, and cerebella were washed three times with ice cold HBSS. 1 ml DNase solution was added and cerebella were homogenised by pipetting up and down approximately 10 times using a larger rounded Pasteur pipette, then a smaller and then a very small one. Then, 5 ml ice cold HBSS were added. The supernatants with separated cells were filled into a new Greiner tube and were centrifuged for 15 minutes at 4 °C. The supernatants were aspirated with small amounts of solution left. Cells were resuspended in 2 ml X1 medium and the numbers of living cells were determined by trypan blue exclusion method. Subsequently, 1×10^5 living cells were seeded per 10 mm diameter well.

X1 Medium

- BME-Earle medium containing 2.2 mg/ml NaHCO₃ (Biochrom, Berlin, Germany)
- 10 % FCS
- penicillin, streptomycin (see 2.4.2)
- NEAS (see 2.4.2)
- 2 mM L-glutamine
- 0.1 % BSA
- 10 µg/ml insulin
- 4 nM L-thyroxine
- 100 µg/ml holo-transferrin
- 30 nM sodium selenite
- 1 mM sodium pyruvate
- protease inhibitor cocktail

HBSS (pH 7.4)185 mg/l $\text{CaCl}_2 \times 2 \text{H}_2\text{O}$ 97.57 mg/l MgSO_4

8 g/l NaCl

400 mg/l KCl

60 mg/l KH_2PO_4 350 mg/l NaHCO_3 47.88 mg/l Na_2HPO_4

1 g/l D-Glucose

Trypsin/DNase solution (pH 7.8)

HBSS

1 % Trypsin

0.05 % DNase I

DNase solution

HBSS

0.05 % DNase I

2.4.6 Neurite outgrowth assay

Cerebellar granule neurons were prepared from 1-4 day old CD1 mice as described previously (2.4.5). Cells were seeded onto PLL coated coverslips (2.4.4) and were maintained in serum-free medium. To determine neurite outgrowth in the presence of supernatants from transfected COS7 cells, supernatants were applied 12 hours after co-transfection of COS7 cells with CHL1/ADAM constructs and 1-2 hours after seeding the neurons. CHL1-Fc protein was added to the medium as a positive control. 18 hours after addition of either CHL1-Fc protein or supernatants of transfected COS7 cells, neurite outgrowth was determined. To visualise neurite outgrowth, cells were fixed with 4 % glutaraldehyde for 5 minutes and stained with either Richardson's Blue or with anti-NF200 antibody (see 2.1.6). For each experimental setup, neurites of at least 50 cells with processes longer than the cell body diameter were measured using a Zeiss Axiophote microscope with digital equipment.

Neurite outgrowth assays in co-cultures of cerebellar neurons with transfected COS7 cells were performed by plating 5×10^4 COS7 cells in 30 mm plates on PLL coated coverslips. One day after transfection of COS7 cells, freshly prepared cerebellar neurons were added at

densities of 10^5 cells per plate. The co-culture was maintained in serum-free medium. After 24 hours, the number of attached neurons per visual field and neurite lengths were determined as described above. Each neurite outgrowth experiment was performed with cerebella from 3 mice and each condition was determined in triplicates. The experiments were repeated at least 3 (n=9) or 4 (n=12) times as indicated. Data were given as mean values \pm standard error of the mean (S.E.M.).

2.4.7 Cell survival assay

Cerebellar granule neurons were seeded in 96 well plates coated with PLL at densities of 10^5 cells per well. After cell attachment, either serum-free medium or supernatants from transfected COS7 cells were applied. After day 1, 3, and 5, the numbers of surviving cells were counted by Trypan blue exclusion staining. Data are from three independent experiments in triplicates and as mean values \pm standard error of the mean (S.E.M.). A Student *t* test was used to determine the significance and a *p* value < 0.01 was considered as highly significant.

3. Results

3.1 Expression and catalytic activity of recombinant ADAM8

For *in vitro* cleavage assays, a soluble, catalytically active form of ADAM8 was required. Initially, cDNA comprising the pro and the metalloprotease domain (A8-ProMP) was expressed in cell culture, and the secreted protease was isolated from cell culture supernatants. In order to scale up the amount of isolated protease, a respective cDNA construct was expressed in *E.coli*. Cloning of the corresponding cDNA construct as well as improvement of expression conditions and purification methods of ADAM8 from *E.coli* was performed by the diploma student Simone Reipschläger. The determination of proteolytic activity of soluble ADAM8 was performed either by using recombinant MBP or by using specific peptides.

3.1.1 Expression of recombinant, catalytically active ADAM8 in cell culture

For expression of soluble ADAM8 in cell culture, a cDNA comprising the pro and metalloprotease domain of mouse ADAM8 was amplified by polymerase chain reaction with the primers mA8MPSec.s and mA8MPSec.as. The reverse primer contained the sequence encoding a birch pollen profilin (BiPro) tag at the 3'-end for detection in immunoblots with a monoclonal antibody. The resulting PCR product was ligated into the respective cloning sites – EcoRI and XhoI – of the expression vector pSecTag2 B. This vector expressed ADAM8 as a fusion protein with a Sec-tag at the N-terminus and a His-tag at the C-terminus. The Sec-tag encoded for a secretion signal for secretion of expressed proteins into the extracellular space. The His-tag encoded 6 histidine residues required for purification with Nickel-NTA. In two clones sequenced, a point mutation afar from the consensus sequence caused an exchange from serine to glycine. From later analysis, it was inferred that this mutation did not affect catalytic activity.

Expression of A8-ProMP was analysed by immunoblotting using a monoclonal antibody against the BiPro-tag cloned to the 3'-end of the cDNA construct. Initially, COS7 cells were transfected with LipofectAMINETM. Supernatants were concentrated by ultrafiltration and applied to SDS-PAGE and immunoblotting. A8-ProMP was detectable as two bands – a 60 kDa fragment corresponding to the entire A8-ProMP, and a fragment with 35-40 kDa corresponding to the metalloprotease domain after prodomain removal. However, in many purification experiments only the smaller band was detectable indicating activation of ADAM8 by prodomain removal (Figure 3.1.1A).

As a control for proteolytic activity, cleavage assays with MBP were performed. Recombinant MBP was incubated overnight with protease solution in the presence or absence of metalloprotease inhibitors. Cleavage was analysed by SDS-PAGE using 15 % gels which were subsequently silver stained. The 20 kDa protein was cleaved by ADAM8 to several smaller fragments, and cleavage was inhibited in the presence of the metalloprotease inhibitors EDTA and 1,10 *ortho*-phenanthroline (OPT) or the hydroxamate inhibitor batimastat (BB-94) (Schlomann et al., 2002), Figure 3.1.1B, Figure 3.2.1B). A more sensitive method for detection of catalytic activity was a peptide cleavage assay with a peptide derived from the MBP cleavage site (Schlomann et al., 2002). This peptide Suc-H-(Mcp)-GSLPQKSH-K-(Dpa)-R-amide had the fluorophor Mcp bound to the N-terminal end and the quencher Dpa to a lysine residue at the C-terminal end. After cleavage, the fluorescence of the fluorophor was detectable. For the assay, peptide and protease solution were incubated in the presence or absence of metalloprotease inhibitors. Fluorescence increase was monitored over a time course of 2 hours (Figure 3.1.1C). The MBP peptide was obtained from a collaborating group, synthesised by an organic chemist, who is now retired. After we ran out of the peptide, alternative peptides had to be examined, as a Dpa modified lysine residue was not commercially available. Different proposed ADAM peptide substrates were tested according to the fluorescamine method to get alternative peptides for determination of catalytic activities (see 3.1.3).

When only small amounts of recombinant, catalytically active ADAM8 were needed, transfection of cells in small scales and concentration of catalytic activities by ultrafiltration was sufficient. For systematic *in vitro* assays, larger amounts of purified, catalytically active ADAM8 were required. In order to scale up A8-ProMP expression, different cells and transfection methods were used. Initially, cells were transfected with the green fluorescent protein (GFP) encoding the expression vector pGFP-N3. Transfection efficiencies were determined by estimating the ratio of GFP containing cells and the total number of transfected cells using a fluorescence microscope. Finally, COS1 cells were transfected according to the DEAE/Dextran transfection method with an efficiency of about 60 %. Liposome transfections with LipofectAMINETM were even more effective, but were not performed due to the high price of the transfection reagent. HEK293T cells constitutively expressing another soluble ADAM8 construct had seemed to express less protease in comparison to transiently transfected cells. For that reason, a stable cell line constitutively expressing the A8-ProMP cDNA construct in pSecTag2 B vector was not generated but transient transfections were performed. For purification of A8-ProMP from cell culture supernatants either

chromatography with ConA sepharose or Nickel-NTA was performed (Figure 3.1.1A). Instead of 60 mm cell culture plates, several 140 mm plates were used for transfection in order to get larger volumes of A8-ProMP containing supernatants.

The lectin concanavalin A (ConA) is a metalloprotein used for separation and purification of glycoproteins. As A8-ProMP contained glycosylation sites, it was purified with conA sepharose. A8-ProMP bound tightly to ConA sepharose and was not completely eluted from the matrix under native conditions at concentrations of α -D-methylmannoside up to 0.4 M. Therefore, in cleavage assays ConA pellets with bound protease were used instead of eluted protease solution (see also 3.4).

A8-ProMP was expressed as a fusion protein with a His-tag consisting of 6 histidine residues at the C-terminal end. For enrichment of proteins with a His-tag, chromatography with Nickel-NTA was performed under native conditions in a batchwise procedure. The chelate nitrilotriacetic acid (NTA) was bound to a matrix and charged with nickel for specific binding of histidine containing proteins. After elution from the matrix with an imidazole containing buffer, the A8-ProMP containing solution was dialysed against a HEPES buffer and concentrated by ultrafiltration depending on the elution volume.

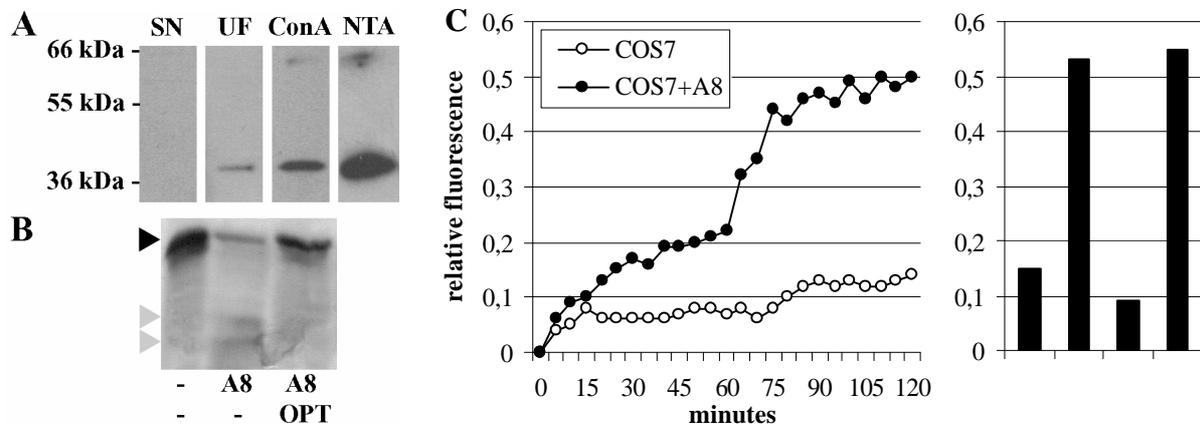


Figure 3.1.1: Expression and catalytic activity of soluble ADAM8 in cell culture. **A** COS1 cells were transfected with cDNA encoding A8-ProMP. 20 μ l of untreated (SN), ultrafiltrated (UF, 10 x concentrated) and purified supernatants (\sim 50 x concentrated) either with ConA sepharose (ConA) or Nickel-NTA (NTA) were subjected to 7.5 % SDS-PAGE and immunodetected with an anti-BiPro antibody. A8-ProMP was expressed in low concentrations, and was not detectable in untreated (SN) and only the processed form in 10 x concentrated supernatants (UF). The proform and the processed form were detected in affinity purified, 50 x concentrated supernatants (ConA, NTA) with most of the protease present in its activated form. **B** MBP protein was incubated with A8-ProMP purified with Nickel-NTA. MBP was cleaved to fragments of 9 and 11 kDa, whereas cleavage was inhibited by OPT. **C** A fluorogenic peptide derived from the MBP cleavage site was incubated with ultrafiltrated supernatants of cells transfected with A8-ProMP (COS7+A8) and of untransfected COS7 cells (COS7). Fluorescence increase which corresponded to peptide cleavage was monitored over a time course of two hours. ADAM8 from supernatants of transfected cells significantly cleaved the peptide (left panel). In an equivalent cleavage assay, inhibitors were added to the samples. The right panel shows fluorescence increase after two hours. Supernatants from cells transfected with A8-ProMP cleaved the MBP-peptide, whereas OPT inhibited cleavage. In accordance with published data, TIMP1 did not inhibit ADAM8 activity.

In figure 3.1.1, an example for A8-ProMP isolation from cell culture supernatants and for determination of ADAM8 catalytic activity is shown. Supernatants of COS1 cells transiently transfected with cDNA encoding A8-ProMP were subjected to ultrafiltration, to purification with ConA sepharose or Nickel-NTA and analysed in immunoblots with an anti-BiPro antibody. No specific ADAM8 bands were detected in corresponding silver stained gels indicating low amounts of ADAM8. It was not possible to scale up ADAM8 expression significantly by cloning a new cDNA construct encoding a Sec-tag for additional secretion of expressed protein or by improving the cell system and transfection method. Isolation of larger amounts of protease was only possible by increasing the number of transfected cells.

3.1.2 Expression of ADAM8 in *E.coli*

Parallel to expression in cell culture, ADAM8 was expressed in bacteria in order to scale up protease expression. cDNA encoding for A8-ProMP was amplified by polymerase chain reaction with primers containing cleavage sites for the endonucleases EcoRI and XhoI. The reverse primer contained the sequence encoding the BiPro-tag for detection in immunoblots. The resulting PCR product was ligated into respective cloning sites of a pASK-3+ vector. This vector contained a tetA promoter for induction of protein expression with anhydrotetracyclin. A8-ProMP was expressed as a fusion protein with a Strep-tag at the C-terminal end for purification by affinity chromatography. The vector was transformed into the *E.coli* strain BL21(DE3)pLysS. Bacteria were grown at 30 °C and induced at OD 0.5 with 0.2 µg/ml anhydrotetracyclin. Six hours after induction, cells were harvested. A8-ProMP was extracted from the cytoplasm of bacteria and purified with the Strep-tag[®]/Strep-Tactin[®] system. Samples were analysed by immunoblotting and Coomassie-Brilliant-Blue stained SDS gels (Diploma thesis, Simone Reipschläger).

In agreement with the results from cell culture, A8-ProMP was present in its proform and in the processed form indicating that autocatalytic prodomain removal occurs also in bacteria. The processed form detected with ~35 kDa was smaller than that isolated from cell culture, probably because of slightly different length of cloned cDNA constructs and because of glycosylation of the metalloprotease domain from cell culture. Catalytic activity of the isolated A8-ProMP was determined by cleavage of MBP or ADAM8 specific peptides according to the fluorescamine method (3.1.3). According to published data for ADAM8 from cell culture (Amour et al., 2002, Schlomann et al., 2002), MBP cleavage by A8-ProMP from *E.coli* was inhibited by EDTA, OPT and BB-94 (Figure 3.1.2B). However, cleavage was not inhibited by the hydroxamate inhibitor marimastat (BB-5216). Although A8-ProMP

expression in *E.coli* was increased upon induction with anhydrotetracyclin, the amount of isolated protein was still low with a maximal yield of 800 µg per litre bacterial culture.

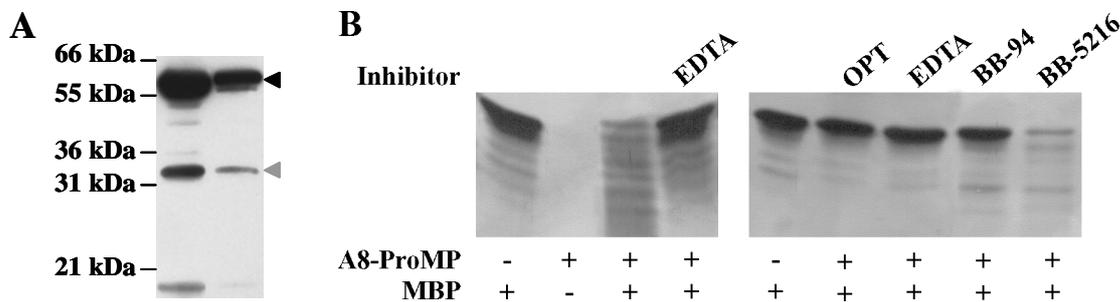


Figure 3.1.2: Expression and catalytic activity of soluble ADAM8 in *E.coli*. **A.** Different elution fractions from purified A8-ProMP from *E.coli* were analysed in immunoblots with an antibody detecting the BiPro-tag at the C-terminal end. The proform and the processed form were detected with 60 kDa (black arrow) and 35 kDa (grey arrow) indicating autocatalytic prodomain removal in *E.coli*. The function of an additional fragment detected with ~ 20 kDa was unknown. **B.** MBP was incubated with soluble A8-ProMP isolated from bacteria and purified with the Strep-tag[®]/Strep-Tactin[®] system in the presence and absence of inhibitors. Cleavage of MBP by A8-ProMP was inhibited in the presence of OPT, EDTA, BB-94, but not by BB-5216.

3.1.3 Determination of catalytic activity using peptides and fluorescamine

In order to have a peptide substrate in addition to the quenched peptide derived from MBP for the determination of ADAM8 catalytic activity, a peptide cleavage assay with fluorescamine was established and several peptides were examined as ADAM8 peptide substrates.

Schlomann et al. (2002) found that the ADAM8 prodomain is removed by autocatalysis. Within the hinge region between the prodomain and the metalloprotease domain, three different autocatalytic cleavage sites were determined in a sequence comprising 13 amino acids (Figure 3.1.3A). Three 10mer peptides each containing one of these cleavage sites in the central sequence were synthesised. The peptides NH₂-NDLGPRALEI-COOH (A8.1), NH₂-PRALEIYRAQ-COOH (A8.2) and NH₂-RAQPRNWLIP-COOH (A8.3) were synthesised with an automatic peptide synthesiser by Fmoc/tBu strategy in a batchwise procedure. In case of peptides A8.1 and A8.2 Wang resin was used and in case of peptide A8.3 2-Chlorotrityl-resin. Correct synthesis was confirmed by MALDI-TOF MS and reverse phase HPLC. The cleavage assay method with the fluorophor fluorescamine is based on binding of the fluorophor to alpha amino groups of new N-terminal ends resulting from peptide cleavage. Disadvantage of this assay method is low selectivity, as it binds to all N-terminal amino groups as well as to epsilon amino groups of lysine residues in a sample. Depending on the purity of the protease solution, large background fluorescence can be detected which can mask fluorescence increase from peptide cleavage.

As the synthesised peptides had free N-terminal ends, they were expected to bind fluorescamine. Therefore, buffer conditions and fluorescamine concentrations as well as peptide concentrations were tested with these peptides alone and an initial protocol for the cleavage assay was set up. Initially, 150 μ l reaction batches containing protease solution, ConA or cell pellets, 50-100 μ M peptide and a buffer containing protease inhibitor cocktail, 50 mM HEPES (pH 7.4), 0.2 M NaCl, 0.01 M CaCl₂ and 0.01 % Brij-35 were mixed. Immediately, and after 1 and 2 hours, 25 μ l were mixed with 5 μ l 1 % fluorescamine and 70 μ l buffer. 50 μ l of this mixture were mixed with 150 μ l buffer, and fluorescence was measured in black-coated 96 well plates. Later on, the protocol for the cleavage assay was modified by leaving out the second dilution step and decreasing the sample volume to 100 μ l. For 100 μ l samples, protease solution, ConA or cell pellets were mixed with 100-200 μ M peptide, protease inhibitor cocktail and a buffer only containing 20 mM HEPES and 0.015 % Brij-35. Immediately, after 1, 2 and 3 hours, 25 μ l of the sample were mixed with 5 μ l 1 % fluorescamine and 70 μ l HEPES buffer, and fluorescence was directly measured. According to this protocol, soluble peptides derived from CD23, IL-6R, L-Selectin, TGF- α , TNF- α and TNF-R1 were tested as ADAM8 substrates (Diploma thesis, Simone Reipschläger). For both protocols, assays were performed several times because of the sensitivity of the test method resulting in large variations of fluorescence values.

For the three peptides derived from the ADAM8 autocatalytic cleavage site, approximately linear increase of fluorescence with increase of available N-terminal amino groups was expected. Indeed, for peptides A8.1 and A8.3, a constant increase of fluorescence with increasing peptide concentrations was observed, whereas fluorescence did not increase in case of peptide A8.2. Probably, fluorescamine could only bind to free amino groups but not to secondary amino groups such as the proline residue at the N-terminal end in peptide A8.2 (Figure 3.1.3C). The peptides cleavage assays were performed several times with recombinant ADAM8 from cell culture. Protease and peptides were incubated in the presence or absence of the metalloprotease inhibitor EDTA (10 mM). Even if the absolute fluorescence values differed between several cleavage assays, tendencies were similar. The peptides A8.1 and A8.2 were significantly cleaved, whereas peptide A8.3 was not (Figure 3.1.3D). Although two peptide substrates were determined, for determination of catalytic activity, a peptide derived from CD23 was preferred as it seemed to be cleaved with higher specificity and as it had a modified N-terminal amino group.

In order to design peptides with cleavage sites located in the centre, peptides A8.1 and A8.2 contained two cleavage sites: a “main” one in the central sequence and a second one either at

the N-terminal side (A8.2) or at the C-terminal side (A8.1) (Figure 3.1.3B). As both peptides were cleaved, it was not clear if the two different “main” cleavage sites in the centres were cleaved or if in both peptides one cleavage site was preferred independent from the position. Thus, it is not possible to draw a conclusion about a preferred autocatalytic cleavage site.

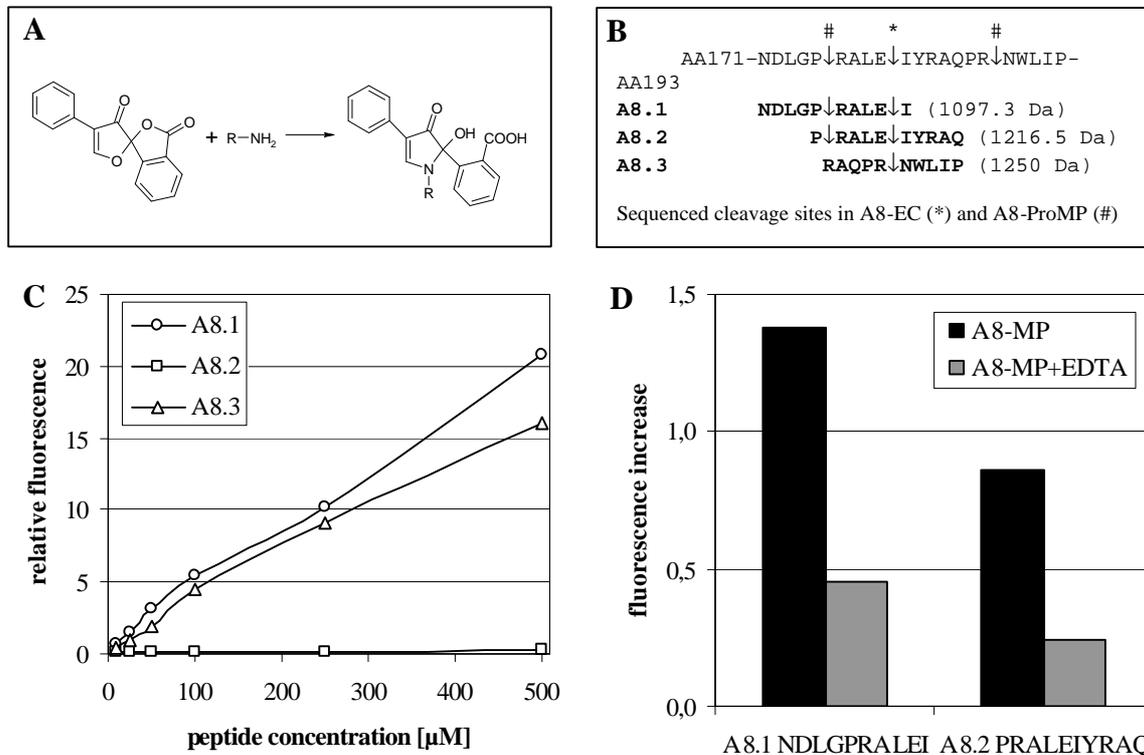


Figure 3.1.3: Determination of catalytic activity. **A.** The fluorophor fluorescamine binding to N-terminal alpha amino groups and to epsilon amino groups of lysine residues was used for determination of ADAM catalytic activities. **B.** Three peptides derived from different autocatalytic cleavage sites of ADAM8 were synthesised and examined as new ADAM8 peptide substrates. **C.** Different peptide concentrations were mixed with fluorescamine and fluorescence was measured. In case of peptides A8.1 and A8.3, fluorescence increased with increasing peptide concentrations as a result of increased number of available N-terminal amino groups. Fluorescamine did not bind to the secondary amino group of proline in peptide A8.2. **D.** In peptide cleavage assays, the peptides A8.1 and A8.2 were cleaved. In all experiments, no fluorescence increase was detected for peptide A8.3. As in most cases fluorescence even decreased with time, results for A8.3 are not included into the figure. For A8.1 and A8.2, average values from two measurements were taken into account.

3.2 Detection of CHL1 as a physiological substrate of ADAM8

The neuronal cell adhesion molecule Close Homologue of L1 (CHL1) was found as an ADAM8 substrate in an *in vitro* cleavage assay with recombinant proteins. On the basis of this result, the protease-substrate relationship between ADAM8 and CHL1 was examined for its physiological relevance in further *in vivo* experiments. The results of these experiments are published in the Journal of Biological Chemistry (Naus et al., 2004). In the following chapter, I summarised the results. Some figures are equal to those in the publication, others were slightly modified.

3.2.1 Cleavage of recombinant CHL1 by ADAM8

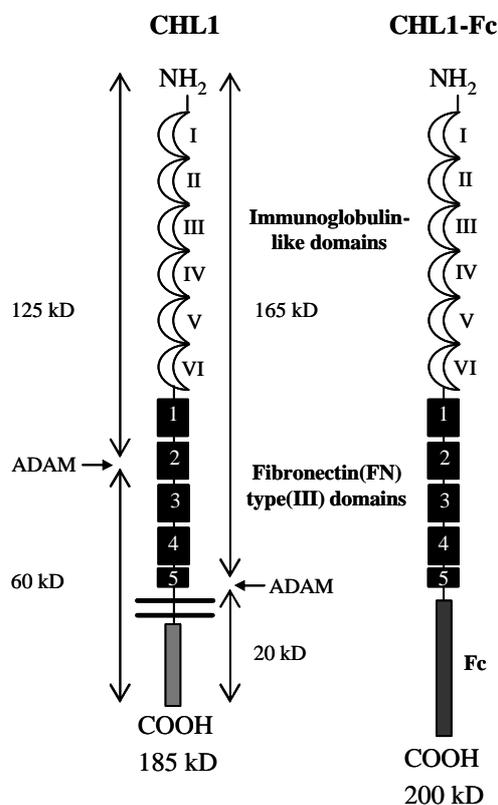


Figure 3.2.1-1: Structure of CHL1. CHL1 has five fibronectin type III (FNIII) domains (black boxes) of which the fifth is only partially conserved and six immunoglobulin-like domains (roman numerals). Full length membrane-bound CHL1 (left) has a molecular mass of 185 kDa and the fusion protein CHL1-Fc a mass of 200 kDa. Two cleavage sites within the FNIII domains resulting in fragments of 125 kDa and 165 kDa were determined in this work (black arrows). From: Naus et al., 2004.

In *in vitro* cleavage assays, the extracellular domains of three different neuronal cell adhesion molecules, NCAM, L1 and CHL1 (Figure 3.2.1-1), were incubated with recombinant, catalytically active ADAM8. The extracellular domains of these molecules were fused to immunoglobulin G Fc-tags for detection with a monoclonal antibody in immunoblots. After incubation, samples were analysed in silver stained gels and in immunoblots. The silver stained gels showed that the CHL1-Fc protein was cleaved into two products in the presence of ADAM8 – a prominent 165 kDa fragment corresponding to the entire extracellular portion of CHL1 and a less prominent of 125 kDa, whereas NCAM and L1 were not cleaved (Figure 3.2.1-2A). The CHL1 cleavage was confirmed in immunoblots using an antibody against the extracellular portion of CHL1. Non-cleaved CHL-Fc protein was detectable with a size of about 200 kDa. For the cleaved CHL1-Fc protein, the fragments with 125 kDa and 165 kDa were detected together with additional bands of 40 kDa and 80 kDa.

These smaller fragments corresponded to the larger cleavage fragments of CHL1-Fc containing the residual Fc-portion (Figure 3.2.1-2B). The processing of A8-ProMP into active protease by autocatalytic prodomain removal was confirmed by immunostaining with an antibody against the BiPro-tag at the C-terminal end. In the immunoblot, two forms of ADAM8 corresponding to proform and processed form were detected in approximately equal amounts (Figure 3.2.1-2C).

Extensive cleavage of CHL1-Fc protein by soluble ADAM8 was inhibited by broad-range inhibitors of metalloproteases, BB-94 and OPT, but not by excess concentrations of tissue inhibitors of metalloproteases (TIMP) 1, 2, 3, and 4, as demonstrated by a silver stained gel and by quantitative analysis of the stained gels (Figure 3.2.1-2D). For the quantitative analysis, the band intensities of uncleaved CHL1-Fc were determined for each cleavage condition. These intensities were compared to 1 μ g of uncleaved CHL1-Fc protein which intensity was set to 100 % and were given as means. The observed inhibition profile was in accordance with previous cleavage studies on ADAM8 (Amour et al., 2002; Schlomann et al., 2002).

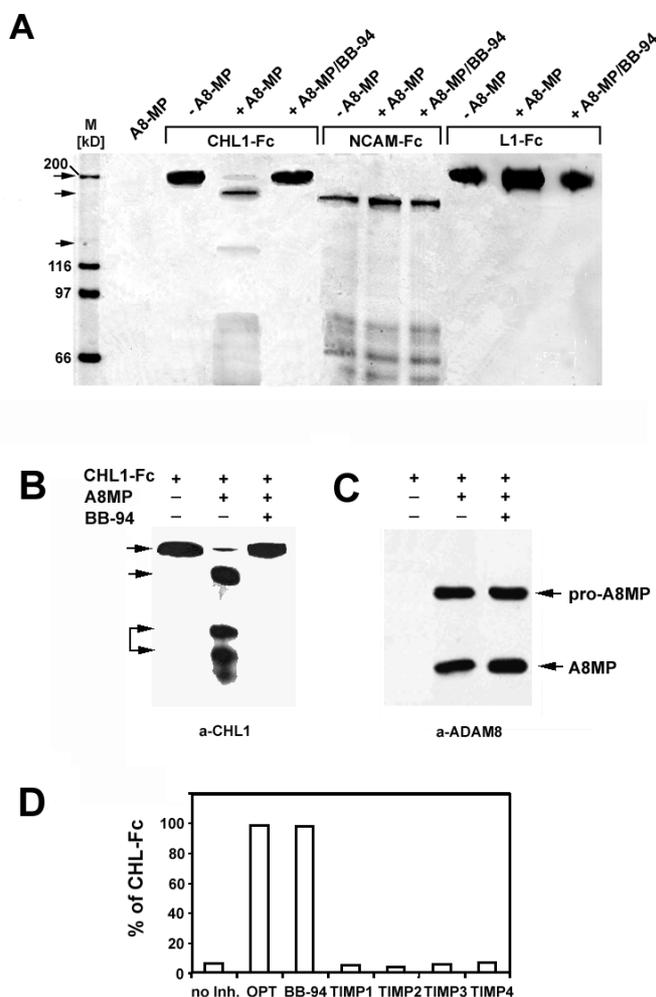


Figure 3.2.1-2: Cleavage analysis of CHL1-Fc, NCAM-Fc and L1-Fc.

A. The silver stained gel shows that CHL1-Fc was cleaved into fragments of 165 kDa and 125 kDa in the presence of ADAM8 (+A8-MP), whereas NCAM-Fc and L1-Fc were not cleaved. Cleavage of CHL1-Fc was inhibited in the presence of the inhibitor BB-94 (+A8-MP/BB-94).

B. In the corresponding immunoblot, complementary smaller fragments were detected in addition to the 165 kDa and 125 kDa fragments. The double arrow indicates the 125 kDa and the corresponding ~80 kDa fragment. **C.** The proform (Pro-A8) and the processed form of ADAM8 (A8-MP) isolated from supernatants of transfected cells were detected in immunoblots. **D.** Quantification of CHL1-Fc cleavage in the presence of inhibitors. From: Naus et al., 2004

The smaller cleavage fragments of CHL1-Fc with about 40 kDa and 80 kDa were subjected to amino terminal sequencing. For the 165 kD fragment, the cleavage site was determined at LVP↓GAEHIV located within the fifth FN III-like domain at amino acid position 1040. For the 125 kD fragment, the cleavage site within the second FN III-type domain was determined at amino acid position 753 in the sequence WKP↓QGAPE.

3.2.2 Cleavage of CHL1 by ADAM8 in cell culture

The ectodomain shedding of CHL1 was further analysed in cell culture experiments. CHL1 was co-transfected into COS7 cells either with wild type ADAM8 or the catalytically inactive EQ-ADAM8, in which the glutamate residue Glu₃₃₀ was exchanged by a glutamine (Schlomann et al., 2002). In addition, ADAM10 and ADAM17 were co-transfected with CHL1 in order to examine the specificity of CHL1 cleavage. Cell lysates as well as cell supernatants of transfected cells were analysed in immunoblots.

In advance, peptide cleavage assays with cells transiently transfected with cDNAs encoding full length ADAM8, EQ-ADAM8, ADAM10 and ADAM17 were performed in order to determine catalytic activities of the expressed proteases (Figure 3.2.2A). In cell lysates, protease activities were determined for ADAM8 and ADAM10, but not for mock transfected cells, EQ-ADAM8 and ADAM17 using a MBP derived peptide substrate. A TNF- α peptide substrate was used to monitor ADAM17 activity. This peptide was significantly cleaved by ADAM17 but not by cell lysates of mock transfected cells. These data indicated that the mouse cDNAs used to express ADAM8, ADAM10 and ADAM17 encoded functional proteases in COS7 cells.

In immunoblots, the full length CHL1 transmembrane protein was detectable as 185 kD band, slightly more in cells transfected with EQ-ADAM8 than in cells transfected with ADAM8 with an antibody against the extracellular portion of CHL1. Co-transfection of ADAM8 significantly increased the amount of soluble 165 kD and 125 kD fragments of CHL1 in supernatants of cells transfected with ADAM8, but not in supernatants of cells transfected with EQ-ADAM8, ADAM10 and ADAM17 (Figure 3.2.2B, upper panels). It was concluded from that experiment, that ADAM8 expression in CHL1 transfected COS7 cells resulted in significantly higher amounts of soluble CHL1 compared to the basal CHL1 shedding and that CHL1 cleavage was specific for ADAM8 as demonstrated by co-transfection experiments with cDNAs for CHL1 and ADAM10 or CHL1 and ADAM17.

In addition, the supernatants of ADAM8/CHL1 co-transfected cells were analysed after high speed spin (spin) and under non-reducing conditions without mercaptoethanol (nr). Under

both conditions the 125 kDa band as well as the corresponding membrane stub fragment with ~60 kDa was not detectable in the immunoblot (Figure 3.2.2B, upper panels). To analyse the dependence of ADAM8 mediated shedding on activators of protein kinase C, well described for ADAM17, the co-transfection experiments were performed in the presence of phorbol ester TPA and pervanadate, a phosphatase inhibitor. No significant effect on CHL1 shedding was observed, even when applied in high concentrations (Figure 3.2.2C).

The presence of ADAM8, EQ-ADAM8, ADAM10 and ADAM17 in the co-transfected cells was confirmed by immunoblot analysis using an anti-BiPro antibody or a polyclonal anti-ADAM10 antibody. For ADAM8 and ADAM17, two forms corresponding to proforms and catalytically active processed forms were detected, whereas for ADAM10 mainly the processed form was detectable. EQ-ADAM8 was present as catalytically inactive proform. Equal loading was confirmed by detection of the house-keeping protein GAPDH (Figure 3.2.2B, lower panels).

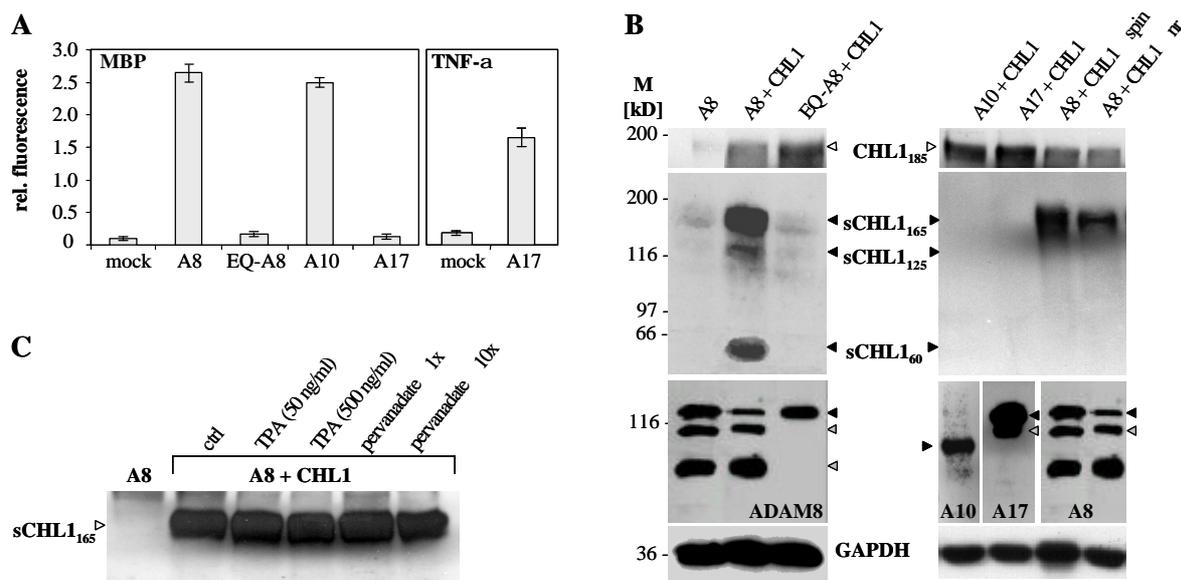


Figure 3.2.2: Cleavage of CHL1 by ADAM8 in COS7 cells. **A.** Peptide cleavage assays were performed with cells transfected with different full length ADAM cDNAs. Catalytic activities were determined for ADAM8 and ADAM10 with a MBP derived peptide and for ADAM17 with a TNF- α derived peptide. **B.** CHL1 was co-transfected with different ADAM constructs, and shedding was analysed in immunoblots. The 165 kDa, the 125 kDa and a complementary 60 kDa fragment were detected in supernatants of CHL1/ADAM8 co-transfected cells. After high spin and under non-reducing conditions, the 125 kDa fragment and the membrane stub fragment were absent. Co-transfections with EQ-ADAM8, ADAM10 or ADAM17 did not result in CHL1 cleavage. The upper panel shows a short exposure of the blot with the 185 kDa form, whereas the panel beneath shows the smaller forms after longer exposure. ADAM proteases were detected in immunoblots. Black arrowheads indicate proforms and grey arrowheads processed forms of ADAM proteins. Equal loading was confirmed with detection of GAPDH. **C.** In cells co-transfected with CHL1/ADAM8, no significant effect of shedding was observed when activators TPA and pervanadate were applied. From: Naus et al., 2004

3.2.3 CHL1 shedding in ADAM8 overexpressing WR and in ADAM8 KO mice

The correlation between ADAM8 expression and CHL1 shedding was further analysed *in situ*. Proteolytic processing of CHL1 in brain extracts from 30 day Wobbler (WR) mutant mice, and from mice deficient in ADAM8 was compared to wild type litter mates (Figure 3.2.3). Brain homogenates prepared from cerebellum and brain stem were subjected to SDS-PAGE and immunoblotting. For ADAM8 detection with an antibody against the cytoplasmic domain, ConA preparations were used. CHL1 was detected with an antibody against the extracellular domain.

In cerebellum/brain stem from wild type mice, ADAM8 was present in its catalytically active form, and CHL1 processing into the 125 kD and 165 kD fragments was observed. In cerebellum/brain stem from Wobbler mice, an upregulation of ADAM8 was demonstrated as a consequence of neurodegeneration and astrogliosis (Schlomann et al., 2002; Figure 3.2.3A). Consistently, an increased proteolytic processing of the 185 kD form of CHL1 into both soluble fragments was detected (Figure 3.2.3A). In homozygous ADAM8 deficient mice, the ADAM8 protein was not detectable. Processing of CHL1 in brain extracts of these mice was dramatically reduced, emphasising a significant contribution of ADAM8 to the proteolytic processing of CHL1 in this particular brain region *in situ* (Figure 3.2.3B). Equal loading was confirmed by detection of the house-keeping protein GAPDH.

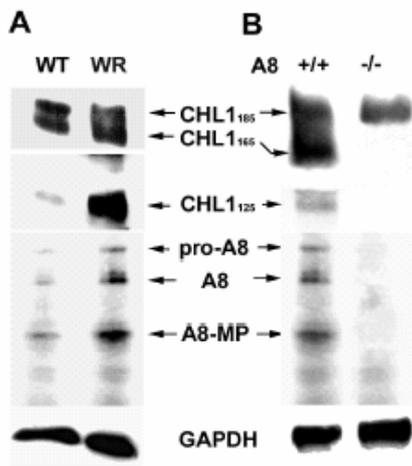


Figure 3.2.3: Cleavage of CHL1 in WR mutant mice (A) and ADAM8 deficient mice (B). **A.** In brain homogenates from WR mice, ADAM8 expression as well as CHL1 cleavage was increased in comparison to wild type mice brain homogenates. The upper panel shows a short exposure of the blot with 185 kDa and 165 kDa forms of CHL1, whereas the panel beneath shows the 125 kDa form after longer exposure of the blot. **B.** In brain homogenates of ADAM8 deficient mice, CHL1 cleavage was significantly reduced in comparison to that in wildtype litter mates. From: Naus et al., 2004

3.2.4 Effect of CHL1 shedding on neurite outgrowth in neuronal cell cultures

To analyse the physiological function of soluble CHL1 generated by ADAM8-dependent ectodomain shedding, neurite outgrowth and cell survival assays with mouse cerebellar neurons in the presence of conditioned media from CHL1 and ADAM8 co-transfected COS7 cells were performed. As described previously, recombinant L1-Fc or CHL1-Fc proteins were able to stimulate neurite outgrowth from cerebellar or hippocampal neurons (Chen et al., 1999).

COS7 cells were transfected with ADAM protease, CHL1 or both and maintained in serum-free medium for 24 hours. Protease expression was determined in lysates by immunostaining as shown in 3.2.2. The COS7 supernatants were applied to cerebellar neurons and 18 hours later, neurite outgrowth was quantified by determination of neurite length of at least 50 individual neurons after staining with Richardson's blue. Supernatants from ADAM8 or CHL1 transfected cells caused a basal level of neurite outgrowth ($20 \mu\text{m} \pm 1.6$ for ADAM8, $22 \mu\text{m} \pm 1.9$ for CHL1). In contrast, supernatants from cells co-transfected with ADAM8 and CHL1 induced significantly increased neurite outgrowth up to $43 \mu\text{m} \pm 4$. A comparable rate to the basal level of neurite outgrowth was achieved with EQ-ADAM8/CHL1 ($21 \mu\text{m} \pm 2$), ADAM10/CHL1 ($19.5 \mu\text{m} \pm 2.5$) or ADAM17/CHL1 ($21 \mu\text{m} \pm 3$). In corresponding micrographs of cerebellar granule neurons stained with neurofilament NF200 antibody, increased neurite outgrowth caused by supernatants from ADAM8/CHL1 co-transfected cells was apparent. Supernatants were immunostained with anti-CHL1 antibody to detect the soluble 165 kDa form of CHL1. This form was mainly present in supernatants from cells co-transfected with CHL1 and ADAM8.

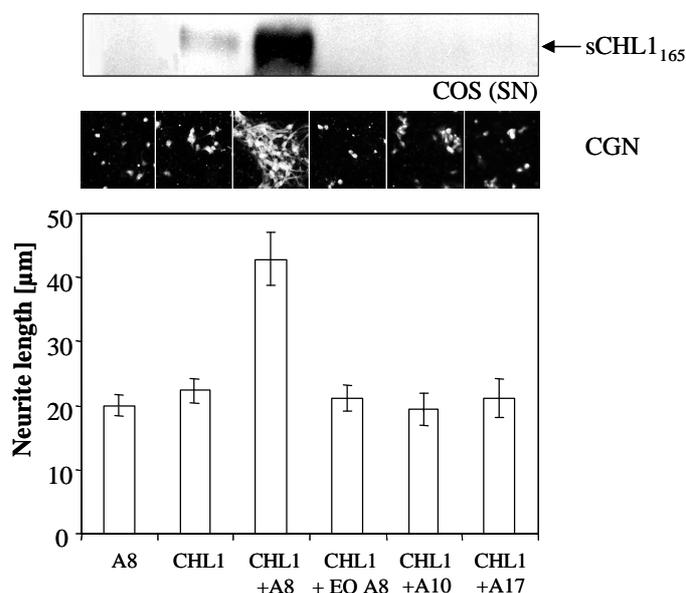


Figure 3.2.4: Effect of supernatants from transfected COS7 cells on neurite outgrowth. Cerebellar granule cells were treated with supernatants of COS7 transfected with CHL1 and/or ADAM protease as indicated. Neurite outgrowth was strikingly increased when neurons were applied with supernatants from cells transfected with CHL1 and ADAM8. In these supernatants, the concentration of the soluble 165 kDa form of CHL1 was increased. From: Naus et al., 2004

3.2.5 Enhancement of neurite outgrowth by substrate-coated soluble CHL1

In order to determine whether CHL1 proteolysis enhances neurite outgrowth when offered as a substrate, co-culture experiments were performed. COS7 cells were transfected with full length ADAM protease, CHL1 or both. One day after transfection, freshly prepared cerebellar granule neurons were plated on top of the cells. To one co-culture co-transfected with ADAM8 and CHL1, BB-94 (200 nM) was repeatedly added to inhibit catalytic activity of ADAM8. After 24 hours, the number of substrate-attached cells with neurite length longer than their diameter was determined in at least 50 randomly chosen visual fields by phase-contrast microscopy. Furthermore, neurite outgrowth was determined as described for the previous experiment (Figure 3.2.5).

In accordance with the previous experiment, the number of substrate-attached cells was significantly larger when granule neurons were plated together with COS7 cells transfected with ADAM8 and CHL1 (375 ± 20). A lower number of substrate-attached cells and significantly lower levels of neurite outgrowth were observed when either ADAM8 or CHL1 were transfected alone (100 ± 5 and 180 ± 7 , respectively), or when ADAM10 (160 ± 10) and ADAM17 (168 ± 3) were co-transfected with CHL1. In this experiment, EQ-ADAM8 was not used. Instead, BB-94 was used for inhibition of CHL1 cleavage in CHL1/ADAM8 co-transfected cells (170 ± 15). When shedding of CHL1 was inhibited by BB-94, substrate attachment was reduced slightly below the level obtained with CHL1 alone. This difference suggests that in addition to ADAM8, another protease which is inhibited by BB-94 could be involved in ectodomain shedding of CHL1, although at a much lower level.

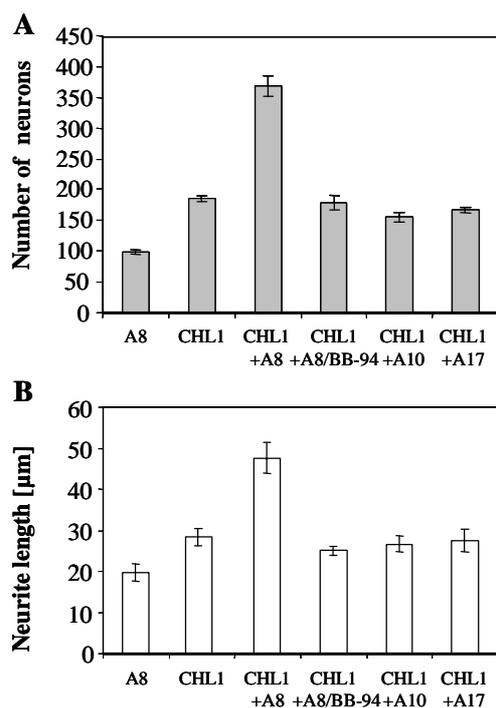


Figure 3.2.5: Effect of CHL1 shedding on neuronal survival and neurite outgrowth in co-culture with COS7 cells. Freshly prepared cerebellar granule neurons were plated on top of COS7 cells one day after transfection with CHL1 and/or ADAM protease as indicated. The number of substrate-attached neurons and neurite length was significantly increased in co-culture with COS7 cells transfected with CHL1 and ADAM8 in comparison to co-cultures with COS7 cells transfected with CHL1 and other ADAM constructs. From: Naus et al., 2004

3.2.6 Reduction of cell death by soluble CHL1

To determine the time course of cell death, cerebellar granule neurons were maintained in serum-free media from COS7 cells transfected either with CHL1 alone or co-transfected with CHL1/ADAM8 or CHL1/EQ-ADAM8 (Figure 3.2.6). After 1, 3 and 5 days, the numbers of surviving cells were determined by trypan blue exclusion method.

The number of surviving cells declined to $19\% \pm 1.5\%$ after 5 days in culture when the conditioned medium was derived from either only CHL1 or CHL1/EQ-A8 transfected cells. Similar values were obtained when neurons were treated with supernatants from CHL1/ADAM10 and CHL1/ADAM17 co-transfected cells (data not shown). In contrast, a significantly higher proportion ($36\% \pm 1.8\%$) of cells survived when conditioned medium from COS7 cells co-transfected with ADAM8 and CHL1 was applied to neuronal cultures. Thus, survival of neurons was increased by $\sim 50\%$ due to the presence of soluble CHL1 derived by proteolytic processing.

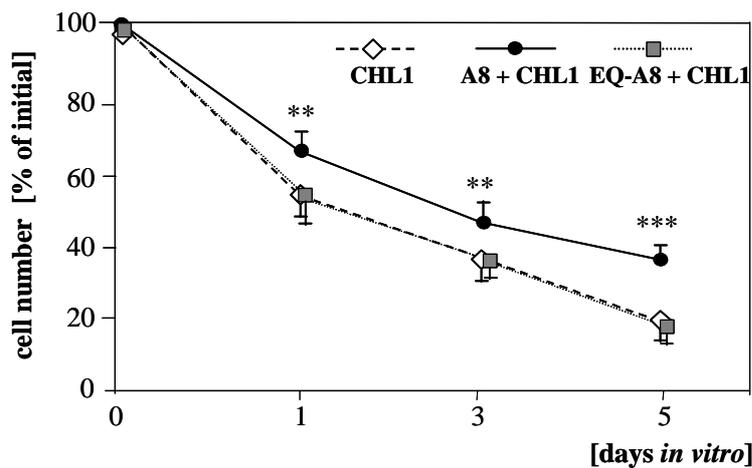


Figure 3.2.6: Effect of supernatants from COS7 cells on cell death. Granule neurons were maintained in supernatants of COS7 cells transfected with either CHL1, CHL1/ADAM8 or CHL1/EQ-ADAM8. Strikingly more cells survived in supernatants from CHL1/ADAM8 co-transfected cells. From: Naus et al., 2004

3.3 Peptide substrate screening

In order to determine new ADAM8 substrates, a collection of fluorogenic peptides was designed and incubated with soluble forms of ADAM8. This assay provided indications for new ADAM8 substrates and furthermore information about a possible ADAM8 cleavage site or at least minimal amino acid requirements for ADAM8 cleavage. On the basis of the results of the cleavage assay, corresponding proteins of new peptide substrates are examined in further physiological studies – similar to those performed for CHL1 – for their biological relevance as ADAM8 substrates. A manuscript about the peptide screening and *in vivo* studies examining APP as a biological substrate is in preparation (Naus et al., in preparation).

3.3.1 Peptide screening for new ADAM8 substrates

Peptides were tested as peptide substrates of soluble forms of mouse ADAM8 protease. These peptides were derived from mouse proteins and included sequences of other metalloprotease peptide substrates as well as sequences from membrane proximal regions of proteins supposed to be released from the cell surface by metalloproteases. Peptides were synthesised on continuous cellulose membranes using the SPOT-synthesis technique with an amino-benzoic fluorescent moiety at the N-terminal end. Punched out as disks for microtiter plates, they were incubated with protease solution. Proteolytic cleavage of a peptide resulted in release of the N-terminal part with the fluorophor and therefore with an increase in fluorescence in the reaction solution. After incubation, the reaction solution was transferred into a 96 well plate for detection of the N-terminally labelled peptide fragment measured in a fluorescence microtiter plate reader. The peptides were provided by JPT Peptide Technologies GmbH (Berlin)(http://www.jerini.de/content/pep/protease_profiling/proteasespots.htm).

My initial interest was in characterising the role of proteolytically active ADAM8 in inflammation with a focus on the nervous system. Therefore, most of the peptides tested were derived from proteins involved in inflammatory processes and immune response. Among the chosen proteins were cytokines (CD40L, CX3CL1, KL, TGF- α , TNF- α , TRANCE), cytokine receptors (IL-1R-2, TNF-R1, TNF-R2), cell adhesion molecules (L-Selectin, PSGL-1) and immunoglobulin receptors (Fc- γ -RIII, CD23). The amyloid beta A4 protein precursor (APP) was also taken into the set of tested proteins as involved in Alzheimer's disease, a dementia type of neurodegenerative disorders involving inflammatory processes. In table 3.3.1 chosen peptides with corresponding proteins and their accession numbers in the Swiss-Prot/TrEMBL protein database were listed as well as the proteases supposed to cleave the proteins and corresponding references. For synonyms of protein names see 6.5.

Protein	Accession Number	Peptide substrate	Proteases involved	References
APP , amyloid beta (A4) protein precursor	P12023	EVRRHQKLVFF	ADAM8, 9, 10 ADAM17 ADAM33	(Amour et al., 2002) (Buxbaum et al., 1998) (Zou et al., 2004)
CD23 , low affinity immunoglobulin ϵ Fc receptor precursor	P20693	SNQLAQKSQV AEQKQMKQAQD LRNAQSQNSK VAKLWIEILI	MP ADAM8	(Marolewski et al., 1998) (Mayer et al., 2002) (Fourie et al., 2003)
CD40-L , CD40 ligand	P27548	NSFEMQRGDE	ADAM10	(Amour et al., 2002) Swissprot
CD163 , macrophage hemoglobin scavenger receptor precursor	Q99MX8	HGTGHPTLTA PKMTSESHGT DASIQCCLPKM SDCGHKEDAS PAKPWSHSDC ESSLWDCPAK	MP	(Droste et al., 1999)
CX3CL1 , fractalkine	O35188	QAATRRQAVG	ADAM17	(Garton et al., 2001) Swissprot
Fc-g R-III , low affinity immunoglobulin gamma Fc receptor III	P08508	SLVWYHTAFS PATTSSISLV VTITVQDPAT TQHQSKEPVTI	MP	(Harrison et al., 1991)
IL-1R-2 , interleukin-1 receptor type II precursor	P27931	TTVKEVSSSTF	ADAM8	(Amour et al., 2002)
KL , kit ligand precursor	P20826	PPVAASSLRN	ADAM8, 9 ADAM33	(Amour et al., 2002) (Zou et al., 2004)
L-selectin precursor	P18337	QETNRSFSKI	ADAM17	(Peschon et al., 1998),
MBP , myelin basic protein	P04370	YGSLPQKAQG	ADAM8 ADAM10 ADAM28	(Schlomann et al., 2002) (Chantry et al., 1989) (Howard et al., 2001)
PSGL-1 , P-selectin glycoprotein ligand 1 precursor	Q62170	LIPVKQCLLI TLPGSSDLIP PGNSPAPTLP KKGLIVTPGN HLPDSGLKKG	MP	(Davenpeck et al., 2000)
TGF-α , transforming growth factor alpha	P48030	AVVAASQKKQ	MP ADAM17	(Arribas et al., 1997) (Peschon et al., 1998)
TNF-α , tumour necrosis factor alpha	P06804	AQTLTLRSSH	ADAM8, 9, 10 ADAM17	(Amour et al., 2002) (Black et al., 1997) (Moss et al., 1997)
TNF-R1 , tumour necrosis factor receptor 1	P25118	NPQDSGTAVL PPLANVTNPQ CMKLCPLPPL	MP ADAM17	(Peschon et al., 1998) (Pinckard et al., 1997) (Cui et al., 2002)
TNF-R2 , tumour necrosis factor receptor 2	P25119	PTLSAIPRTL APESPTLSAI	MP ADAM17	(Pinckard et al., 1997) (Reddy et al., 2000)
TRANCE , TNF-related activation-induced cytokine	O35235	VGPQRFSGAP	ADAM17 ADAM19	(Lum et al., 1999) (Chesneau et al., 2003) Swissprot

Table 3.3.1: Peptide substrate collection

The peptide assays were performed at least twice. In most experiments bacterial soluble A8-ProMP was used, and in several cases as controls soluble A8-ProMP from cell culture supernatants. Protease and peptides platelets were incubated in the presence and absence of the metalloprotease inhibitor EDTA (10 mM). The solution was monitored immediately ($t=0$) and after 2, 6 and 24 hours. In case of constant increase of fluorescence and none in the presence of EDTA, a protease specific cleavage was presumed. The peptide for MBP was used as a control and for normalisation, as this peptide was already known as an ADAM8 peptide substrate (Schlomann et al., 2002). According to Amour et al. (2002), homologues peptides derived from human APP, IL-1R-2 and KL were cleaved by mouse ADAM8, whereas a CD40-L peptide was not. Because of the close homology of tested mouse and human peptides for APP, IL-1R-2, KL and CD40-L, these also served as controls. As a control for selectivity of the test method, several peptides containing arginine or lysine residues and several peptides not containing these amino acids were incubated with trypsin. As expected, only for those peptides containing arginine or lysine residues fluorescence increase was observed.

The average fluorescence increases after 24 hours were compared (Figure 3.3.1). The fluorescence increase which corresponded to protease activities was expressed in units normalised with MBP set to 1 unit. As the manufacturers of the peptide platelets claimed that differences up to 25 % could be observed between results obtained for spots with the same sequences as a result of errors in synthesis, pipetting, evaporation, bleaching and measurement, the results were not interpreted quantitatively. They were rather indicative for further *in vivo* analysis of potential ADAM8 substrates.

Relative fluorescence increase of 1 unit and more was supposed to signify a protease specific peptide cleavage, whereas increase of less than 0.5 units indicated no protease-substrate relationship. Fluorescence increases of 0.5 to 1 units were assessed more carefully even if they probably indicated cleavage at lower levels. Low average increases up to 0.2 units the most were observed for the negative controls with EDTA. Only for the APP peptide, an average increase of 0.48 for the negative controls was observed (data not shown). Therefore, increases of more than 0.5 units probably indicated specific cleavage. But the possibility that these indicated false positive peptide substrates could not be excluded completely.

In agreement with data published in Amour et al. (2002), the APP peptide was significantly cleaved by ADAM8 and the IL-1R-2 peptide with lower specificity, whereas CD40-L peptide was not. In contrast to their results, the peptide derived from KL was not cleaved. This was not expected, as the used peptides were very similar. The mouse KL peptide used in this assay

had an additional asparagine residue at the C-terminus, whereas a leucine residue at the N-terminal end was missing (LPPVAASSLR, Amour et al., 2002). Summarising the results, 15 of 34 tested peptides were cleaved by ADAM8, with four of them at lower levels, and 19 peptides were not. Peptides derived from APP, CD23, CX3CL1, Fc- γ -RIII, L-Selectin, PSGL-1, TGF- α and TNF- α were cleaved significantly, and peptides derived from CD163, IL-1R-2, TNF-R1 and TRANCE were probably cleaved with lower specificity. In case of PSGL-1 and CD23, two peptides were cleaved. As the two peptides for PSGL-1 shared overlapping amino acids, a cleavage site might be located in this overlapping region. CD23 was known to be released from the cell surface by cleavage at three different cleavage sites (Mayer et al., 2002). Possibly, ADAM8 cleaves at two of these sites. This peptide screening was an *in vitro* approach not taking into account *in vivo* conditions. Therefore, this approach was only indicative for further analyses which are necessary to assess the corresponding proteins of peptide substrates as physiologically relevant ADAM8 substrates.

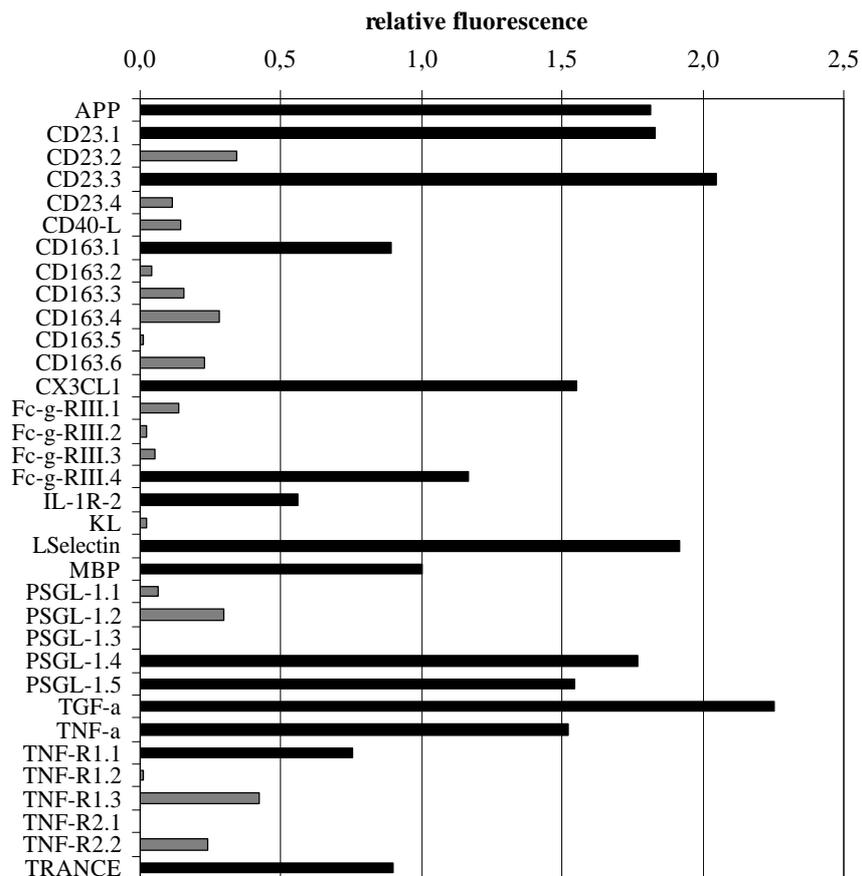


Figure 3.3.1: Peptide screening for new ADAM8 substrates. Average fluorescence increases of tested peptides measured after 24 hours were compared. Fluorescence increases corresponded to protease activity and were given as units normalised with MBP peptide set to 1. Fluorescence increase > 1 was assumed to indicate significant cleavage and increase 0.5-1 to indicate cleavage with lower specificity. Increase < 0.5 (grey) indicated no protease-substrate relationship. Peptides derived from APP, CD23, CX3CL1, Fc- γ -RIII, L-Selectin, PSGL-1, TGF- α and TNF- α were cleaved significantly.

3.3.2 Screening of ADAM8 catalytic specificity

Mutant MBP peptides derived from the MBP cleavage site (Schlomann et al., 2002) were tested as ADAM8 substrates in order to find specific amino acids necessary for substrate cleavage. According to the wild type peptide substrate, peptides with mutated amino acids close to the cleavage site from position P3 to position P3' were designed (Figure 3.3.2A). The experimental procedure was equal to the previous peptide screening (see 3.3.1)

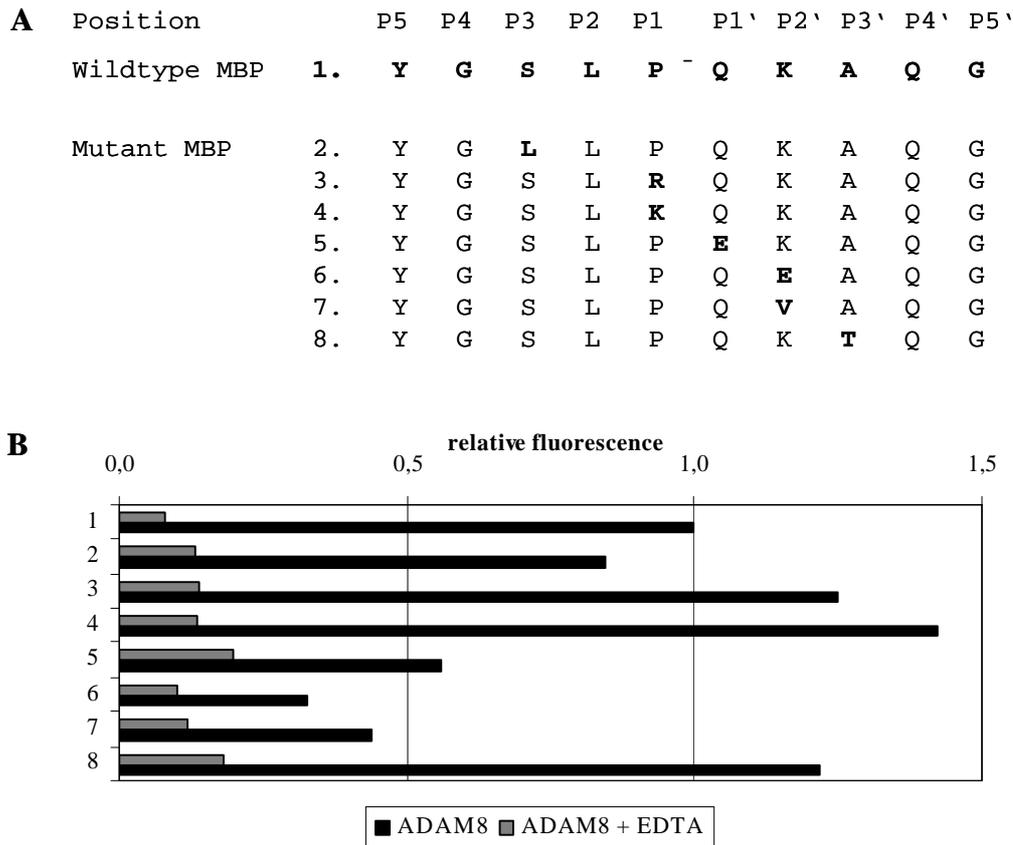


Figure 3.3.2: Screening of MBP mutant peptides. **A.** A peptide derived from the MBP cleavage site and 7 mutant peptides were incubated with catalytically active ADAM8. Mutated amino acids are given in bold letters. **B.** Average fluorescence increases of tested peptides measured after 24 hours were compared. Fluorescence increases corresponded to protease activity and were given as units normalised with fluorescence increase of the wild type MBP peptide set to 1. The peptides with mutations in the positions P1' and P2' were cleaved at significantly lower levels.

Based on variable fluorescence increases, it was concluded that ADAM8 cleaved the MBP mutant peptides with different efficiencies depending on the amino acid exchange (Figure 3.3.2B). The mutant peptides with an exchanged leucine for a serine in position P3 (peptide 2), an exchanged threonine for a leucine in position P3' (peptide 8) and basic residues – either arginine or lysine – for a proline in position P1 (peptides 3, 4) were cleaved by ADAM8. Possibly, there was a slightly enhanced cleavage for the mutants with basic amino acids in

position P1 in comparison to the MBP wild type peptide. Mutations in positions P1' and P2' had significant effects on peptide cleavage (peptides 5, 6, 7). An exchange of a glutamine residue to an acetic glutamate residue in position P1' as well as the exchange of the basic lysine residue in position P2' to either an acetic (glutamate) or a neutral, aliphatic amino acid (valine) resulted in decreased levels of peptide cleavage. From these results, it was hypothesised for the MBP peptide that basic residues in positions P1 and P2' increased the efficiency of ADAM8 cleavage, whereas an acetic residue in position P1' and P2' decreased it. Experiments with further mutant MBP peptides are necessary to support this hypothesis.

So far, no consensus sequence is known for ADAM8 substrates, partly because only a few cleavage sites are known. Assuming that the cleavage sites of the MBP mutant peptides were equal to that of the wild type peptide, and that other cleavage sites of screened peptide substrates were equal to those of the human homologues or to data in the Swissprot/TrEMBL database, a number of ADAM8 substrate cleavage sites can be compared (Table 3.3.2). From these sequences, no obvious consensus cleavage site can be deduced but some regularity is observed. There are no methionine, cysteine, tryptophan residues in the positions P3 to P3', and only in case of IL-1R-2 with a glutamate in position P3 an acetic residue. In the respective region, several basic residues are located.

A QK motif at positions P1' and P2' in the peptides derived from APP, CD23 and MBP was also present in the TGF- α peptide (AVVAASQKKQ) suggesting cleavage next to this motif. Examination of other cleaved peptides with unknown cleavage sites stated previous observations. In these peptides, there were also no methionine, cysteine and tryptophan residues present. Only in three peptides derived from L-Selectin (QETNRSFSKI), TNF-R1 (NPQDSGTAVL) and PSGL-1 (HLPDSGLKKG) acetic residues occurred, whereas it was possible that they were not located within positions P3 to P3' of the cleavage site. For the PSGL-1 peptide (HLPDSGLKKG) a cleavage site within the C-terminal amino acids afar from the aspartate residue was probable, as another PSGL-1 peptide overlapping with this region (KKGLIVTPGN) was also cleaved.

Examination of sequences of the non-cleaved peptides (see 3.3.1) supports the observed regularity. The peptides derived from KL (PPVAASSLRN) and from TNF-R2 (PTLSAIPRT) were the only non-cleaved peptides fulfilling the "criteria" of the cleaved peptides: absence of acetic, methionine, cysteine, tryptophan residues and presence of basic amino acids.

Protein	Cleaved peptides	Reference
APP	EVRH↓QKLVFF	YEVHH↓QKLVFF (Amour et al., 2002)
CD23.1	SNQLA↓QKSQV	GDQMA↓QKSQS (Mayer et al., 2002)
CD23.1	LRNAQ↓SQNSK	DLSSFK↓SQELNE (Mayer et al., 2002)
CX3CL1	QAATR↓RQAVG	QAATR↓RQAVG Swissprot
IL-1R-2	TTVKEVS↓STF	TVKEAS↓STFSWG (Amour et al., 2002)
MBP	YGSLP↓QKAQG	YGSLP↓QKAQG (Schlomann et al., 2002)
MBP	YGSLR↓QKAQG	YGSLP↓QKAQG (Schlomann et al., 2002)
MBP	YGSLK↓QKAQG	YGSLP↓QKAQG (Schlomann et al., 2002)
MBP	YGSLP↓QKTQG	YGSLP↓QKAQG (Schlomann et al., 2002)
MBP	YGLLP↓QKAQG	YGSLP↓QKAQG (Schlomann et al., 2002)
TNF- α	A↓QTLTLRSSS	SPLA↓QAVRSSRK (Amour et al., 2002)
TNF- α	AQTLT↓LRSSS	SPLAQA↓VRSSRK (Amour et al., 2002)
TNF- α	AQTLTLRS↓SS	SPLAQAVRS↓SSRK (Fourie et al., 2003)
TRANCE	VGPQR↓FSGAP	VGPQR↓FSGAP Swissprot

Table 3.3.2: Proposed cleavage sites of new substrate peptides. Some cleavage sites were derived from human homologous sites. MBP wild type and MBP mutant peptides were supposed to be cleaved equally. A QK motif at the cleavage site in positions P1' and P2' is present in APP, CD23.1 and MBP. The listed peptides do not contain methionine, cysteine and tryptophan residues in positions P3 to P3' and only IL-1R-2 contains an acetic residue in this region.

3.3.3 Search for physiological ADAM8 substrates

Peptides cleaved in the *in vitro* peptide screening assays do not necessarily mean that corresponding proteins are biologically relevant substrates *in vivo*. Based on results of the peptide assays, the full length proteins are examined in further experiments as physiologically relevant substrates. So far, this analysis was initiated for APP, TNF-R1 und PSGL-1. In a collaboration with Dr. Stefan Lichtenthaler (MPI für Biochemie, München), APP cleavage by ADAM8 was tested in transfected cells. TNF-R1 cleavage was analysed in mouse tissues as well as in different primary cells with ELISA test assays which were mostly performed by Dirk Wildeboer (Doctoral thesis, Universität Bielefeld). The PSGL-1 cDNA cloning is in progress and subsequent co-transfection experiments with PSGL-1 and ADAM8 will be performed.

APP

In the group of Stefan Lichtenthaler, cDNAs encoding ADAM8, catalytically inactive EQ-ADAM8 or ADAM10, and the control vector pTarget were transfected into cells expressing APP. The cell lysates were analysed in immunoblots in order to detect ADAM8 or EQ-ADAM8, respectively. The effect of ADAM expression on APP shedding and release from the cell surface was determined by ELISA tests detecting soluble forms of APP in the cell supernatants. Basal levels of APP release were detected in supernatants of EQ-ADAM8 and pTarget transfected cells, whereas APP release was significantly increased in supernatants of

cells transfected with ADAM8 and ADAM10 at comparable levels. These results indicated a physiological role of ADAM8 in APP shedding in addition to ADAM10 (data not shown).

TNF-R1

For analysis of TNF-R1 shedding, tissues of wobbler and ADAM8 KO mice were used. In cerebellum and brain stem of WR mice, ADAM8 is increasingly expressed upon neurodegeneration. Therefore, these tissues and cortex tissue as control for an unaffected brain region were used for analysis of TNF-R1 shedding by ADAM8. Tissues prepared of 30 day-old mice were subjected to ELISA tests detecting soluble TNF-R1 (sTNF-R1, Figure 3.3.3). In cerebellum and brain stem of wobbler mice (A8^{+/+}, wr/wr), TNF-R1 release was strikingly increased in comparison to both cortex of WR mice (A8^{+/+}, wr/wr) and cerebellum/brainstem of wild type mice (A8^{+/+}, wt). In ADAM8 KO mice without the WR phenotype (A8^{-/-}, wr/+), TNF-R1 release in cortex as well as in cerebellum/brain stem was comparable to that in the corresponding wild type (A8^{+/+}, wt), whereas levels were slightly increased in ADAM8 KO mice with WR background (A8^{-/-}, wr/wr). These data indicated that ADAM8 upregulated upon neurodegeneration enhanced TNF-R1 release (A8^{+/+}, wr/wr vs. A8^{-/-}, wr/wr). Furthermore, it can be hypothesised that under inflammatory conditions TNF-R1 is also released by another protease, as release was increased in ADAM8 KO/WR mice (A8^{-/-}, wr/wr) in comparison to ADAM8 KO mice without WR phenotype (A8^{-/-}, wr/+).

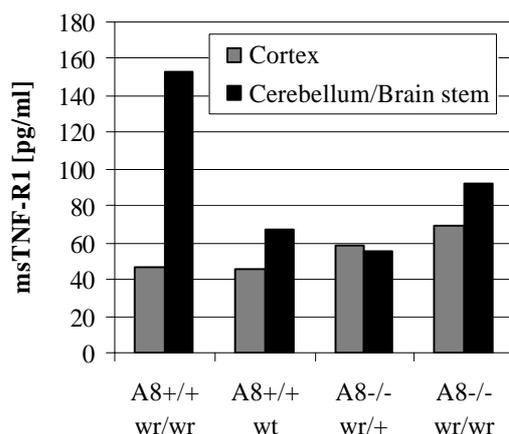


Figure 3.3.3: Effect of ADAM8 on TNF-R1 shedding. TNF-R1 release was analysed in cerebellum/brain stem and cortex of mice with different genetic WR and ADAM8 backgrounds. In cerebellum/brain stem of wobbler mice (A8^{+/+}, wr/wr) increased ADAM8 expression resulted in significantly increased release of TNF-R1 from the cell surface in comparison to the release in cortex of wobbler mice (A8^{+/+}, wr/wr) and in tested brain tissues of wildtype mice (A8^{+/+}, wt).

3.4 ADAM8 and ADAM19 activities in primary human brain tumours

As ADAMs are markers for different kinds of tumours, the analysis of their expression patterns as well as the definition of their proteolytic activities in tumours is a challenging task. In a parallel project in which the doctoral student Dirk Wildeboer determined ADAM expression patterns in different kinds of primary human brain tumours, I have worked out a method for the detection of ADAM activities in protein extracts from these tumours. A manuscript about the distinct expression levels and activities of human ADAMs in primary brain tumours was submitted to the American Journal of Pathology (Wildeboer, D., Naus, S., Sang, Q.X., Bartsch, J.W., Pagenstecher, A., submitted).

Gliomas are the most common primary brain tumours with diffuse infiltrative growth regularly preventing complete resection of the tumour. Most low grade gliomas over time develop into malignant tumours that present cell anaplasia, neovascularisation and finally necrosis (Kleihues and Ohgaki, 2000). In a project of Dirk Wildeboer, expression levels of human ADAMs known to be expressed in the brain were determined in primary brain tumours (astrocytoma WHO grade I-III (AI-III), glioblastoma (GBM), oligoastrocytoma II (OAI), oligodendroglioma WHO grade II and III (OII/III), ependymoma WHO grade II and III (EII/III) and primitive neuroectodermal tumour (PNET)). With RT-PCR, significantly increased mRNA levels of ADAMs 8, 11, 15, 17, 19 and 28 were determined. Subsequent quantification by real-time PCR revealed only weak mRNA changes for ADAM11, 15 and 17, whereas ADAM8, 19 and 28 were significantly upregulated in respective tumours. Strongest upregulation of gene expression was detected in GBM and OIII for ADAM8, in GBM and AIII for ADAM19 and in GBM for ADAM28. Expression of ADAM28 was strikingly increased in many of the tested tumours. ADAM8 and 19 expressions were further examined. Immunoblotting revealed low expression levels of ADAM8 in normal brain and mainly of the remnant 60 kDa form, whereas the amounts of proform and processed forms were strikingly increased in the examined tumour tissues. The proform of ADAM19 was detected in most of the samples but levels of the processed form were significantly increased in malignant tumours.

In order to determine whether increased mRNA and protein levels of ADAM8 and ADAM19 in different human primary brain tumours corresponded to increased proteolytic activity, peptide cleavage assays with human brain tumour homogenates and peptides for ADAM8 and ADAM19, respectively, were performed. The peptide Dnp-SHHGDQMAQKSQSTQI-COOH derived from CD23 and obtained from M. Moss was used for determination of ADAM8

catalytic activity. This peptide was significantly cleaved by recombinant ADAM8 (Diploma thesis, Simone Reipschläger) as well as a peptide derived from the homologues murine sequence (see 3.3.1, CD23.1). For determination of ADAM19 catalytic activity, the peptide substrate Ac-RPLESNAV-COOH (CRDA19) representing an autocleavage site within the cysteine-rich domain of human ADAM19 was used (Kang et al., 2002).

Peptide cleavage assays with fluorescamine were performed (see 3.1.3) Protease activities were extracted from the brain tissue samples. Membrane preparations were either not effective or too time-consuming to maintain proteolytic activity. Finally, to purify the sample and to enrich protease activity, ConA sepharose chromatography was performed immediately after lysis. ConA pellets with bound proteins were used for peptide assays providing low levels of enriched proteins and corresponding amino groups in solution. ConA pellets and peptides were incubated in the presence of inhibitor cocktail containing inhibitors for complete inhibition of serine and cysteine proteases. As aspartic proteases with pH optima at acetic pH exhibit no or only little catalytic activity under physiological conditions, no specific inhibitors for these proteases were added. Remaining proteolytic activity was assumed to be metalloprotease activity. EDTA was used as an unspecific metalloprotease inhibitor proving metalloprotease activity. To distinguish ADAM activities from those of MMPs, a combination of recombinant human TIMP1 and human TIMP3 was used and preincubated with the tumour homogenates. Immediately and after 1, 2 and 3 hours incubation, samples of the reaction batches were mixed with fluorescamine and fluorescence was measured. Fluorescence increases after three hours were normalised with samples containing peptides and normal brain extracts without inhibitors which were set to 1 (Figure 3.4).

Cleavage assays were performed with homogenates of normal brain (NB), pilocytic astrocytoma (AI), anaplastic astrocytoma (AIII), glioblastoma (GBM) and oligoastrocytoma II (OAI) (Figure 3.4). CD23 peptide cleavage was increased in all tested tumour tissue homogenates compared to normal brain. EDTA strongly inhibited CD23 cleavage indicating metalloprotease activity. Preincubation with the TIMP1/3 combination inhibited CD23 peptide cleavage in the OAI sample, whereas it did not affect cleavage in extracts from AI, AIII and GBM. As ADAM8 is not inhibited by any of the four known TIMPs (Amour et al., 2002; Schlomann et al., 2002), decreased cleavage in OAI indicated the presence of another metalloprotease which was inhibited by TIMP1 and/or TIMP3. Cleavage of the CRDA19 peptide was detected in AIII and GBM. EDTA inhibited cleavage indicating metalloprotease activity. TIMP1/3 inhibited CRDA19 cleavage in GBM but not in AIII indicating an additional metalloprotease activity which was inhibited by TIMP1 and/or by TIMP3. The

results of the peptide cleavage assay obtained with ADAM19 corresponded to results of the real-time PCR in which ADAM19 expression was increased in AIII and GBM.

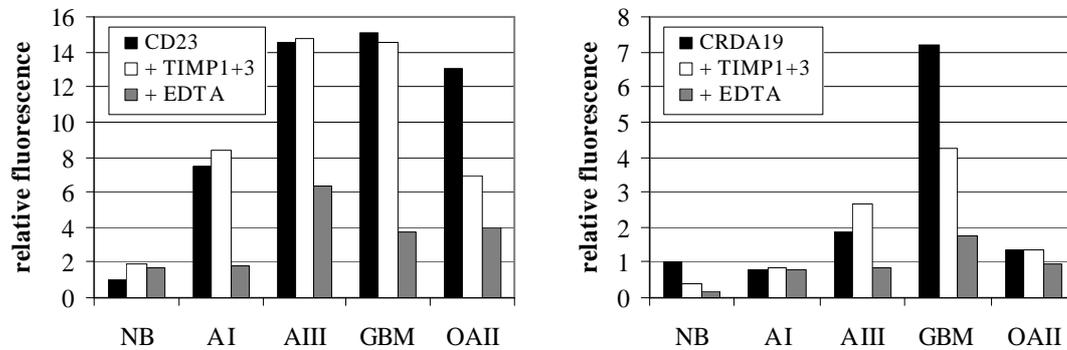


Figure 3.4: Cleavage of CD23 (A) and CRDA19 (B) peptides by human primary brain tumour homogenates. Fluorescence increase of samples with normal brain tissue and peptides was set to 1. **A.** The CD23 peptide was cleaved in all tested tumour tissues compared to normal brain. EDTA inhibited cleavage indicating metalloprotease activity. TIMP1/3 did not affect cleavage in AI, AIII and GBM, but inhibited CD23 cleavage in OAII indicating activity of another metalloprotease which is inhibited by TIMP1 and/or TIMP3. **B.** The CRDA19 peptide was cleaved in AIII and GBM. EDTA inhibition indicated metalloprotease activity. In AIII cleavage was not inhibited by TIMP1/3, but peptide cleavage in GBM was affected indicating an additional metalloprotease activity which is inhibited by TIMP1 and/or TIMP3.

4. Discussion

4.1 Expression of soluble, catalytically active ADAM8

A soluble form of ADAM8 comprising the pro and metalloprotease domain was expressed in cell culture as well as in bacteria and was isolated and purified either from cell culture supernatants or from cytoplasm of *E.coli*. ADAM8 from cell culture as well as from *E.coli* was catalytically active as shown either by MBP protein cleavage or by peptide cleavage assays with peptides derived from MBP, CD23 and the ADAM8 autocatalytic cleavage site.

ADAM8 as all other ADAMs is expressed as a catalytically inactive zymogen and is activated by prodomain removal. In contrast to other ADAMs, ADAM8 does not contain the consensus sequence RX(K/R)R between pro and metalloprotease domain, a consensus cleavage site for furin-like convertases. Instead, ADAM8 is activated by autocatalysis. The full length form of catalytically inactive EQ-ADAM8 expressed on cells was not activated. The EQ-ADAM8 prodomain was released when the complete extracellular domain of active ADAM8 was co-expressed by the same cells. It was neither activated by a co-expressed soluble form of ADAM8 comprising the pro and metalloprotease domain nor by the complete extracellular domain applied to supernatants of transfected cells (Schlomann et al., 2002) indicating activation in *cis* in the Trans-Golgi network and the disintegrin and cysteine-rich domains being important for activation. It was suggested that homophilic interactions of disintegrin domains, e.g. by two ADAM8 molecules, might be necessary for autocatalysis bringing two molecules in close proximity. Schlomann et al. (2002) observed complete processing of the entire extracellular domain transfected into COS7 cells, whereas the soluble form of ADAM8 comprising pro and metalloprotease domain was only partially processed. In the work presented here, A8-ProMP from cell culture was also partially processed, but the relative amounts of proform (60 kDa) and processed form (35-40 kDa) varied. In some experiments, the predominant portion was the processed form, whereas prodomain removal was much weaker in other experiments. Possibly, prodomain removal is also dependent on ADAM8 concentrations arguing for activation independent from the disintegrin and cysteine-rich domains when ADAM8 is present in high concentrations. This could also explain A8-ProMP prodomain removal in *E.coli*. As the prodomain removal was supposed to take place in the Trans-Golgi network, it was not clear whether A8-ProMP expressed by *E.coli* would be activated. Nevertheless, proform and activated processed form were present in different relative amounts in the cytoplasmic fractions of induced bacterial cultures (Diploma thesis, Simone Reipschläger). From these results we conclude that activation of A8-ProMP in *E.coli*

is independent from the Trans-Golgi network, from the disintegrin domain and furthermore, from glycosylations. Possibly, autocatalytic prodomain removal requires chaperones, and similar proteins present in *E.coli* can act as chaperones.

For structural analysis of interaction of the prodomain with the catalytic site, A8-ProMP is not suitable, as it is autocatalytically processed. Therefore, a catalytically inactive form should be used, e.g. a soluble form comprising the EQ mutation.

Soluble forms of ADAMs have been expressed in different cellular systems. Similarly to the work presented here, a cDNA construct encoding the extracellular domain of ADAM19 with a MycHis-tag was transiently transfected into COS7 cells, and supernatants were purified via the His-tag (Chesneau et al., 2003). For other work, CHO, COS1 or COS7 cells were used to express soluble forms of ADAMs either containing the complete extracellular portion or the pro and metalloprotease domains. For purification either IgG-Fc, FLAG or Myc-tags were used (Amour et al., 2002; Chesneau et al., 2003; Fourie et al., 2003; Kang et al., 2002). Moreover, Sf9 insect cells were infected with recombinant baculovirus for expression of ADAM19 extracellular domain and pro and metalloprotease domains of ADAM8, 15, 17 and 28 (Chesneau et al., 2003; Fourie et al., 2003). ADAM metalloprotease domains were so far not expressed in *E.coli*, probably for the reason of inefficiency. As the prodomain is supposed to be important for proper folding, recombinant metalloprotease domains have to be expressed with the prodomain. For most ADAMs, additional recombinant furin-like convertases would be required for prodomain removal converting the proform into an active ADAM protease.

Concerning ADAM expression, bacteria were used to express the disintegrin and cystein-rich domain of ADAM8 (Schlomann et al., 2002) and the prodomains of ADAM17 (Gonzales et al., 2004), ADAM8 and ADAM10 (personal communication, M.Moss, Biozyme Inc., NC, USA). Several MMPs such as MMP7, MMP9, MMP12 and MMP13 were expressed in bacteria (Kroger and Tschesche, 1997; Oneda and Inouye, 1999; Parkar et al., 2000; Pathak et al., 1998). The ADAM prodomains as well as the MMPs were isolated from inclusion bodies and were refolded. The expression rates for the MMPs expressed in bacteria were much higher than those observed for A8-ProMP. As ADAM8 was also expressed at low levels in cell culture, high expression of soluble forms of ADAM8 is possibly a general problem and could be due to a short half-life of the protein itself.

4.2 Peptides as tools for systematic search for new ADAM substrates

A peptide cleavage assay with fluorogenic peptides was established in order to perform a systematic search for new ADAM8 substrates. The peptides were derived from known peptide substrates of other metalloproteases or from juxtamembraneous regions of supposed metalloprotease substrates. This approach was indicative for further analysis which would be necessary to assess the corresponding proteins of peptide substrates, as physiological relevance of ADAM8 substrates might be dependent on exposure of cleavage sites, posttranslational regulation, interactions with binding partners, and localisation in the cell membrane in addition to a specific cleavage site.

So far, only a few publications exist in which peptides were used for systematic search for ADAM substrates. In order to determine catalytic properties, peptide assays with synthetic peptides derived from proteins known to be cleaved by other ADAMs or metalloproteases were performed with recombinant ADAM9, ADAM19 and ADAM33 (Chesneau et al., 2003; Roghani et al., 1999; Zou et al., 2004). In another study, synthetic peptides were used to compare catalytic properties of ADAM8 with those of other ADAMs and MT-MMPs. From six tested peptides, ADAM8 cleaved peptides derived from APP, IL-1R, KL and TNF- α (Amour et al., 2002), whereas ADAM10 cleaved peptides derived from APP, CD40-L and TNF- α (Amour et al., 2000). In the specified examples, peptide cleavage was analysed by chromatography and mass spectrometry. Corresponding proteins of some peptide substrates were further examined in co-transfection experiments. So far, only one work using fluorogenic peptides for systematic search of ADAM substrates was published (Fourie et al., 2003). A collection of about 50 peptides was labelled at the C-terminal end with a quencher and at the N-terminal end with a fluorophor, so that cleavage of the peptide could be monitored by an increase in fluorescence. These peptides were incubated with ADAM8, ADAM15, ADAM17 and ADAM28 in order to examine different specificities of these proteases. ADAM17 specificity seemed to differ from that of the other analysed ADAMs. In subsequent *in vivo* studies, CD23 and CD27-L were also cleaved by ADAM8 in co-transfection experiments.

Most publications cited here were published in the last three years. The approach of using peptides for determination of ADAM catalytic specificity is quit new and was hardly used for systematic search for new potential ADAM substrates. The fact that in recent studies some of the corresponding proteins of peptide substrates which were further examined were also cleaved *in vivo* in cell culture experiments supports the efficiency of this approach. However, there were differences in peptide cleavage *in vitro* and cleavage of the full length protein *in*

in vivo. Therefore, these studies also show that further analysis is necessary as some proteins such as TNF- α , KL and APP were not cleaved *in vivo*, although derived peptides were cleaved by ADAM19 and ADAM33, respectively (Chesneau et al., 2003; Zou et al., 2004).

In the work presented here, peptides derived from APP, CD23, CX3CL1, Fc- γ -RIII, L-Selectin, PSGL-1, TGF- α and TNF- α were significantly cleaved by ADAM8 and peptides derived from CD163, IL-1R-2, TNF-R1 and TRANCE at lower levels. Therewith, CX3CL1, Fc- γ -RIII, L-Selectin, PSGL-1, TGF- α , CD163, IL-1R-2 and TRANCE were determined as potential new substrates of ADAM8, whereas APP, CD23, IL-1R-2 and TNF- α were stated as those (Amour et al., 2002; Fourie et al., 2003). Further studies have to examine the physiological role of these proteins as potential ADAM8 substrates.

4.3 Substrate specificity of ADAMs

In order to examine regularities in the amino acid composition of ADAM8 peptide substrates, mutant MBP peptides derived from the MBP cleavage site were designed. These peptides had point mutations in a region of 6 amino acids around the cleavage site. According to Schlomann et al. (2002) the disintegrin domain influenced autocatalytic prodomain removal of ADAM8, e.g. by mediating the interaction of at least two ADAM8 monomers. In addition, interaction of other substrates with the disintegrin domain of ADAM8 might be important for physiological cleavage. In this assay a soluble protease lacking the disintegrin domain and short peptide substrates probably only interacting with the catalytic centre were used. The fact that peptide substrates were cleaved specifically supports the importance of a consensus sequence or at least preferred amino acids around the cleavage site. A specific cleavage site might be essential for cleavage but not sufficient.

The mutant MBP peptides were cleaved with different efficiencies. Assuming that cleavage occurred at the same sites in all peptides it could be concluded for the MBP peptide that ADAM8 preferred a cleavage site with basic residues in positions P1 and/or P2' and one without acetic residues around the cleavage site, basically not in position P1'. Mayer et al. (2002) suggested a similar hypothetical consensus sequence $XXR/K\downarrow XKX$ from human CD23 cleavage sites. A QK motif at positions P1' and P2' in the peptides derived from APP, CD23 and MBP was also present in the TGF- α peptide (AVVAASQKKQ) suggesting cleavage next to this motif.

Examination of the screened peptide collection revealed regularity for cleaved peptides, which were not applicable for most of the non-cleaved ones. The cleaved peptides contained basic amino acid residues, whereas they did not contain cysteine, methionine or tryptophan

residues. Only five of the cleaved peptides contained acetic residues which were possibly not located in positions P3 to P3' of proposed cleavage sites. In contrast, only two peptides did contain basic residues, but no acetic, cysteine, methionine or tryptophan residues and were not cleaved – peptides derived from KL and TNF-R2. In this work, the KL peptide LPPVAASSLR was not cleaved by mouse A8-ProMP, whereas in another work the similar peptide PPVAASSLRN was cleaved by an equivalent ADAM8 form at the cleavage sites A↓A and A↓S (Amour et al., 2002). The reason for this contradictory result is not clear as there were hardly any differences in the ADAM8 forms and peptides used. The soluble forms of ADAM8 comprised the pro and metalloprotease domain and had either a His-tag or a human IgG-Fc-tag. The peptides used shared 9 amino acids and differed by only one amino acid.

It is possible that peptides are cleaved specifically by ADAM8 at QK motifs in positions P1'/P2', but others also with less specificity when sufficient criteria are fulfilled such as the presence of basic amino acids and the absence of acetic, cysteine, methionine or tryptophan residues. For several peptide substrates, different cleavage sites were determined depending on the ADAM protease used (Table 4.3). This supports the notion that substrate interaction with the ADAM catalytic sites and subsequent cleavage is not totally dependent on a specific consensus sequence. In peptides derived from TNF- α , three different cleavage sites for ADAM8, and two different cleavage sites for ADAM9 and ADAM19, respectively, were determined. Three different cleavage sites were sequenced for ADAM9 in a KL peptide. For MBP, only one motif next to the QK motif was determined for ADAM8, ADAM10, ADAM28 and ADAM33 emphasising the importance of this motif. ADAM8, ADAM9 and ADAM33 cleaved an APP peptide also at the QK motif, whereas ADAM10 cleaved the APP peptide two amino acids closer to the C-terminus. Comparison of ADAM cleavage sites in different substrate peptides also shows that different ADAMs cleave the same substrate peptides with the cleavage sites being the same or in close proximity. This could indicate that ADAM catalytic activities towards peptides are similar, although kinetic properties could be different. Molecular interactions of either ADAM disintegrin or ADAM cytoplasmic domains with substrates or cellular localisation of protease and substrate might be more important for substrate specificity. Possibly, adapter molecules play a role by bringing protease and substrate into close proximity. Cleavage might then occur in exposed loop regions with amino acid sequences meeting minimal criteria. Further studies are necessary to determine substrate specificities of ADAMs. In addition to testing ADAM specificities towards systematically mutated peptides, it will be important to examine further criteria besides consensus sequences.

Protein	Sequence	A8	A9	A10	A19	A28	A33
APP	YEVHH↓QK↓LVFF	H↓Q ^a	H↓Q ^c	K↓L ^a			H↓Q ^g
KL	LPPVA↓A↓S↓SLR	A↓S ^a	A↓A ^e A↓S ^e S↓S ^e	NC ^d	A↓S ^b		A↓S ^g
MBP	YGSLP↓QKAQG	P↓Q ^{e*}		P↓Q ^{d*}		P↓Q ^{d*}	P↓Q ^g
TNF-α	PLA↓QA↓VR↓S↓SS	A↓Q ^a A↓V ^a S↓S ^c	A↓Q ^e S↓S ^e	A↓V ^a	R↓S ^b S↓S ^b		

Table 4.3: ADAM cleavage sites in APP, KL, MBP and TNF-α. ^aAmour et al., 2002; ^bChesneau et al., 2003; ^cFourie et al., 2003; ^dHoward et al., 2001; ^eRoghani et al., 1999; ^fSchlomann et al., 2002; ^gZou et al., 2003; *cleavage site determined from cleavage of recombinant protein

4.4 Physiological relevance of new ADAM8 substrates

4.4.1 ADAM8 in nerve regeneration

Data shown in the publication (Naus et al., 2004) were the first evidence that shedding of CHL1 by ADAM8 had a physiological role in the nervous system. ADAM8 proteolytic activity was specific for CHL1, whereas NCAM and L1 were not cleaved. Furthermore, CHL1 cleavage was specific for ADAM8. ADAM10 and ADAM17 did not cleave CHL1, although both ADAMs are expressed at higher levels in the nervous system and also under healthy conditions. ADAM10 is responsible for ectodomain shedding of other neuronal cell adhesion molecules in the CNS such as L1 and N-Cadherin (Mechtersheimer et al., 2001; Reiss et al., 2005), but not for CHL1 shedding.

Specificity of ADAM8 for CHL1 is possibly defined by structural differences between CHL1 and other cell adhesion molecules. The cleavage site for the 165 kDa fragment is located in the fifth FNII domain which is not conserved in NCAM and L1. Shedding of CHL1 by ADAM8 was performed under reducing and non-reducing conditions. As under non-reducing conditions the 125 kDa band was absent, it was supposed that the cleavage site was located between two cysteine residues forming a cysteine bridge under non-reducing conditions. This would argue for the 165 kDa fragment as the physiologically more important one. In this case, the unconserved fifth FNII domain might be essential for determining ADAM8 specificity.

CHL1 release by ADAM8 was not enhanced by activators of protein kinase C such as TPA or the phosphatase inhibitor pervanadate, whereas a number of publications report induction of ADAM17 ectodomain shedding by both these activators. These results indicated that ADAM8 intracellular regulation differed from that of ADAM17 involving protein kinase C epsilon (Wheeler et al., 2003) and furthermore, that ADAM17 was not responsible for CHL1

shedding. These findings underline the clear cut specificity of the protease-substrate relationship between ADAM8 and CHL1.

In the CNS, CHL1 and ADAM8 are expressed in neurons and oligodendrocytes arguing for CHL1 cleavage occurring in *cis* on the same cell surface. CHL1 expression was highly upregulated in injured and regenerating central and peripheral neurons and was additionally expressed in reactive astrocytes of the optic nerve (Chaisuksunt et al., 2000a; Chaisuksunt et al., 2000b; Rolf et al., 2003; Zhang et al., 2000). Under pathological conditions, ADAM8 is additionally expressed by astrocytes and microglia induced by the cytokine TNF- α (Schlomann et al., 2000). Thus, under pathological conditions cleavage of CHL1 could also occur in *cis* on astrocytes. Soluble forms of CHL1 promoted neuronal cell survival and controlled neurite outgrowth (Chen et al., 1999; Naus et al., 2004) indicating involvement of CHL1 in nerve regeneration or remodelling with an unknown functional role. In cell migration assays, CHL1 mediated enhanced cell migration towards collagen I via integrins $\alpha(1)\beta(1)$ and $\alpha(2)\beta(1)$ (Buhusi et al., 2003). Similar to L1, integrin-dependent cell migration could be enhanced by fragments released from membrane-bound CHL1 (Thelen et al., 2002). As ADAM8 specifically cleaved CHL1, ADAM8 might affect cell adhesion by cleavage of CHL1, but also by direct binding to integrins via the disintegrin domain (Schlomann et al., 2002) thereby activating intracellular signalling pathways.

Under pathological conditions, cleavage of CHL1 by ADAM8 on astrocytes can lead to enhanced ECM remodelling by reactive astrocytes, e.g. by extension of processes, an important characteristic of many neuropathological states. Based on pathological conditions in the nervous system of the wobbler mouse, it is likely that CHL1 shedding is also important for activation of glial cells. As the pro-inflammatory cytokine TNF- α induced ADAM8 expression in the brain stem and spinal cord of the wobbler mouse, which in turn lead to enhanced CHL1 cleavage, TNF- α indirectly regulated CHL1 release via upregulation of ADAM8 (Naus et al., 2004).

4.4.2 Possible role of ADAM8 as an α -secretase in Alzheimer's disease

Amyloid beta A4 protein precursor (APP) is cleaved proteolytically in two distinct pathways. It is cleaved in the amyloidogenic pathway by β - and γ -secretases to release amyloid β (A β) peptide forming non-soluble plaques. In a non-amyloidogenic pathway, APP is cleaved within the amyloidogenic A β domain by an α -secretase to release a non-amyloidogenic p3 peptide and, furthermore, a large ectodomain of APP (sAPP α) with neuroprotective and memory-enhancing function (Furukawa et al., 1996; Mattson et al., 1999; Meziane et al., 1998).

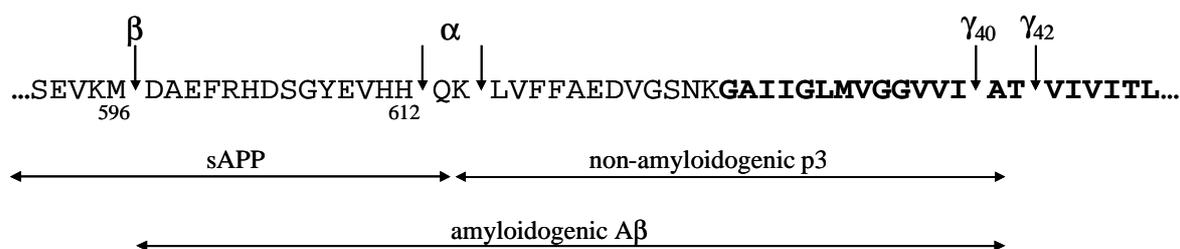


Figure 4.4.2: Amyloidogenic and non-amyloidogenic processing pathways of APP. The amino acid sequence depicts a part of the extracellular domain of human APP (normal letters) and a part of the transmembrane region (bold letters). In the amyloidogenic pathway, APP is cleaved by the β -secretase BACE (beta-site APP-cleaving enzyme) and by γ -secretases to release amyloid A β peptides of mainly 40 and 42 amino acid residues (γ 40 and γ 42). The γ -secretase is a complex of proteins including presenilin 1 cleaving APP in the transmembrane region. In the non-amyloidogenic pathway, cleavage by α -secretases in the amyloidogenic A β domain precludes the generation of A β peptides and enables the generation of a non-amyloidogenic p3 peptide and a large sAPP with neuroprotective function (De Strooper and Annaert, 2000).

Based on cleavage of a peptide derived from APP by ADAM8, *in vivo* studies were performed showing that ADAM8 transfected into APP expressing cells released APP from the cell surface. These results indicated that ADAM8 is an α -secretase for APP shedding. ADAM9, 10 and 17 are known as α -secretases cleaving APP into non-amyloidogenic forms (Buxbaum et al., 1998; Koike et al., 1999; Lammich et al., 1999) preventing accumulation of amyloidogenic A β peptides to cerebral plaques that cause Alzheimer's disease. In addition, ADAM8 and ADAM33 were shown to cleave a peptide derived from the α -secretase cleavage site (Amour et al., 2002; Zou et al., 2004). But in co-transfection experiments, ADAM33 did not cleave APP but worked as a negative regulator of APP shedding (Zou et al., 2004). Moreover, no difference in production of APP cleavage products in cultured hippocampal neurons from ADAM9 KO mice was detected compared to wild type neurons, arguing against an important role of ADAM9 as α -secretase (Weskamp et al., 2002). However, mice slightly overexpressing ADAM10 produced more neurotrophic sAPP and less amyloidogenic A β peptides suggesting that activation of ADAM10 α -secretase activity might be a therapeutic target for treatment of Alzheimer's disease (Postina et al., 2004).

Therefore, analysis of the exact roles of different ADAMs as potential α -secretases or as negative regulators is of medical interest. Possibly, endogenous α -secretase is composed of several ADAM enzymes (Asai et al., 2003). The upregulation of ADAM8 expression in the nervous system under inflammatory conditions argues for ADAM8 belonging to this set of α -secretases (Schlomann et al., 2000). ADAM8 might contribute to enhanced production of neuroprotective sAPP under pathological conditions in order to compensate neurodegeneration as an additional α -secretase. This could mean that ADAM8 has a neuroprotective function in the CNS during inflammation.

4.4.3 ADAM8 in immune and inflammatory response

TNF- α is a potent cytokine with critical functions in the activation and regulation of immune and inflammatory responses via two different membrane-type TNF receptors. TNF-R1 and TNF-R2 share similarities in the extracellular domains but differ in the intracellular domains and their signal transduction. Upon binding of TNF- α to TNF-R1, a caspase cascade as well as nuclear factor- κ B (NF- κ B) is activated via the intracellular “death domain” of TNF-R1. The activation of caspases leads to apoptosis, the NF- κ B pathway to the production of cytokines and proteins with potential anti-apoptotic activity. Thus, the activation of TNF-R1 by TNF- α induces potential inflammatory and anti-inflammatory pathways (Hehlgans and Mannel, 2002).

In order to determine the role of TNF- α in brain injury, TNF-R1-KO mice were generated. Damage to neurons caused by focal cerebral ischemia and epileptic seizures was enhanced in TNF-R1-KO mice, and activation of microglial cells was suppressed (Bruce et al., 1996). These findings demonstrated that there was a potent effect of TNF- α on the injured CNS. TNF-receptor-associated periodic syndrome (TRAPS) is a hereditary autoinflammatory disease with periodic fever attacks and severe localised inflammation. It is likely that TRAPS is caused by mutations in the extracellular domain of TNF-R1 leading to impaired shedding of TNF-R1 possibly because of conformational changes in TNF-R1 (Galon et al., 2000). Knock-in mice expressing a mutated non-sheddable TNF-R1 developed Toll-like receptor dependent innate immune hyperreactivity leading to efficient reactions against intracellular bacterial infections but also to disbalanced, pathological inflammatory reactions such as spontaneous hepatitis, enhanced susceptibility to endotoxic shock, exacerbated TNF-dependent arthritis, and experimental autoimmune encephalomyelitis (Xanthoulea et al., 2004).

Therefore, TNF- α was supposed to serve function in neuroprotection and regulation of immune response via TNF-R1 by activation of intracellular signalling pathways upon binding to this receptor. Furthermore, soluble TNF-R1 might have antagonistic function by acting as a new ligand for other receptors or by capturing TNF- α . ADAMs might be involved in these processes either directly by shedding of TNF- α or its receptors or indirectly by their activation induced by TNF- α . ADAM17 was shown to act as a sheddase for TNF- α and for the receptors TNF-R1 and TNF-R2 (Black et al., 1997; Moss et al., 1997; Peschon et al., 1998). The results of the peptide assay presented in this work indicate that ADAM8 can act as a sheddase for TNF- α and the receptor TNF-R1. Furthermore, ADAM8 was shown to be involved in TNF- α response as a target gene of TNF- α induction. ADAM8 detected in

neurons and oligodendrocytes in the CNS under normal conditions was up-regulated in the CNS upon neurodegeneration followed by activation of glia cells, astrocytes and microglia. The inducer for enhanced transcription was the cytokine TNF- α (Schlomann et al., 2000).

In *in vivo* experiments, it was examined whether ADAM8 is involved in immune response as a sheddase of TNF-R1. In ADAM8 overexpressing brain tissues of WR mice, enhanced soluble TNF-R1 release was detected in comparison to normal brain tissues and also to brain tissues of ADAM8 deficient WR mice indicating ADAM8 acting as a TNF-R1 sheddase. The neurodegenerative phenotype of ADAM8 deficient mice with a genetic WR background was enhanced. These results argue for neuroprotective functions of ADAM8 in the nervous system which are in accordance with the observations made in the TNF-R1-KO mice. The following scenario is possible (see figure 4.4.3): TNF- α released from the cell surface binds to TNF-R1 inducing ADAM8 transcription by intracellular signalling. Expressed ADAM8 protein on the cell surface is able to cleave TNF-R1. Released, soluble forms of TNF-R1 capture soluble TNF- α and act as antagonistic ligands inducing anti-inflammatory signalling pathways. Thereby, target cells are desensitised to high TNF- α concentration under inflammatory conditions and a dose-dependent cellular response is maintained (Moss and Bartsch, 2004).

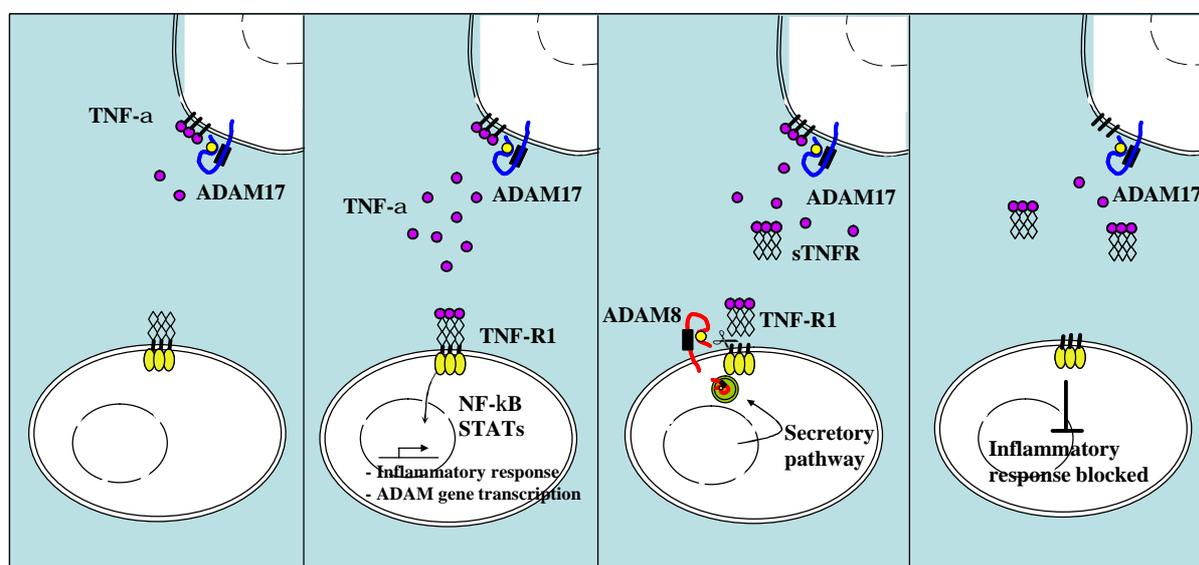


Figure 4.4.3: Hypothetical feedback mechanism for TNF- α response. TNF- α is released from the cell surface by ADAM17 with soluble forms of TNF- α mediating inflammatory response. Via the receptor TNF-R1, TNF- α stimulates ADAM8 transcription. Under pathological conditions increased concentrations of soluble TNF- α lead to enhanced ADAM8 expression. We hypothesise that TNF-R1 is released from the cell surface by ADAM8. By a feedback mechanism, the inflammatory response via TNF-R1 signalling would be blocked. Soluble TNF-R1 could capture TNF- α or act as anti-inflammatory signalling molecule (modified from Moss and Bartsch, 2004).

TNF-R1 shedding involved interactions with regulatory ectoproteins. The amino peptidase regulator of TNF-R1 shedding (ARTS-1) – a type II integral membrane protein – bound to the TNFR1 extracellular domain. The formation of a TNF-R1-ARTS-1 molecular complex with respective proteases might represent a mechanism for regulation of TNF-R1 shedding (Cui et al., 2002). Possibly, ARTS-1 interacts with ADAM8 recruiting TNF-R1 into a complex, thereby regulating shedding of TNF-R1.

Der P1 is the major allergen of the house dust mite *Dermatophagoides pteronyssinus*. It is a cysteine protease known to initiate allergic reactions in some people upon inhalation via cleavage of CD23 on B-cells leading to up-regulated IgE synthesis (Schulz et al., 1995). As peptides derived from CD23 were shown to be cleaved by ADAM8 (Fourie et al., 2003), ADAM8 was supposed to be involved in allergic diseases in the lungs such as asthma (King et al., 2004). Possibly, Der P1 is also able to enhance IgE production indirectly via ADAM8. It is hypothesised that Der P1 can release the prodomain of ADAM8 and thereby converting it to its active form. This could lead to enhanced CD23 cleavage and increasing IgE production (personal communication, F. Shakib, Nottingham University, UK).

4.4.4 ADAM8 in growth regulation

The epidermal growth factor receptor (EGF-R) is a tyrosine kinase receptor with important function in development and in diseases such as cancer. EGF-R signalling via G-protein-coupled receptors (GPCR) requires activation of EGF-R ligands (Fischer et al., 2003) and dimerisation of EGF-R. For TGF- α and EGF, it was shown that two EGF-R dimerise through peptide loops that are only exposed upon ligand binding. Thus, two separate binding events must occur for dimerisation of EGF-R and for forming a functional signalling dimer. With limited concentration of EGF-R ligands, dimerisation is more probable when soluble forms of ligands bind to EGF-R, as the receptors are less mobile when ligands are still tethered to adjacent cells. Therefore, EGF-R signalling might be regulated via shedding of EGF-R ligands (Blobel, 2005). Several ADAMs, including ADAM10, 12 and 17 have been reported, to be implicated in shedding of at least six of the seven known EGF-R ligands TGF- α , amphiregulin, HB-EGF, epiregulin, EGF, betacellulin and epigen (Sahin et al., 2004).

ADAM17 deficient mice resembled mice lacking TGF- α or EGF-R and had defects in maturation and morphogenesis of endothelial cells (Peschon et al., 1998). HB-EGF is expressed in the developing heart in monolayers of endocardial cells overlaying the endocardial cushion. HB-EGF and ADAM17 deficient mice as well as knock-in mice with an uncleavable HB-EGF had similar defects in heart development. Severe defects in

morphogenesis in the endocardial cushion resulted in enlarged heart valves. EGF-R was supposed to have an inhibitory effect on the proliferation of the endocardial cushion. Therefore, HB-EGF released from the cell surface by ADAM17 might block proliferation of cells in the endocardial cushion (Jackson et al., 2003; Yamazaki et al., 2003).

Based on the peptide assays presented in this work ADAM8 is a potential TGF- α sheddase. In the ADAM8 deficient mouse no major defects or abnormalities were evident during development arguing against an important role of ADAM8 as an EGF-R ligand sheddase during development. As upregulated in several kinds of cancers, ADAM8 might be involved in regulation of EGF-R signalling in cancer tissues by TGF- α release (Ishikawa et al., 2004). Soluble TGF- α might stimulate cell proliferation and therefore tumour growth via EGF-R. However, the effect of soluble TGF- α could also be inhibitory for cell proliferation similar to the effect of soluble HB-EGF in endocardial cushion.

4.4.5 Possible involvement of ADAM8 in leukocyte extravasation

Leukocyte extravasation from blood into underlying tissues to sites of infection and inflammation is a multistep process involving leukocyte tethering and rolling on endothelial cells, tight adhesion and migration into endothelial tissue. P-Selectin and P-Selectin glycoprotein ligand-1 (PSGL-1) are important proteins mediating leukocyte rolling. P-Selectin is a cell adhesion molecule located in intracellular vesicles in normal endothelial cells. Upon induction by paracrine inflammatory signals, P-Selectin is secreted to the cell surface of endothelial cells. PSGL-1 is constitutively expressed on most circulating leukocytes and a ligand for P-Selectin, thereby mediating tethering of leukocytes to endothelial cells. To ensure cell adhesion, expression of integrin subunits on leucocytes is induced by platelet activating factor (PAF) (McEver and Cummings, 1997; Figure 4.4.5).

PSGL-1 was shown to be less present on the cell surface of human neutrophils, monocytes, and eosinophils upon stimulation with PAF and phorbol ester PMA. This was supposed to be the result of enhanced release from the cell surface, as soluble forms were detected in supernatants of activated neutrophils and in human bronchoalveolar lavage fluids upon induction with allergic asthmatic subjects. PSGL-1 release was inhibited by EDTA, but not by phenanthroline, batimastat and marimastat. The activation-induced down-regulation of PSGL-1 resulted in significant reductions in binding of neutrophils to immobilised P-Selectin. It was discussed whether a divalent cation-dependent sheddase or another mechanism was responsible for PSGL-1 release (Davenpeck et al., 2000).

If a metalloprotease was responsible for PSGL-1 cleavage, the broad-range MMP inhibitor phenanthroline should also inhibit release in addition to EDTA. ADAM8 catalytic activity is inhibited by EDTA, phenanthroline and batimastat, but not by marimastat as shown in the presented work and by Schlomann et al. (2002). Furthermore, ADAM8 shedding activity is not induced by PMA. Nevertheless, the expression profiles of ADAM8 and PSGL-1 in the same cell types argue for a distinct protease-substrate relationship. ADAM8 mRNA was detected in different hematopoietic cell lines like macrophages, granulocytes, monocytes and B-cells (Yoshiyama et al., 1997) and was upregulated under inflammatory conditions (Schlomann et al., 2000). Therefore, release of PSGL-1 by ADAM8 might be possible in *cis* on leukocytes in an enhanced manner under inflammatory conditions.

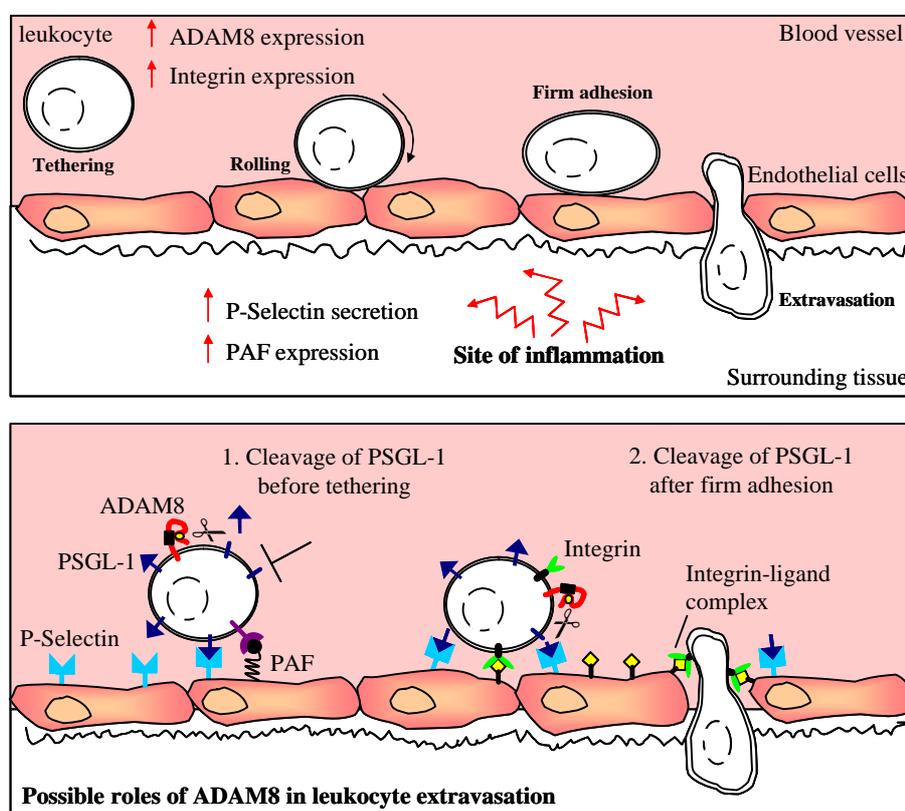


Figure 4.4.5: Possible roles of ADAM8 in leukocyte extravasation. Leukocytes normally circulate in the blood unattached to the cells. Upon inflammatory signals, release of P-Selectin to the cell surface of endothelial cells, PAF expression in epithelial cells as well as ADAM8 expression in leukocytes is induced. Weak adhesion between P-Selectin on endothelial cells and PSGL-1 on leukocytes causes tethering and rolling of leukocytes. By interaction of PAF on endothelial cells with a receptor on leukocytes, integrin expression in leukocytes is induced. The interactions of these integrins with cell adhesion molecules on endothelial cells enable firm adhesion and subsequent extravasation of leukocytes into tissues. PSGL-1 is a potential substrate for ADAM8. Cleavage of PSGL-1 before tethering to endothelial cells would decrease attachment of leukocytes to endothelial cells and extravasation to sites of inflammation. Cleavage of PSGL-1 after firm adhesion of leukocytes to endothelial cells possibly enables easier extravasation.

Release of PSGL-1 from the cell surface of monocytes before binding to P-Selectin on epithelial cells could decrease tethering, binding and extravasation into tissue to sites of inflammation. ADAM8 mediating this shedding would have anti-inflammatory function. However, it is more likely that shedding would occur after PSGL-1 had bound to P-Selectin as secretion of P-Selectin to the cell surface upon paracrine inflammatory stimuli is faster than activation of monocytes and upregulation of ADAM8 expression. Cleavage of a PSGL-1/P-Selectin complex might occur when monocytes are already attached to epithelial cells via induced integrins. PSGL-1 is not expressed on mature macrophages arguing against a role of the PSGL-1/P-Selectin complex for further extravasation. Therefore, it can be hypothesised that cleavage of this complex is necessary for further migration of macrophages into tissues. In that case, ADAM8 would have pro-inflammatory functions.

4.5 Role of ADAMs in malignant brain tumours

In several studies, increased levels of ADAMs were detected in cancer cells indicating involvement of ADAMs in cancer. ADAMs have potential implication for metastasis of cancer cells via cell adhesion and protease activity. Besides identification of ADAMs as molecular markers for diagnosis and prognosis of cancer, little work was done on characterising the role of ADAMs in cancer progression. In the work presented here (Wildeboer et al., submitted), ADAM expression and catalytic activities of ADAMs were examined in primary brain tumours with different grades of malignancy.

From twelve cerebrally expressed ADAM genes examined, strong upregulation of gene expression was observed for ADAM8 and ADAM19 in malignant brain tumour tissues, whereas ADAM28 expression was upregulated in most examined tumour tissues. Immunoblotting revealed processed forms of ADAM8 and ADAM19 in tissues with upregulated gene expression. The CD23 peptide was increasingly cleaved by all tested tumour tissue homogenates compared to normal brain as a result of increased ADAM8 expression. In homogenates of oligoastrocytoma, grade II, an additional protease which was not inhibited by TIMP1 or TIMP3 was supposed to cleave the CD23 peptide. Cleavage of the CRDA19 peptide was detected in the malignant glioma tumours AIII and GBM. Cleavage in GBM was supposed to be specific for ADAM19, whereas in AIII another metalloprotease did probably also cleave the peptide. Thus, the role of ADAMs in the progression of brain tumours can be based on catalytic activity towards proteins that are involved in the biological behaviour of brain tumours. Shedding of immune-mediating receptors or ligands such as TNF- α and TGF- α could lead to tumour induced immunosuppression. Cleavage of molecules involved in

growth control such as neuregulin or EGF receptors could influence progressive malignancy of astrocytic brain tumours. EGF receptors were upregulated in malignant astrocytomas (Kleihues et al., 2002). NRG-1 shed by ADAM19 was shown to activate erbB-2/erbB-3 receptors (Gollamudi et al., 2004), and erbB-2 was detected in highly malignant gliomas (Andersson et al., 2004).

Characteristic for gliomas is the diffuse infiltration of surrounding brain tissue. As a number of MMPs have been shown to contribute to diffuse infiltration of astrocytic tumour cells (e.g. Belien et al., 1999; Deryugina et al., 1997; Lakka et al., 2002), related ADAMs are also candidates to contribute to infiltration. ADAM28 showed strong upregulation in infiltrative astrocytic tumours, whereas ADAM8 and 19 were predominantly upregulated in malignant tumours while infiltrative low grade astrocytoma and oligoastrocytoma only showed modest upregulation. In migration experiments with matrigel as a substrate, ADAM8 cDNA transfected to either COS7 or NIH3T3 cells enhanced invasive activity in comparison to mock transfected cells (Ishikawa et al., 2004). Similar experiments with transfected glioma cells could demonstrate, whether ADAM8 contributes to migration and infiltration of glioma cells. Some MMPs and also membrane-type proteases such as MT-MMPs and ADAMs have been shown to be implicated in the angiogenic switch that occurs in the course of progressive malignancy (Bauvois, 2004; Deryugina et al., 2002; Fang et al., 2000). ADAM19 expression correlated to neovascularisation suggests that it can be involved in this process e.g. by shedding of pro-angiogenic factors such as TGF- α or TNF- α .

4.6 Conclusion and perspective

In the work presented here, several new potential ADAM8 substrates were determined and for some the physiological relevance has been proven. In future, the remaining potential substrates identified on the basis of peptide assays will be further examined in *in vivo* studies to validate the biological relevance as ADAM8 substrates.

The identification of ADAM substrate is a prerequisite for understanding the biological role of ADAMs in development, homeostasis and diseases. Ectodomain shedding by ADAMs has been recognised to be important in several signalling pathways such as EGF-R signalling or TNF-signalling and also in neuronal diseases such as Alzheimer's disease and MS making ADAMs to potential drug targets in treatment of different diseases. Enhancing ADAM8 catalytic activity in Alzheimer's disease could increase α -secretase activity which could have a positive effect on slowing down the accumulation of amyloidogenic peptides to plaques causing dementia. The EGF-R signalling pathway can be a target for cancer treatment.

Knowledge about the exact roles of ADAMs in general or of ADAM8 in particular in EGF-R signalling could help to find potential drug targets for treatment of cancer progression. As ADAM8 is involved in TNF- α signalling and CD23 cleavage, ADAM8 is implicated in allergic reaction such as in asthma and could serve as a drug target.

Although catalytic activity of ADAM8 is a potential drug target for several diseases, its exact substrate repertoire and regulation mechanisms need to be analysed in order to design drugs – either activators or inhibitors of catalytic activity – specifically for ADAM8 and for specific pathways. Broad range metalloprotease inhibitors such as batimastat were used in clinical trials treating tumours and metastasis. Batimastat influenced activities of several metalloproteases in an unspecific manner with too many side effects. Thus, it is of importance to design more specific inhibitors. Structural analysis will be helpful to determine differences in the catalytic sites and to design specific inhibitors. From recent data, we know that the ADAM8 prodomain is an effective inhibitor of ADAM8, ADAM10 and ADAM17 catalytic activities (personal communication, M.Moss, Biozyme Inc., NC, USA). A derivative of the respective inhibitory amino acid sequence would be a promising inhibitor for *in vivo* studies.

5. References

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Major parts of this work is/will be published in the following articles.

Original contribution

Naus, S., Richter, M., Wildeboer, D., Moss, M., Schachner, M., and Bartsch, J. W. (2004). Ectodomain shedding of the neural recognition molecule CHL1 by the metalloprotease-disintegrin ADAM8 promotes neurite outgrowth and suppresses neuronal cell death. *J Biol Chem* **279**, 16083-90.

Wildeboer, D., Naus, S., Sang, Q.X., Bartsch, J.W., Pagenstecher, A. (2005). Distinct expression levels and activities of human Metalloprotease-Disintegrins including ADAM8 and ADAM19 in primary brain tumours. Submitted.

Naus, S., Reipschläger, S., Wildeboer, D., Lichtenthaler, S., Bartsch, J.W. A “selective” degradomics approach to identify novel candidate substrates of the Metalloprotease-Disintegrin ADAM8. In preparation.

Book chapter

Bartsch, J.W., Naus, S., Rittger, A., Schlomann, U., Wildeboer, D. ADAM8/MS2/CD156a – A metalloprotease-disintegrin involved in immune responses. The ADAM Family of Proteases. Series: Proteases in Biology and Disease, Vol. 4. Hooper, Nigel M.; Lendeckel, Uwe (Eds.).

6. Appendix

6.1 DNA sequences

6.1.1 Mouse ADAM8

GTGCTCCAGATCCCATCATGCTTGGCCTCTGGCTGCTCAGCGTCTTATGGACACCAGCAGTAGCCCTGGACCTC
 CTTTGCCCCATGTGAAACAGTATGAAGTGGTTTGGCCTCGGGCCTAGCTGCATCCCGCTCCCGCAGAGCCCTGC
 CCTCCACTGGGGCCAGTACCCAGAGAGTCTGAGCTATGCTCTTGGGACCAGCGGGCACGTTTTCCACCCTGCACC
 TTCGAAAGAACAGGGACCTGCTGGGCTCAAGCTACACAGAGACCTACTCAGCTGCCAATGGCTCTGAGGTGACAG
 AGCAACTGCAGGAGCAGGACCATTGCCTCTACCAAGGCCATGTGGAAGGGTACGAGGGCTCAGCTGCCAGTATTA
 GCACCTGTGCTGGCCTCAGGGGCTTTTTCCGAGTTGGGTCCACTGTCCACTTGATTGAGCCTCTGGATGCTGATG
 AAGAGGGGCAACATGCGATGTATCAGGCAAAGCATCTGCAACAGAAGGCTGGGACCTGTGGGGTCAAAGATACCA
 ACCTGAATGACCTAGGGCCTCGGGCATTAGAAATATACAGGGCTCAGCCACGGAACCTGGCTGATACCCAGAGAAA
 CCCGCTATGTGGAGTTGTATGTGGTTGCAGACAGCCAAGAGTTCCAGAAGTTGGGGAGCAGAGAGGCCGTGCGCC
 AGCGAGTGTGGAGTTGTAAACCACGTGGACAAGCTTTATCAGGAACTCAGTTTTCGAGTTGTCTGGTGGGCC
 TGGAGATCTGGAACAAGGACAAATTCTACATCAGCCGCTATGCCAACGTGACACTGGAGAACCTTCTTGTCTTGG
 GGAACAGAACTTGCAAGGGCAGCACCCACATGACAACGTGCAACTTATCACGGGGTTGATTTTCATTGGGAGCA
 CTGTTGGACTGGCTAAGGTGTCTGCCCTGTGTTCCCGTCACTCCGGAGCTGTGAATCAGGACCACTCCAAGAACT
 CCATTGGTGTAGCCTCCACCATGGCCCATGAGCTGGGCCACAACCTGGGCATGAGCCATGATGAGGACATTCCAG
 GATGCTACTGTCTGAACCACGGGAGGGTGGTGGCTGCATCATGACCGAAAGCATCGGCTCCAAGTTCCCCAGGA
 TATTGAGAGGTGTAGCAAGATTGACCTAGAGTCATTCTGACAAAACCCAGACAGGCTGCCTGACCAATGTTT
 CAGATGTCAACCGGTTCTGTGGTGGCCCTGTGTGTGGAACCTGTTTGTGGAGCATGGAGAGCAGTGTGACTGTG
 GCACACCTCAGGACTGTCAAACCCCTGCTGCAATGCCACCACTTGCCAGCTGGTCAAGGGGGCAGAGTGTGCCA
 GTGGTACCTGTTGTATGAATGCAAGGTGAAGCCAGCTGGAGAGGTGTGTCTCAGTAAGGACAAATGTGACC
 TGGAGGAGTTCTGTGATGGCCGGAAGCCAACATGTCCCGAAGATGCCTTCCAACAGAATGGCACTCCCTGCCAG
 GGGGCTACTGCTTTGATGGGAGCTGTCCACCCTGGCACAGCAGTGCCGGGATCTGTGGGGGCCAGGTGCTCGGG
 TAGCAGCCGACTCCTGCTATACTTTAGCATCCCTCCGGGCTGCAATGGGAGGATGTAATCTGGCAGGATCAACC
 GGTGTGGAGCGCTGTACTGTGAGGGAGGCCAGAAGCCCTTGAACGCTCCTTCTGCACTTTCTCCTCCAACCATG
 GAGTCTGCCATGCTCTTGGCACAGGCAGCAACATTGACACCTTTGAGCTGGTATTGCAGGGCACCAAGTGCGAGG
 AGGGAAAGTTTTGCATGGATGGAAGCTGCCAGGACCTCCGTGTATACAGATCTGAAAACCTGCTCTGCTAAATGCA
 ACAACCATGGGGTATGCAACCACAAGAGGGAGTGCCACTGTGACAAGGGCTGGGCACCACCAACTGTGTACAGC
 GGCTGGCAGATGTATCAGATGAACAAGCAGCGTCTACGAGCCTCCAGTCAGTGTGGTGTGGTCTTGGTGTATCC
 TGGTGGCTGCGATGGTCATCGTGGCAGGCATCGTCTATACCGAAAGGCTCCGAGACAAAATCCAGAGGAGGAGTG
 TGGCACCCAAGCCTATCTCGGGGCTCTCCAACCCCTATTCTACACAAGGGACAGCAGCCTGCCAGCTAAGAACA
 GGCCTCCAGACCCTTCTGAGACAGTTTCTACCAACCAGCCCCAAGACCCATAGTGAACCAAAAGAGGCCCTCCCC
 CTGCACCTCCAGGTGCTGTGTCCAGTTCACCACTCCAGTTCTGTTTATGCCCCAAAAGATACCAAATCAGTTTA
 GACCTGATCCTCCCACCAAGCCCTCCCAGAGCTGAAACCCAAGCAGGTCAAGCCAACCTTTGCACCCCGACAC
 CACCAGTCAAGCCCGGACTGGAGGGACGGTGCCTGGAGCAACTCAGGGAGCTGGTGGACCAAAGGTTGCTCTGA
 AGTCCCCATCCAGAAGAGGTGACCAGCTAGGGCACCCAGGGCCATCGTTTGTGGACGTTTGGAGATACCACTG
 CTCCTATAAATGTGTTCTTTCAGCAACACCACAACCACCCAGGCTGGACCTGCACACGCTGTTCTACCTACCTCA
 GGAATAATCTTTCTGTGGCTCTTGTGTCCAGATGCCCTCATGAACCAGTGGCCTCTCACTGGCCTTGGCCAGCT
 TAGACCATAACATCAGCGTATGCTAGTTGCTAGGCAGGGGCGGTGTACCCTGCAGTGGGCGTGATCAAGTCTGTCT
 GTCTCTGGGTGGACAGAATGCCAGGAGTCCCCTCCCAGCAACTTCTGCACTGGCCCTCACACAGTGACCTCTCTC
 CAAGGCACAGTTCCTGTATCTACATCCCCTCGTGTATGAGACTTAGGAAAAGGAAAAGACGTCGTTCTTGGGGTG
 TTGGGACAAGAAGTTGCCGTCTCTCCGGATTGAAACAGATTAGTTGTTGAAATTATGACTAAACACTATTCACT
 AATAAAGATTCTATTTTATCAATAGATTATAGAT

- DNA region cloned into pSecTag 2B vector encoding the pro and metalloprotease domain

6.2 Protein sequences

6.2.1 Mouse ADAM8

MLGLWLLSVLWTPAVAPGPPLPHVKQYEVVWPRRLAASRSRRALPSHWGQYPELSYALGTSGHVFTLHLRKNRD
LLGSSYTETYSAANGSEVTEQLQEQDHCLYQGHVEGYEGSAASISTCAGLRGFFRVGSTVHLIEPLDADEEGQHA
MYQAKHLQKAGTCGVKDTNLDL**GPRALEIYRAQPRNWLIP**RETRYVELYVVADSQEFQKLGSRVAVRQRVLEV
VNHVDKLYQELSFVRVVLVGLEIWNKDKFYISRYANVTLENFLSWREQNLQGHQHPHDNVQLITGVDFIGSTVGLAK
VSALCSRHS GAVNQDHSKNSIGVASTMAHELGHNLGMSHDEDIPGCYCPREPREGGCIMTESIGSKFPRIFSRCS
KIDLESFVTKPQTGCLTNVPDVNRFVGGPVCNLFVEHGEQCDCGTPQDCQNPCCNATTCQLVKGAECASGTCCH
ECKVKPAGEVCRLSKDKCDLEEFCDGRKPTCPEDAFQQNGTPCPGGYCFDGSCTLAQQCRDLWGPARGARVAADSC
YTFSIIPPGCNGRMYSGRINRCGALYCEGGQKPLERSFCTFSSNHGVCHALGTGSNIDTFELVLQGTKCEEKVC
DGSCQDLRVYRSENC SAKCNNHVCNHNKRECHCHKGWAPPNCVQRLADVSDEQAASTSLP**VSVVVVLVILVAAMV**
IVAGIYIYRKAPRQIQRRSVAPKPISGLSNPLFYTRDSSLPAKNRPPDPSETVSTNQPPRPVVKPKRPPPPAPPGA
VSSSPLPVPVYAPKIPNQFRPDPPTKPLPELKPQVKPTFAPPTPPVKPGTGGTVPGATQGAGEPKVALKVP IQK
R

- transmembrane domain
- region covered by synthetic peptides
- autocatalytic cleavage sites (Schlomann et al., 2002)

6.2.2 Mouse APP

MLPSLALLLLAAWTVRALEVPDTGNAGLLAEPQIAMFCGKLNMMHNVQNGKWESDPSGKTCIGTKEGILQYCQE
VYPELQITNVVEANQPVTIQNWCKRGRKQCKTHTHIVIPYRCLVGEFVSDALLVPDKCKFLHQERMDVCEETHLHW
HTVAKETCSEKSTNLHDYGMLLPCGIDKFRGVEFVCCPLAEESSDVSADAEEDSDVWVGADTDYADGGEDKV
VEVAEEEEVADVEEEEEADDEDVEDGDEVEEEAEPEYEEATERTTSTATTTTTTTSVSEVVREVCSEQAETGPC
RAMISRWFYDVTGKCVFFYGGCGGNRNNDTDEEYCMVCGSVSTQSLKTTSEPLPQDPDKLPPTAASTPDAV
DKYLETPGDENEHAHFQKAKERLEAKHRERMSQVMREWEAEERQAKNLPKADKKAVIDQHFQEKVESLEQEEANER
QQLVETHMARVEAMLNDRRLALENYITALQAVPPRPHVFNMLKKYVRAEQKDRQHTLKHFEHVRMVDPKKAAQ
IRSQVMTHLRVIYERMNQSLSLLYNVPAVAEEIQDEVDLQKEQNYSDDLANMISEPRI SYGNDALMPSLTET
KTTVELLPVNGEFSLLDLPWHPFGVDSVPANTENEVEPVDARPAADRGLTTRPGSGLTNIKTEEISEVKMDAEF
GHDSGF**EVHRQKLVFF**AEDVGSNK**GAIIGLMVGGVVIATVIVITLVML**KKKQYTSIHGGVVEVDAAVTPEERHLS
KMQQNGYENPTYKFFEQMQN

- transmembrane domain
- region covered by synthetic peptides
- α -secretase cleavage sites (e.g. Amour et al., 2002; Roghani et al., 1999)
- alternative α -secretase cleavage site for ADAM10 (Amour et al., 2002)

6.2.3 Mouse CHL1

MMELPLCGRGLILSLIFLLKLKLSAAEIPLSVQQVPTIVKQSYVQVAFPFDEYFQIECEAKGNPEP IFSWTKDDKP
FDLSDPRI IAANNSGTFKIPNEGHI SHFQGYRCFASNRLGTA VSEEIEFIVPGVPKFPKEKIEPIDVEEGDSIV
LPCNPPKGLPPLHIYWMNIELEHIEQDERVYMSQRGDLYFANVEENDSRNDYCCFAAFPKLRTIVQKMPMKLTVN
SSNSIKQRKPKLLLPQAQMSLSAKTVLKGDTLLLECFAEGLPTPHIQWSKPGSELPEGRATIEVHEKTLKIENI
SYQDRGNRYRCTANLLGKASHDFHVTVEEPPRWKKKQSAVYSTGSSGILLCEAEGEPQPTIKWRLNGLPIEKHP
FPGDFMFPREISFTNLLPNHTGVYQCEASNIHGTILANANIDVIDVIPLIKTKNEENYATVVGYSAFLHCEYFAS
PKATVVWEVADETHPLEGDRYHTHENGTLEIYRTTEEDAGSYSCWVDNAMGKAVITANLDIRNATKLRVSPKNPR
IPKSHVLELYCESQCDSHLKHSKLSWSKDGEAFEMNGTEDGRIVIDGAYLTI SNITAEDQGVYSCSAQTS LDST
SKKTQVTVLGVGDPPETFTCQKDKNRSVRLREAGDDHNSKASTIVEFEGNREEPGKWEELTRVQGEETDVVLS
LAPYVRYQFRVTAVNEVGRSHASLPSDHHETPPAAPDKNPQNIRVQASQPKEMI IKWEPLKSMEQNGPGLEYKVS
WK**PQ**GAPPEWEEIEVTNHTLRVMTPTVYAPYDVVKVQAINQLGSSDPQPVTLYSGEDYPSTAPVIQRVDMNSTL
VKVTWSSIPKETVHGLLRGYQINWWTKSLLDGRTHPKEVNILRFSGQRNSGMVPSLDPFSEFHLTVLAYNSKGA
GPESEPIYFQTPGVPPEQPSFLKVIKVDKDTATLSWGLPKKLNGLTG YLLQYQIINDTYELGELNEINVTTPSK
SSWHLNLSNTTKYKLYLRACTSRGCGKPISEEGATLGEKSGKIRKITEGVNVTQK IHPVEVLV**PGA**EHI VHLMT
KNWGDNDISIFQDVIETRGREYAGLYDDISTQGWFI GLMCAIALLTLILLTICFVKRNRGKYSVKEKEDLHPDPE
VQSAKDETFGEYSDSDEKPLKGLRSLNRNMQPTESADSLVEYEGEGDQSI FNEDGSF IGAYTGAKEKGSVESNGS
STATFPLRA

- cleavage site resulting in 125 kDa fragment (Naus et al., 2004)
- cleavage site resulting in 165 kDa fragment (Naus et al., 2004)

6.2.4 Mouse TNF-R1

MGLPTVPGLLLSLVLLALLMGIHPSGVTGLVPSLGDREKRDSLCPQGGKYVHNSKNNICCTKCHKGTYLVSDCPSP
 GRDTVCRECEKGTFTASQNYLRQCLSCKTCRKEMSQVEISPCQADKDTVCGCKENQFQRYLSETHFQCVDCSPCF
 NGTVTIIPCKETQNTVCNCHAGFFLRESECVPCSHCKKNEE**CMKLCCLPPPLANVTNPQDSGTAVLLPLVILLGLCL**
LSFIFISLMCRYPRWRPEVYSIIICRDPVPVKEEKAGKPLTPAPSPAFSPTSGFNPTLGFSTPGFSSPVSSSTPI
 IFGPSNWHFMPVSEVVPTQGADPLLYESLCSVPAPTSVQKWEDSAHPQRPDNADLAILYAVVDGVPPARWKEFM
 RFMGLSEHEIERLEMQNGRCLREAQYSMLEAWRRRTPRHEDTLEVVGVLVLSKMNLACLENILEALRNPAPSSTT
 RLPR

- transmembrane domain
- region covered by synthetic peptides

6.3 DNA nucleotide letter code

A	deoxyadenylate
C	deoxycytidylate
G	deoxyguanylate
T	thymidylate

6.4 Amino acid letter code

A	alanine
C	cysteine
D	aspartic acid
E	glutamic acid
F	phenylalanine
G	glycine
H	histidine
I	isoleucine
K	lysine
L	leucine
M	methionine
N	asparagine
P	proline
Q	glutamine
R	arginine
S	serine
T	threonine
V	valine
W	tryptophan
X	any amino acid
Y	tyrosine

6.5 Abbreviations

AI	pilocytic astrocytoma, astrocytoma WHO grade I
AIII	anaplastic astrocytoma, astrocytoma WHO grade III
AA	amino acid
A8-EC	soluble, recombinant form of ADAM8 containing the whole extracellular portion of ADAM8
A8-ProMP	soluble, recombinant form of ADAM8 containing the pro and metalloprotease domain
A β	amyloid β peptide; depending on the protease cleaving the amyloid beta A4 protein precursor (APP), either an amyloidogenic A β peptide or a non-amyloidogenic p3 peptide is generated; A β forms accumulate in the brain to non-cleavable plaques causing Alzheimer's disease
Abl	proto-oncogene tyrosine-protein kinase ABL1; synonyms: p150, c-ABL
ADAM	a disintegrin and metalloprotease
ADAM-TS	a disintegrin and metalloproteinase with thrombospondin motifs
ALS	amyotrophic lateral sclerosis
APP	amyloid beta A4 protein precursor; synonyms: APP, ABPP, Alzheimer's disease amyloid protein homolog, amyloidogenic glycoprotein (AG)
APS	ammonium persulfate
AR	amphiregulin precursor; synonyms: AR, schwannoma-derived growth factor (SDGF)
ARTS-1	amino peptidase regulator of TNFR1 shedding
BB-5216	marimastat
BB-94	batimastat
bp	base pairs
BSA	bovine serum albumin
BTC	betacellulin precursor
CD	clusters of differentiation; groups of monoclonal antibodies that identify the same cell surface molecule; the cell surface molecule is designated CD followed by a number;
CD23	low affinity immunoglobulin epsilon Fc receptor; synonyms: lymphocyte IGE receptor, Fc-epsilon-RII, CD23 antigen
CD36	platelet glycoprotein IV, synonyms : GPIV, GPIIIB, CD36 antigen, PAS IV, PAS-4 protein

CD163	macrophage hemoglobin scavenger receptor precursor
CD27-L	tumour necrosis factor ligand superfamily member 7; synonyms: CD27 ligand, CD27-L, CD70 antigen
CD30-L	tumour necrosis factor ligand superfamily member 8; synonyms: CD30 ligand, CD30-L
CD40-L	tumour necrosis factor ligand superfamily member 5; synonyms: CD40 ligand CD40-L, TNF-related activation protein (TRAP), T cell antigen GP39
CHAPS	3-[(3-cholamidopropyl)- dimethylammonio]-1-propanesulfonate
CHL1	close homologue of L1
CNS	central nervous system
CSF-1-R	macrophage colony stimulating factor I receptor precursor; synonyms: CSF-1-R; Fms proto-oncogene, c-fms, CD115 antigen
CX3CL1	fractalkine precursor; synonyms: CX3CL1, neurotactin, CX3C membrane-anchored chemokine, small inducible cytokine D1
CXCL16	small inducible cytokine B16 precursor; synonyms: transmembrane chemokine, CXCL16, scavenger receptor for phosphatidylserine and oxidised low density lipoprotein (SR-PSOX)
DBU	1,8-Diazabicyclo[5.4.0]undec-7-ene
DEAE	diethylaminoethyl-
DIPEA	ethyl-diisopropylamine
DMEM	Dulbecco's modified eagle medium
DMF	N,N-dimethylformamide
DMSO	dimethylsulfoxide
DNA	desoxyribonucleic acid
Dnp	di nitro phenol
Dpa	L-2-amino-3-(2,4-dinitrophenyl) aminopropionic acid
ECL	enhanced chemiluminescence
<i>E.coli</i>	<i>Escherichia coli</i>
EDTA	ethylene diamine-N,N,N',N'-tetraacetic acid
EGF	pro-epidermal growth factor precursor
EGF-R	epidermal growth factor receptor
EGTA	ethylene glycol bis(beta-aminoethylether)-N,N,N',N'-tetraacetic acid
ELISA	enzyme-linked immunosorbent assay

Ephrin A2	ephrin-A2 precursor; synonyms: EPH-related receptor tyrosine kinase ligand 6, LERK-6, ELF-1, CEK7-ligand, CEK7-L
EQ-ADAM8	catalytically inactive full length form of ADAM8 with an E/Q exchange in the catalytic centre
erbB-2	receptor tyrosine-protein kinase erbB-2; synonyms: p185erbB2, c-erbB-2
erbB-3	receptor tyrosine-protein kinase erbB-3; synonym: c-erbB3
F	farad; unit of electric capacitance
Fc- γ RIII	low affinity immunoglobulin gamma Fc region receptor III precursor; synonyms: IgG Fc receptor III, Fc-gamma RIII, FcRIII
FCS	fetal calf serum
Fmoc	9-fluorenylmethyloxycarbonyl
Fyn	proto-oncogene tyrosine-protein kinase FYN; synonym: P59-FYN
GBM	glioblastoma
GHS-R	growth hormone secretagogue receptor type 1; synonyms: GHS-R GH-releasing peptide receptor (GHRP), ghrelin receptor
GPI	glycosylphosphatidylinositol
GPV	platelet glycoprotein V precursor; synonyms: GPV, CD42D
Grb2	growth factor receptor-bound protein 2; synonyms: GRB2 adapter protein, SH2/SH3 adapter GRB2
GPIb- α	platelet glycoprotein Ib alpha chain precursor; synonyms: glycoprotein Ibalpha, GP-Ib alpha, GPIbA, GPIb-alpha, CD42B-alpha, CD42B
HB-EGF	heparin-binding EGF-like growth factor precursor
HBSS	hanks balanced salt solution
HEPES	N-(2-hydroxyethyl)piperazine-N'-(2-ethanesulfonic acid)
HER-4	receptor tyrosine-protein kinase erbB-4 precursor; synonyms: p180erbB4 tyrosine kinase-type cell surface receptor HER4
HPLC	high pressure liquid chromatography
IFN- γ	interferon gamma precursor
Ig	immunoglobulin
IGFBP	insulin-like growth factor binding protein precursor; synonyms: IGFBP, IGF-binding protein (IBP)
IL-4	interleukin-4 precursor; synonyms: IL-4, B-cell stimulatory factor 1 BSF-1, lymphocyte stimulatory factor 1, IGG1 induction factor, B-cell IGG differentiation factor, B-cell growth factor 1

IL-13	interleukin-13 precursor; synonyms: IL-13, T-cell activation protein, P600
IL-1R-2	interleukin-1 receptor, type II precursor; synonyms: IL-1R-2, IL-1R-beta
IL-6R-1	interleukin-6 receptor alpha chain precursor; synonyms: IL-6R-alpha IL-6R 1
IL-15R- α	interleukin-15 receptor alpha chain precursor; synonyms: IL-15Ralpha, IL-15RA
IPTG	isopropyl- β -thiogalactopyranosid
IRF-1	interferon regulatory factor 1; synonym: IRF-1
Da	dalton; unit of molecular mass approximately equal to the mass of a hydrogen atom (1.66×10^{-24} g)
KL	kit ligand precursor; synonyms: c-kit ligand, stem cell factor (SCF), mast cell growth factor (MGF), hematopoietic growth factor KL, steel factor
L1	neural cell adhesion molecule L1 precursor; synonym: N-CAM L1
LB	Luria Bertani
Lck	proto-oncogene tyrosine-protein kinase LCK; synonyms: P56-LCK, LSK, T cell-specific protein-tyrosine kinase
LPS	lipopolysaccharide
L-Selectin	L-selectin precursor; synonyms: lymph node homing receptor, leukocyte adhesion molecule-1 (LAM-1), lymphocyte antigen 22 (Ly-22), lymphocyte surface MEL-14 antigen, leukocyte-endothelial cell adhesion molecule 1 (LECAM1), CD62L antigen
MALDI TOF	matrix-assisted laser desorption ionisation time of flight
MBP	myelin basic protein; synonyms: myelin A1 protein
Mcp	DL-2-amino-3-(7-methoxycoumaryl)-propionic acid
MMP	matrix metalloprotease
MS	multiple sclerosis
MT-MMP	membrane-type matrix metalloprotease
Mucin 1	mucin 1 precursor; synonyms: polymorphic epithelial mucin (PEMT), episialin
N1	non-toxic ectodomain of PrP generated by proteolytic cleavage
NB	normal brain
N-Cadherin	neural-cadherin precursor; synonyms: N-cadherin, cadherin-2
NCAM	neuronal cell adhesion molecule 1
NEAS	non-essential amino acids

Notch1	neurogenic locus notch homolog protein 1 precursor; synonyms: Notch 1, Notch, A, mT14, p300
NRG-1	pro-neuregulin-1 precursor; synonym: pro-NGR1
NTA	nitrilotriacetic acid
Ω	Ohm; unit of electric resistance
OAI	oligoastrocytoma II, WHO grade II
OPT	1,10- <i>ortho</i> -phenanthroline
p75NTR	tumour necrosis factor receptor superfamily member 16 precursor; synonyms: low-affinity nerve growth factor receptor, NGF receptor, low affinity neurotrophin receptor p75NTR
PAF	platelet activating factor
PAR-1	proteinase activated receptor 1 precursor; synonyms: PAR-1, thrombin receptor
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PFAR γ	peroxisome proliferator-activated receptor gamma binding protein
PI	phosphatidylinositol
PKC δ	Protein kinase C δ
PKT	Protein tyrosine kinase
P-LAP	Leucyl-cystinyl aminopeptidase; synonyms: cystinyl aminopeptidase oxytocinase, OTase, Insulin-regulated membrane amino peptidase, insulin-responsive amino peptidase (IRAP), placental leucine amino peptidase (P-LAP)
PLL	poly-L-lysine
PMA	phorbol 12-myristate13-acetate; phorbol ester
PMSF	phenylmethylsulfofluoride
PrP	major prion protein precursor; synonyms: PrP, PrP27-30, PrP33-35C
PSGL-1	P-selectin glycoprotein ligand 1 precursor; synonyms: PSGL-1, selectin P ligand
PVDF	polyvinylidene fluoride
RIPA	radio-immunoprecipitation assay
RNA	ribonucleic acid
sAAP α	ectodomain of APP with neuroprotective and memory-enhancing function generated by α -secretase cleavage
SDS	sodiumdodecylsulfate

SDS-PAGE	sodiumdodecylsulfate polyacrylamid gel electrophoresis
SH2	Src homology 2
SH3	Src homology 3
Src	proto-oncogene tyrosine-protein kinase Src; synonyms: p60-Src, c-Src
STAT	signal transducer and activator of transcription
SVMP	snake venome metalloprotease
TBS	tris buffered saline
TBTU	O-benzotriazol-1-yl-N,N,N',N'-tetramethyluroniumtetra-fluoroborate
TEMED	N,N,N',N'-tetramethylethylendiamin
TFA	trifluoroacetic acid
TGF- α	transforming growth factor alpha precursor; synonyms: TGF-alpha, EGF-like TGF (ETGF), TGF type 1
TIMP	tissue inhibitor of metalloproteases
TIPS	triisopropylsilane
TNF- α	tumour necrosis factor precursor; synonyms: TNF-alpha, tumour necrosis factor ligand superfamily member 2, TNF-a, Cachectin
TNF-R1	tumour necrosis factor receptor superfamily member 1A precursor; synonyms: tumour necrosis factor receptor 2 (TNF-R2), p60, TNF-R1, TNF-RI, p55
TNF-R2	tumour necrosis factor receptor superfamily member 1B precursor; synonyms: tumour necrosis factor receptor 2 (TNF-R2), tumour necrosis factor receptor type II, p75, p80 TNF-alpha receptor
TPA	12-O-tetradecanoylphorbol-13-acetate; phorbol ester
TRANCE	tumour necrosis factor ligand superfamily member 11; synonyms: receptor activator of nuclear factor kappa B ligand (RANKL), TNF-related activation-induced cytokine (TRANCE), osteoprotegerin ligand (OPGL), osteoclast differentiation factor (ODF), osteoclastogenesis-inhibitory factor (OCIF)
TRAPS	TNF-receptor-associated periodic syndrome
Tween20	polyoxyethylenesorbitan monolaurate
U	unit; unit for activity of restriction enzymes defined as the amount of enzyme required to digest 1 μ g of bacterial virus lambda DNA in 1 hour in a 50 μ l reaction
WR	wobbler
X-Gal	5-bromo-4-chloro-3-indolyl-beta-D-galactopyranoside

Acknowledgements

I thank Dr. Jörg W. Bartsch for supervision of my Ph.D. project, for support and helpful discussions; moreover, for his patience and a comfortable atmosphere in his research group.

I am thankful to Prof. K.J. Dietz for reviewing my dissertation.

I thank the International Graduate School in Bioinformatics and Genome Research for funding my Ph.D. project.

I thank Dirk Wildeboer for close collaboration in the last 4 years; Simone Reipschläger for providing ADAM8 cDNA constructs for expression in *E.coli*; Elke Redecker and Angela Perz for technical assistance; Dr. Uwe Schlomann for providing ADAM8, ADAM10 and ADAM17 constructs; Prof. Melitta Schachner for providing recombinant CHL1-Fc and CHL1 cDNA constructs; Dr. Olaf Kruse for help with fluorescence measurements; Kerstin Böker for N-terminal sequence determination; Prof. Horst Hinssen for help with chromatographies; Prof. Norbert Sewald and Marco Wißbrock for help with peptide synthesis; Prof. Harald Tschesche for recombinant TIMPs and BB-94; Dr. Marcia Moss for helpful comments on the thesis and my lab work; Dr. Peter Heimann for any technical help.

I am thankful to Dirk Wildeboer, Daniel Eberhard, Steffie Leuchtenberger, Uwe Schlomann, Jörg Bartsch and many others in W7 for help and interesting discussions, for coffee breaks including cakes and cookies, works outings, Christmas parties, Easter breakfasts, ...

I thank my parents for giving me the chance to change my study subject and for support during my eight years at university; at last, I want to thank Klaus Gossens for patient listening and helpful discussions and for just being Klaus.

Hiermit versichere ich, Silvia Naus, dass ich die vorliegende Dissertation eigenständig angefertigt und nur die von mir angegebenen Hilfsmittel verwendet habe.

Bielefeld, 15.04.2005