Identification of candidate substrates for ectodomain shedding by the metalloprotease-disintegrin ADAM8

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Abstract

ADAM proteases are type I transmembrane proteins with extracellular metalloprotease domains. As for most ADAM family members, ADAM8 (CD156a, MS2) is involved in ectodomain shedding of membrane proteins and is linked to inflammation and neurodegeneration. To identify potential substrates released under these pathologic conditions, we screened 10-mer peptides representing amino acid sequences from extracellular domains of various membrane proteins using the ProteaseSpot™ system. A soluble ADAM8 protease containing a pro- and metalloprotease domain was expressed in E. coli and purified as active protease owing to autocatalytic prodomain removal. From 34 peptides tested in the peptide cleavage assay, significant cleavage by soluble ADAM8 was observed for 14 peptides representing membrane proteins with functions in inflammation and neurodegeneration, among them the β-amyloid precursor protein (APP). The in vivo relevance of the ProteaseSpot™ method was confirmed by cleavage of full-length APP with ADAM8 in human embryonic kidney 293 cells expressing tagged APP. ADAM8 cleaved APP with similar efficiency as ADAM10, whereas the inactive ADAM8 mutant did not. Exchanging amino acids at defined positions in the cleavage sequence of myelin basic protein (MBP) revealed sequence criteria for ADAM8 cleavage. Taken together, the results allowed us to identify novel candidate substrates that could be cleaved by ADAM8 in vivo under pathologic conditions.

Keywords: ADAM protease; candidate substrates; ectodomain shedding; fluorescence assay; peptide cleavage.

Introduction

ADAMs (a disintegrin and metalloprotease) or MDCs (metalloprotease disintegrin cysteine-rich proteins) form a family of type I transmembrane proteins. Owing to their multidomain structure consisting of pro-, metalloprotease, disintegrin-like, cystein-rich, EGF-like, transmembrane and cytoplasmic domains, ADAMs are capable of four physiological functions: cell adhesion, cell fusion, cell signalling and proteolysis.

They are implicated in physiological processes such as fertilisation, myogenesis and neurogenesis, and are also involved in a number of pathological processes by releasing cytokines and their receptors under inflammatory conditions (Moss and Bartsch, 2004). To date, 40 members are known in different species (table of ADAMs on http://www.people.virginia.edu/~jw7g/). Approximately half of these ADAMs contain the catalytic consensus sequence HEXXHHXXGXXHD in their metalloprotease domains and are therefore predicted to be catalytically active. Proteolysis of membrane-anchored precursor proteins is a key event in signalling cascades (Blobel, 2005) and this process has been termed ectodomain shedding (Peschon et al., 1998). A number of ADAM substrates have been defined either by their physiological role or by their cellular localisation (Seals and Courtneidge, 2003; Moss and Bartsch, 2004; Blobel, 2005). So far, no consensus sequence on the substrate side has been reported, making prediction of potential cleavage sites difficult. Rather, ADAMs recognise a structural footprint on the extracellular part of the membrane protein, and it is assumed that cleavage occurs in the juxtamembranous regions of these membrane proteins.

Our particular research interest is the ADAM family member ADAM8, originally identified as MS2 or CD156 in mouse macrophages (Yoshida et al., 1990). ADAM8 is expressed in several tissues, such as thymus, cartilage, bone, brain and spinal cord, during embryonic development. As embryonic development of the ADAM8-deficient mouse appears normal, ADAM8 does not seem to be essential for developmental processes (Kelly et al., 2005). Rather, it is more likely that ADAM8 has a specific function in cytokine response. In response to inflammatory stimuli such as lipopolysaccharide (LPS) and tumour necrosis factor α (TNF-α), ADAM8 expression is upregulated in macrophages and in the central nervous system (CNS) in activated glia cells – astrocytes and microglia – indicating its involvement in neuron-glia signalling (Schlomann et al., 2000). In addition to glial cells, ADAM8 is expressed in neurons at low levels, and expression is induced by TNF-α, suggesting that under inflammatory conditions in the CNS, neuronal ADAM8 activity is significantly increased (Schlomann et al., 2000).

In the lung, ADAM8 is upregulated under experimental induction of allergic asthma by inflammatory cytokines.
(King et al., 2004), suggesting that ADAM8 plays a role in the pathogenesis of allergic asthma. All these findings underline the importance of ADAM8 in inflammatory processes.

Recent attention has focussed on ADAM8 as a molecule upregulated in various tumours. ADAM8 is considered a prognostic marker for lung adenocarcinomas (Ishikawa et al., 2004) and renal cell carcinomas (Roemer et al., 2004). In addition, in brain tumours such as glioblastoma, oligoastrocytoma, and ependymoma, ADAM8 expression was increased compared to normal brain controls, and ADAM8 expression in glioblastoma correlates with malignancy and invasive activity (Wildeboer et al., unpublished results). In B-cells, ADAM8 cleaves the low-affinity IgE receptor (CD23, FcεRII) suggesting a role in immune modulation (Fourie et al., 2003). Although ADAM8 seems to impair allergy and inflammation by ectodomain shedding, only a few substrates of ADAM8 and their corresponding cleavage sites are known (Amour et al., 2000; Schlomann et al., 2002; Fourie et al., 2003; Naus et al., 2004). In the present study, we analysed the ability of ADAM8 to cleave 10-mer peptides as representatives of the extracellular domains of membrane proteins involved in immune modulation, inflammation, and cell adhesion.

Results

Expression and purification of active ADAM8 protease from E. coli

The bacterial expression vector pASK-IBA3+ allows for expression of recombinant ADAM8 pro-/metalloprotease (A8ProMP) in the cytoplasma after induction with anhydrotetracycline (aTc). Maximal expression rates were obtained 4–6 h after aTc induction and decreased after overnight induction, probably owing to instability of the protein (Figure 1A). Expression of A8ProMP is reflected by a band of ca. 56 kDa, while a lower band of ca. 33 kDa represents the entire metalloprotease domain of ADAM8 (A8MP) after prodomain removal.

Approximately two-thirds of the total ADAM8 protein expressed in E. coli was found in inclusion bodies, and one-third in the cytoplasmic fraction. This fraction was taken for further purification using Strep-Tactin® columns (Figure 1B) without any additional treatment to increase ADAM8 solubility. After elution from the column, one fraction was collected that contained most of the ADAM8 protein (Figure 1B, C). This fraction contained a significant amount of processed ADAM8, as monitored by Western blotting (Figure 1B) and SDS-PAGE (Figure 1C), and was used in all further studies.

Activity of the recombinant ADAM8 protease

The catalytic activity of recombinant A8MP was tested using a peptide derived from the cleavage sequence of human CD23 (DNP-SHGSDMAQKSQSTQI) in a fluorescamine assay (Figure 2). Within 4 h of incubation, there was significant cleavage of the CD23 peptide, whereas no cleavage was observed in the controls, including a sample with ADAM8 protease in the presence of EDTA.

ADAM8 peptide substrates

Our focus was to characterise the role of catalytically active ADAM8 in (neuro-)inflammation. A total of 34 peptides derived from proteins involved in inflammatory processes and immune response in the nervous system were selected. In most cases they represent membrane-proximal sequences.
cleaved peptides were those derived from strongly and four to a lesser extent. Among the strongly activity was demonstrated.

The time dependence of cleavage was monitored for two selected peptides (APP and CD23.1; Figure 3B). With time incubation with A8MP in the presence of 10 mM EDTA was incubated under reaction conditions. The peptide assay was repeated at least five times and the values given are representative of all experiments performed.

All peptides, with the corresponding proteins and their accession numbers in the Swiss-Prot/TrEMBL protein database, are listed in Table 1.

Cleavage assays were performed with A8MP in the absence or presence of 10 mM EDTA. Fluorescence increases were monitored over 18 h. In cases of consistent and significant fluorescence increase and no response in the presence of EDTA, ADAM8-specific cleavage was concluded.

The relative fluorescence increase corresponding to cleavage efficiency was expressed as relative units and was normalised to the MBP peptide as 1 (Figure 3A). The relative fluorescence increase corresponding to protease activity was monitored over 18 h. The fluorescence increase was expressed in units normalised to the original bovine MBP peptide (1).

According to variable fluorescence increases, it was concluded that ADAM8 cleaved the MBP peptide variants with different efficiencies, depending on the amino acid exchange. The peptides bearing exchange of leucine or serine at position P3 (peptide 2), of threonine for alanine at position P3 (peptide 8) and basic residues -- arginine or lysine -- for a proline at position P1 (peptides 3 and 4) were cleaved by ADAM8. Possibly, there was slightly enhanced cleavage for the variants with basic amino acids in position P1 compared to the original MBP peptide. Amino acid exchanges at positions P1' and P2' had significant effects on peptide cleavage (peptides 5–7). Exchange of a glutamine residue to an acidic glutamic acid residue in position P1', as well as exchange of the basic lysine residue in position P2' to either an acidic (glutamic acid) or a neutral, aliphatic amino acid (valine) resulted in decreased levels of peptide cleavage.

From these results, we conclude that basic residues in positions P1 and P2' increase the efficiency of ADAM8 cleavage, whereas acidic residues in position P1' and P2' decrease cleavage.

**ADAM8 cleavage of MBP peptide variants**

A 10-mer peptide derived from MBP is cleaved by recombinant ADAM8 (Schlomann et al., 2002). To identify specific amino acid requirements necessary for substrate cleavage, peptide cleavage assays with various peptides derived from the MBP cleavage site were performed using the ProteaseSpot™ system. Assays were performed in the absence or presence of 10 mM EDTA (Figure 4). The fluorescence increase corresponding to protease activity was monitored over 18 h. The fluorescence increase was expressed in units normalised to the original bovine MBP peptide (1). According to variable fluorescence increases, it was concluded that ADAM8 cleaved the MBP peptide variants with different efficiencies, depending on the amino acid exchange. The peptides bearing exchange of leucine for serine at position P3 (peptide 2), of threonine for alanine at position P3' (peptide 8) and basic residues -- arginine or lysine -- for a proline at position P1 (peptides 3 and 4) were cleaved by ADAM8. Possibly, there was slightly enhanced cleavage for the variants with basic amino acids in position P1 compared to the original MBP peptide. Amino acid exchanges at positions P1' and P2' had significant effects on peptide cleavage (peptides 5–7). Exchange of a glutamine residue to an acidic glutamic acid residue in position P1', as well as exchange of the basic lysine residue in position P2' to either an acidic (glutamic acid) or a neutral, aliphatic amino acid (valine) resulted in decreased levels of peptide cleavage.

**ADAM8-dependent cleavage of APP in human embryonic kidney 293 cells**

We tested whether transfection of ADAM8 into human embryonic kidney 293 cells increased secretion of membrane-associated APP in a cell-based assay. Human 293 cells were used that stably express a fusion protein consisting of the reporter enzyme alkaline phosphatase (AP) tagged to the N-terminus of full-length APP (AP-APP cells). Upon shedding of the APP ectodomain, the soluble AP-tagged APP can easily be detected by measuring the AP activity in the conditioned medium (Lichtenthaler et al., 2003; Schöbel et al., 2006). The AP-APP cells were transfected with constructs encoding ADAM8 and inactive EQ-ADAM8, bearing a point mutation by which glutamate residue 330 is exchanged to glutamic acid (Schlomann et al., 2002). This mutant form of
Table 1 List of peptides and sequences used for screening ADAM8 candidate substrates.

<table>
<thead>
<tr>
<th>Protein</th>
<th>Peptide substrates</th>
<th>ADAMs involved</th>
<th>References</th>
</tr>
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<tr>
<td>APP</td>
<td>EVRH↓QLVFF</td>
<td>ADAM8, 9, 10, 17, 33</td>
<td>Amour et al., 2002; Buxbaum et al., 1998; Koike et al., 1999; Mohan et al., 2002; Zou et al., 2004</td>
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<td>β-Amyloid precursor protein</td>
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<td></td>
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<tr>
<td>CD16</td>
<td>SLVWYHTAPS</td>
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<td>FcεRII, low-affinity IgG receptor</td>
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<td>Accession no. P08508</td>
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<tr>
<td>CD23</td>
<td>SNQLAQ↓K↓SQV</td>
<td>MP, ADAM8</td>
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<td>FcεRII, low-affinity IgE receptor</td>
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<td>CD40-L</td>
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<td>CD163</td>
<td>HGTPGH↓ILTA</td>
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<td>Macrophage haemoglobin scavenger receptor</td>
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<td>Accession no. AAK16065</td>
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<td>Fractalkine CX3CL1</td>
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<td>IL-1RII</td>
<td>TTVKEVS↓STFa</td>
<td>ADAM8, 17</td>
<td>Amour et al., 2002; Reddy et al., 2000</td>
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<td>Interleukin-1 receptor type II</td>
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<td>Accession no. P27931</td>
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<td>KL</td>
<td>PPVAASSLRN</td>
<td>ADAM8, 9, 17, 33</td>
<td>Amour et al., 2002; Chanesuea et al., 2003; Mohan et al., 2002; Roghani et al., 1999; Zou et al., 2004</td>
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<td>Kit ligand</td>
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<td>L-Selectin</td>
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<tr>
<td>TGF-α</td>
<td>ADLLAVVAAS</td>
<td>MP, ADAM17</td>
<td>Ambas et al., 1997; Peschon et al., 1998</td>
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<td>Transforming growth factor α</td>
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<td>Accession no. P48030</td>
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<td>TNF-α</td>
<td>A↓QTLTLR↓SS</td>
<td>ADAM8, 9, 10, 17, 19</td>
<td>Amour et al., 2002; Black et al., 1997; Chanesuea et al., 2003; Mohan et al., 2002; Moss et al., 1997; Mohan et al., 2002</td>
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<tr>
<td>TNFRi</td>
<td>NPQ↓DSGTAVL</td>
<td>MP, ADAM17</td>
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<tr>
<td>Tumour necrosis factor receptor 1</td>
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<tr>
<td>TNFRiI</td>
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<td>Pinckard et al., 1997; Reddy et al., 2000</td>
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<td>Tumour necrosis factor receptor 2</td>
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<td>TRANCE</td>
<td>VGP↓QRFSGAP</td>
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<td>TNF-related activation-induced Cytokine</td>
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Most sequences represent juxtamembraneous regions of the respective proteins. All peptide sequences were derived from the mouse proteins and their Swiss-Prot/TrEMBL database accession numbers are listed (http://www.expasy.org/sprot/). Arrows indicate the ADAM8 cleavage sites identified in this work by HPLC/mass spectrometry (except a,b,c). References on cleavage of the homologous human peptides or respective membrane proteins are given in the right column. ClustalX was used to determine homologous mouse peptides. MP, undefined metalloprotease.

aData from Amour et al. (2002) from homologous human peptide sequences.

bData from Schlomann et al. (2002).

cEstimated cleavage site.
Peptide screening for potential ADAM8 substrates using the ProteaseSpot/H23008 system. Peptides selected and ADAM8 cleavage sites are listed in Table 1. (A) The fluorescence increase based on proteolytic release of the fluorophore was measured after 18 h. Fluorescence values correspond to ADAM8 activity and are given as units normalised to the myelin basic protein (MBP) peptide set to 1. Fluorescence increase \(^G_{\text{1}}\) is assumed to indicate significant cleavage (black bars), and increases of 0.5–1 (grey bars) indicate cleavage with lower efficiency. An increase \(^F_{\text{0.5}}\) (white bars) indicates no protease-substrate relationship. Cleavage analysis was performed with at least three independent ADAM8 preparations. Peptides derived from APP, CD23, fractalkine CX3CL1, FcγRIII, L-selectin, PSGL-1, TGF-α, TGF-α1, TNF-α were cleaved significantly. (B) Time-dependent increase in fluorescence after 2, 4, 6 and 18 h of incubation of APP and CD23.1 peptides with ADAM8 protease. Values were corrected for the fluorescence values at \(t=0\).

ADAM8 lacks prodomain removal and is catalytically inactive in transfection assays (Naus et al., 2004). Additional controls were full-length ADAM10 and mock vector (Figure 5). In AP-APP cells, ADAM8 was expressed in the active full-length form (Figure 5B), with a 120-kDa band for pro-ADAM8, a 90-kDa band for processed ADAM8 and a band representing the remnant form of ADAM8 of ca. 60 kDa. In contrast, EQ-ADAM8 was not processed because of the lack of autocatalytic prodomain removal (Figure 5B; Schloemann et al., 2002). Compared to mock transfected cells, active full-length ADAM8, but not the inactive EQ-ADAM8, increased AP-APP shedding (Figure 5A), revealing that ADAM8 is able to cleave APP \(\text{in vivo}\). An identical result was observed by immunoblot analysis of the same supernatants (Figure 5B) using monoclonal antibody W02, which detects \(\alpha\)-secretase-cleaved APP (Iida et al., 1996). No significant changes in the amount of full-length APP in the cell lysate were detected. This shows that ADAM8 acts as a true \(\alpha\)-secretase and cleaves APP within the Aβ domain, which fits well with the \text{in vitro} cleavage site shown in Table 1.

Interestingly, ADAM8 stimulated APP release to an extent comparable to ADAM10 (Figure 5A), which is one of the candidate \(\alpha\)-secretases that cleave APP (Lammich et al., 1999). Thus, we have demonstrated that ADAM8 might contribute to \(\alpha\)-secretase activity \(\text{in vivo}\).

**Discussion**

Active ADAM8 was isolated from \(\text{E. coli}\) because of its solubility and ability for autocatalytic prodomain removal. The rate of autocatalysis leading to active ADAM8, however, was variable for different preparations. A similar observation was made for ADAM8 expressed in COS cells. This might be because autocatalysis is less efficient with only the MP domain, whereas soluble ADAM8 containing the disintegrin/cysteine-rich domain is completely activated by prodomain removal (Schloemann et al., 2002), but would likely cause solubility problems in \(\text{E. coli}\). In addition, in terms of inhibitory profile and substrate specificity, ADAM8 from \(\text{E. coli}\) is indistinguishable from soluble ADAM8 expressed in COS cells (Schloemann et al., 2002; Naus et al., 2004), suggesting that glycosylation of the ADAM8 protein in eukaryotes is not essential for catalytic activity. Similar to ADAM8 expressed in COS cells, catalytic activity of \(\text{E. coli}\)-expressed ADAM8 was inhibited by EDTA, ortho-phenanthroline, and BB-94, but not by marimastat (data not shown).

The ability of recombinant ADAM8 protease to cleave 10-mer peptides specifically was previously demonstrated (Amour et al., 2002), a prerequisite for screening new potential substrates of ADAM8 protease by the ProteaseSpot™ method. When selecting the peptides, we considered that many ADAM substrates are cleaved in juxtamembrane regions. In addition, species consistency was maintained by screening peptides derived from mouse membrane proteins with mouse ADAM8 protease. In the present work, peptides derived from APP, CD16, CD23, fractalkine CX3CL1, L-selectin, PSGL-1, TGF-α, TNF-α, CD163, IL-1-RII, TNFRI and TRANCE were significantly cleaved by ADAM8. Although it is difficult to transfer the peptide cleavage data to a physiological situation, we can draw some conclusions. In the
Figure 4  ADAM8-dependent cleavage of MBP peptide variants.
A peptide derived from the original MBP cleavage site and seven variant peptides were incubated with active ADAM8 protease. Amino acid variants are given in bold letters. Average fluorescence values for each peptide were determined after 18 h. Values are given as units normalised to the fluorescence increase for the original MBP peptide set to 1. The peptides with amino acid variations in positions P1 and P2 were cleaved at significantly lower levels.

The case of APP and TGF-α, ADAM8 cleavage sites match with the reported sites. For CD16, as deduced from the molecular mass of the released fragment reported (Harrison et al., 1991), cleavage occurs in the most membrane-proximal region. CD23 peptide cleavage sites of ADAM8 match with those reported earlier from in vivo cleavage (Mayer et al., 2002). Cleavage of L-selectin, TGF-α, TNF-α and TNFRI occurs at cleavage sites slightly (±1 or 2 amino acids) different from the physiological sites (see references in Table 1), but sequences used for the screening were derived from mouse sequences, whereas most of the reported sequences refer to human sequences, which have significant differences. For peptides representing CD23 (SNQLAQKXKQ) and TGF-α (AVVAASQKXKQ), ADAM8 cleavage was observed at two adjacent amino acid residues, suggesting that ADAM8 recognises the peptide, but cleaves with inaccuracy.

Peptides derived from the cleavage site of MBP with amino acid exchanges in positions P3–P5 were cleaved by ADAM8 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 acidic residues around the cleavage site, substantially not in position P1’ and P2’. Mayer et al. (2002) suggested a similar hypothetical consensus sequence XXR/KXX from human CD23 cleavage sites. A motif with QK at positions P1’ and P2’ in the peptides derived from APP, CD23 and MBP was also present in the TGF-α peptide (AVVAASQKXKQ), suggesting physiological cleavage next to this motif. Examination of the screened peptide collection revealed regularity for cleaved peptides that were not applicable for most of the non-cleaved peptides. The cleaved peptides contained basic amino acid residues, whereas they did not contain cysteine, methionine or tryptophan residues. Only five of the cleaved peptides (APP, IL-1-RII, L-selectin, PSGL-1, TNFRI) contained acidic residues, and only in the peptides representing IL-1-RII and TNFRI were these acidic residues located in positions P3–P5 of proposed or determined cleavage sites.

Despite these regularities, ADAM substrate cleavage sites are often promiscuous. In peptides derived from TNF-α, three different cleavage sites for ADAM8, and two different cleavage sites for ADAM9 and ADAM19, respectively, were determined (Roghani et al., 1999; Amour et al., 2002; Chesneau et al., 2003; Fourie et al., 2003). Three different cleavage sites were sequenced for ADAM9 in a KL peptide (Amour et al., 2002). The MBP cleavage next to the QK motif was determined for ADAM8, ADAM10 and ADAM28, emphasising the importance of this motif (Howard et al., 2001; Schlimann et al., 2002). ADAM8, ADAM9 and ADAM33 cleaved an APP peptide also at the QK motif, whereas ADAM10 and ADAM17 cleaved the APP peptide two amino acids closer to the C-terminus (Roghani et al., 1999; Amour et al., 2002; Mohan et al., 2002; Zou et al., 2004). Comparison of 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. Further studies are necessary to determine substrate specificity of ADAMs; however, based on our analysis, the likelihood of peptide cleavage by ADAM8 can be predicted.

Based on cleavage of the APP protein by ADAM8, we have in vivo evidence for ADAM8 as additional α-secretase activity. APP is cleaved proteolytically in two distinct
pathways. In the amyloidogenic pathway, APP is cleaved by β- and γ-secretases to release amyloid β (Aβ) peptide, which forms non-soluble plaques (Haass, 2004). In the anti-amyloidogenic pathway, APP is cleaved within the amyloidogenic Aβ domain by α-secretase to release a non-amyloidogenic p3 peptide and, furthermore, a large ectodomain of APP (APPα) with neuroprotective and memory-enhancing function (Furukawa et al., 1996; Meziane et al., 1998; Mattson et al., 1999).

Figure 5  Shedding of APP by ADAM8 in 293 cells expressing alkaline phosphatase (AP)-tagged APP (AP-APP cells). Cells were transiently transfected with plasmids encoding ADAM8 or ADAM10. As controls, cells were transfected with mock vector (pTarget for the ADAM8 constructs and peak12 for the ADAM10 construct) or with inactive ADAM8 (EQ-ADAM8).

(A) Quantification of AP-APP released into the conditioned medium relative to mock transfected cells. The mean and standard deviation of two independent experiments are reported, each one carried out in duplicate. (B) Western blot analysis confirming the increase in AP-APP shedding upon transfection of ADAM8 and demonstrating the presence of processed ADAM8 protein: 120 kDa, pro-ADAM8; 90 kDa, processed ADAM8 lacking the prodomain; and 60 kDa, ADAM8 lacking the pro- and metalloprotease domain. In contrast, inactive EQ-ADAM8 is only present in its proform. ADAM10-HA can also be detected in its immature (upper band) and mature (lower band) forms. For detection of the different proteins, the indicated antibodies were used. AP-APPα, soluble AP-APP generated by α-secretase; AP-APPα, full-length AP-APP; anti-HA, antibody against the C-terminal HA-epitope tag of ADAM10; anti-BiPro, antibody against the C-terminal BiPro-epitope tag of ADAM8.

Materials and methods

Expression of soluble ADAM8 protease in E. coli and purification

For ADAM8 expression in bacteria, the vector pASK-IBA3+ (IBA, Göttingen, Germany) was used. This vector encodes a Streptag® that allows purification of the expressed protein by Streptactin® MacroPrep® Cartridges (IBA). The cDNA encoding the mouse ADAM8 pro- and metalloprotease domain was amplified with primers mA8_EcoRI.s 5'-CTG TAC GTG CTC CTG CTG GTG CTG CTC AGC-3' and mA8_XhoI.as 5'-CTG GAG GAT CTC CTG AGG CTT AAA CTG AGG AAG GGA CAC GAA CCC GGT GAC ATC TGG-3' containing the sequence for the BiPro-tag at the 3' end. The resulting cDNA was cloned into the respective restriction sites of pASK-IBA3+ and transformed into E. coli strain BL21(DE3)pLysS. Bacteria were grown in the presence of tetracycline and ampicillin. For protein expression, bacteria were grown in LB medium and induced at OD600 0.5 with 0.2 μg/ml anhydrotetracycline (aTc, IBA) for 6 h at 30°C with 50 μM ZnCl2 added to the medium. After harvesting the bacteria, pellets were resuspended in 100 mM Tris, pH 8.0, 150 mM NaCl containing 1 mM phenylmethylsulfonyl fluoride and sonicated, and 5 μg/ml DNase I and 10 mg/ml RNase A were added. The mixture was centrifuged twice at 13 000 g and once at 150 000 g before applying the supernatants to Streptactin® cartridges and FPLC. The purity of soluble ADAM8 was confirmed by SDS-PAGE and protein concentrations were determined using BCA reagent (Pierce, Heidelberg, Germany). Depending on the amount of purified protein, FPLC eluates were concentrated with Amicon Ultra Centrifugal Filter Units (Millipore, Bedford, MA, USA). Pro-
teolytic activity was monitored using a fluorescent cleavage assay with the peptide DNP-SHGDGMAQKSQTQI in the absence or presence of ADAM inhibitors.

**Protease assays**

The ProteaseSpot™ system is based on peptides synthesised on continuous cellulose membranes (JPT Peptide Technologies GmbH, Berlin, Germany). Cellulose disks were cut into equivalent pieces, activated in methanol for 5 min and subsequently washed four times with HEPES buffer (20 mM HEPES, pH 7.4, 0.015% Brij-35). For the reactions, disks were incubated at 37°C with 100 μl of protease solution containing 50–100 ng of ADAM8 protease, complete EDTA-free protease inhibitor cocktail (Roche Applied Science, Mannheim, Germany) and HEPES buffer. For negative controls, 10 μl EDTA was added to the samples. For positive controls, some peptides containing arginine or lysine residues were incubated with a 0.1% (w/v) solution of trypsin (Roche Applied Science).

Fluorescence (excitation at 325 nm and emission at 420 nm) was measured initially, after 2, 4 and 6 h, and after 18 h using a Perkin-Elmer LS50B luminescence spectrometer in black-coated 96-well plates (Nunc, Wiesbaden, Germany).

Protease assays in solution were performed to analyse the catalytic activity of purified ADAM8. The peptide DNP-SHGDGMAQKSQTQI (100 μM) was incubated with 100 μl of protease solution containing 50–100 ng of ADAM8 protease, Complete EDTA-free protease inhibitor cocktail (Roche Applied Science) and HEPES buffer (20 mM HEPES, pH 7.4, 0.015% Brij-35), with 10 μl EDTA added to the negative controls. Immediately, and after 2 and 4 h of incubation, 30 μl samples were mixed with 65 μl of HEPES buffer and 5 μl of 1% fluorescamine (Sigma, Taufkirchen, Germany). Fluorescence (excitation at 386 nm and emission at 477 nm) was measured with a Perkin-Elmer LS50B luminescence spectrometer in black-coated 96-well plates (Nunc).

**Western blot analysis**

For Western blot sample preparation, total bacterial lysate pellets with equal cell amounts were mixed with 1× SDS sample buffer and sonicated. For inclusion body samples, the pellets were resuspended in 100 mM Tris, pH 8.0, 150 mM NaCl. All protein samples were mixed with 5× SDS sample buffer (50% glycerol, 10% SDS, 100 mM β-mercaptoethanol, 0.05% bromophenol blue in 250 mM Tris-HCl, pH 6.8), boiled for 10 min and loaded onto 12.5% SDS-PAGE for soluble ADAM8 detection and 7.5% SDS-PAGE for full-length ADAM8. After gel runs, the proteins were blotted onto nitrocellulose transfer membranes (Protran® BA79, 0.1 μm pore size, Schleicher & Schuell, Dassel, Germany) by semidry electroblotting. After control staining with 0.1% Ponceau S solution and blocking for 2 h with 5% skim milk in TBS containing 0.1% Tween-20, proteins were analysed by immunostaining. Mouse monoclonal anti-BiPro-tag antibody (kindly provided by Dr. B.M. Jockusch, Braunschweig, Germany) was diluted 1:25 in blocking buffer and incubated with the membranes overnight at 4°C. The detection of proteins was performed with anti-mouse-IgG-horseradish peroxidase (1:8000, 1 h at RT, Sigma) using Lumi-LightPLUS (Roche Applied Science) as the chemiluminescent substrate. Kodak X-OMAT X-ray films were exposed to the emitted light. Exposure times depended on the intensity of the reaction. Fixing and developing of the films was performed with Kodak X-ray Developer LR 24 and Kodak X-ray Fixer AL4 (Eastman Kodak, Rochester, NY, USA).

For immunoblot detection of the different AP-APP species and ADAM8 and ADAM10 expression in AP-APP cell lysates and conditioned medium, aliquots of the conditioned medium and cell lysates were boiled for 5 min at 95°C with SDS sample buffer. The samples were subjected to SDS-PAGE on an 8% SDS gel. Proteins were transferred to a PVDF membrane and detected with the following antibodies: mouse monoclonal antibody W02 against AP-APPs (kind gift of K. Beyreuther; directed against amino acids 1–16 of the Aβ sequence; Ida et al., 1996); polyclonal antibody 6687 against the C-terminus of APP detecting full-length APP (Steiner et al., 2000); and antibody anti-BiPro, detecting the C-terminal BiPro epitope tag of ADAM8 and EQ-ADAM8. For detection of ADAM10-HA expression, the 6687-blot was stripped and reprobed with a polyclonal anti-HA antibody (Sigma).

**Determination of peptide cleavage sites by mass spectrometry**

Peptide substrate (100 μM) in 250 μl of buffer containing 25 mM Tris, pH 8.0 and 0.0006% Brij-35 was incubated with 300 ng of ADAM8 metalloprotease domain isolated from a Strep-Tactin® affinity column. Incubations were performed at room temperature or 37°C for 4 h, after which an equal volume of 1% heptafluorobutyric acid (HFBA) was added. Substrate was separated from product by separation on a C18 column from Vydac (Hesperia, CA, USA) using an acetonitrile/water gradient with 0.1% HFBA. Further purification was performed using a Shimadzu LC system (comprising a solvent degasser, two LC-10A pumps and a SCL-10A system controller). The LC was coupled to a QSTAR XL quadrupole time-of-flight tandem mass spectrometer (ABI/MDS-Sciei, Toronto, Canada) equipped with an electrospray source. LC was operated at a flow rate of 200 μl/min with a linear gradient as follows: 100% A held isocratically for 2 min, linearly increased to 60% B over 18 min and then increased to 100% B over 5 min. Mobile phase A consisted of water/acetonitrile (98:2 v/v) with 0.1% acetic acid and mobile phase B of acetonitrile/water (90:10 v/v) with 0.1% acetic acid. A Zorbax C8 reverse-phase column (2.1×50 mm) was used for all LC-MS analyses. MS data acquisition and analysis were performed using the Analyst QS software.

**Cell culture and transfections**

Clonal 293-EBNA cells expressing AP-APP and Bcl-XL/CrmA (clone SABC70) were generated and cultured as described by Schöbel et al. (2006). Transfections were carried out using Lipofectamine 2000 (Invitrogen, Groningen, Netherlands). The medium was replaced with fresh medium 1 day after transfection. After overnight incubation, conditioned medium and cell lysate (in 50 mM Tris, pH 7.5, 150 mM NaCl, 1% Nonidet-P 40) were collected. Aliquots of the conditioned medium were treated for 30 min at 65°C to heat-inactivate the endogenous AP activity. AP activity in the conditioned medium was measured as previously described (Lichtenthaler et al., 2003) and normalised to the protein concentration in the cell lysate. The cDNA encoding C-terminally HA-epitope tagged ADAM10 was cloned into the expression vector peak12.

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References


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