ADAM10 is the Responsible Sheddase of the CD44 Ectodomain in Human Melanoma Cells

Christian Haiduk¹, Thea Eichenberg¹, Christian Ihling², Sven Rothemund³, Miroslav Malesevic⁴, Andrea Sinz², Jan C. Simon¹*, Ulf Anderegg¹#*

¹ Department for Dermatology, Venereology and Allergology, University Hospital Leipzig, Germany
² Institute of Pharmacy, Martin Luther University Halle-Wittenberg, Germany
³ Interdisciplinary Center for Clinical Research, University of Leipzig, Germany
⁴ Max Planck Research Unit for Enzymology of Protein Folding, Germany
*both authors contributed equally
#corresponding author: Ulf Anderegg

Department for Dermatology, Venereology and Allergology, University of Leipzig, Medical Faculty
Johannisallee 30 04103 Leipzig
phone: +49-341-9725881 fax: +49 341 -9725878
e-mail: Ulf.Anderegg@medizin.uni-leipzig.de

Abstract

The liberation of CD44s ectodomain plays an important functional crucial role in the tumor biology of human malignant melanoma (MM) cells. It has been shown that the shedded CD44 ectodomain suppresses the proliferation induced by hyaluronan in human MM cells and that ADAM10 is essential for CD44s shedding. Here we provide evidence that ADAM10 indeed cleaves CD44s directly. Co-transfection of CHO-cells with CD44 and ADAM10 resulted in strong CD44 ectodomain release. Both, native and recombinant CD44, were cleaved at the identical cleavage site discovered by us. Furthermore, this study provides an putative binding site of ADAM10 for CD44s for the first time. Using a peptide library of ADAM10-derived peptides we could identify four main peptide motives in the disintegrin and cysteine-rich domains of ADAM10 which bind soluble CD44 ectodomain. Moreover, one of these peptides can interfere with the CD44 shedding in MM cells. The identification of ADAM10 as sheddase of CD44s and the characterization of its shedding mechanism in human MM cells could be a possible target for the development of new therapeutic strategies.
Introduction

CD44 glycoproteins belong to a family of cell surface receptors with a wide structural variety of isoforms, which are generated by alternative splicing and different glycosylation. Being a type I protein, all CD44 isoforms possess an intracellular, a transmembrane and an extracellular domain. The extracellular domain can be subdivided into a globular link domain binding the CD44 major ligand hyaluronan (HA) and a highly glycosylated and variable stalk region, which is located proximal to the membrane and comprises the restriction sites of CD44. Differing sizes of the stalk region due to the expression of variable exons allows a subdivision of the CD44 splicing variants (referred to as CD44v) (reviewed by [1]). The interaction of CD44 and HA regulates the proliferation, migration and differentiation of benign and malignant cells [1-6]. For example, silencing of CD44 in malignant pleura mesothelioma cells diminished the HA induced proliferation and invasion significantly [7]. Blocking of CD44-HA interaction with function blocking antibodies reduced adhesion, motility and invasion in human breast cancer cells suggesting antibodies against specific CD44 epitopes as therapeutics [8]. However, the underlying mechanisms for the function of CD44 are still objects of intensive research. The CD44 within the cell membrane could either act as an organizing platform for other proteins or interact with growth factors and growth factor receptors [9]. The interaction of the CD44 intracellular domain with ezrin or the tumor suppressor merlin has been shown to function as an alterable switch between cell proliferation and apoptosis in a cell density dependent manner [5].

The standard CD44 isoform (referred as CD44s) is 85-100kDa in size and lacks the epitopes encoded by the variant exons. The widely expressed CD44s binds strongly to HA [10] and is most frequently described in the context of several cancers (reviewed by [11]). In human melanoma cells, the interaction of CD44s with its ligand HA promotes the tumour cell proliferation and metastatic formation. In addition, CD44s is strongly upregulated in melanoma indicating its important role in melanoma progression [2,12].

In cancer research, the process of ectodomain shedding has reached significance since it has been shown to enhance the detachment and migration of tumour cells [13,14]. Moreover, it also modifies intercellular communication of tumour cells with
stromal cells and alters signalling from and to the extracellular matrix [14]. Shedding is described as the cleavage of membrane proteins by specific proteases followed by the release of the shed ectodomain into the intercellular space where this ectodomain can compete for ligand binding. CD44s can be shed by a protease resulting in a soluble full functional ectodomain (solCD44). High serum levels of solCD44s correlate with a higher metastatic potential and a poor prognosis in colorectal cancer and lymphoma [15,16]. On cellular level shedding of the CD44-ectodomain plays a critical role in tumour cell migration [4,17]. On the other hand, in human melanoma solCD44s prevents the HA driven cell proliferation probably by competition with the membrane bound CD44s [3].

Several studies suggested different candidate sheddases to be involved in the cleaving of the CD44s ectodomain, mainly the type I-transmembrane matrix metalloproteinase (MT1-MMP), and the a disintegrin and metalloproteinase proteins 10 and 17 (ADAM10, ADAM17) [17-23]. Using a recombinant Tet-off system in the melanoma cell line A375, Nakamura and colleagues could induce a CD44s cleavage by the MT1-MMP [21]. The corresponding cleavage site at Gly192 was identified and resulted in a 37kDa fragment of soluble CD44. However, the constitutive shedding of CD44s in melanoma cells results in a liberated CD44s ectodomain of 65-70 kDa in size and origins from the cleavage at Ser249 [21] by an other protease than MT1-MMP. Thus, ADAMs were alternatively suggested to regulate the constitutive CD44s shedding [17,21]. Murai et al. demonstrated that downregulation of ADAM10 by RNA interference resulted in a suppression of CD44s cleavage in human glioblastoma cell line U251MG [20]. Subsequently, also ADAM17 was shown to induce proteolysis of CD44s [17]. Our group could show that ADAM10, but neither ADAM17 nor MT1-MMP is involved in the constitutive shedding of CD44s in several human melanoma cell lines. After blocking of ADAM10, ADAM17 and MT1-MMP by appropriate siRNAs, only silencing of the ADAM10 mRNA expression resulted in a significant reduction of CD44s shedding. Specific inhibitors blocking the activity of ADAM10 or ADAM10 and ADAM17 confirmed the involvement of ADAM10 in CD44s shedding. As a consequence, decreasing solCD44s by ADAM10 knockdown in MM cells significantly induced cell proliferation [18]. However, having identified ADAM10 as the crucial enzyme for the constitutive shedding of CD44s, the molecular mode of action such as interaction pattern, exact cleavage site and binding motifs had not been identified. In the present study we describe the cleavage site in the CD44s protein in human melanoma and show that ADAM10 is able to cleave these sequence motives. Furthermore, we demonstrate that ADAM10 directly interacts with CD44s and we could identify four putative binding sites in the ADAM10 disintegrin and cysteine-rich domain, which have the ability to bind CD44s.

Results

Coexpression of ADAM10 and CD44s in CHO cells leads to the liberation of solCD44s

The shedding of the CD44s ectodomain in the extracellular space can be measured by
analyzing the amount of solCD44s in the supernatant of cells, which can be determined by ELISA. Almost all melanoma cell lines shed CD44s albeit in markedly different amounts. In high-shedding melanoma cell lines like HT144, MV3, and Bro the concentration of solCD44s may exceed 120ng/10^6 cells in 24 hours. Whether a direct interaction of ADAM10 and CD44s may initiate the cleavage of the CD44s ectodomain or if other proteins are required still remains unclear. To address this issue, we cotransfected these two proteins in CHO cells, which resulted in a CD44s ectodomain release comparable to that of the high shedding melanoma cell line Bro (Fig.1). The shedding capacity of the cotransfected CHO cells was in a range of 80 ng/10^6 cells in 24 hours. Transfection of either ADAM10 or CD44s alone resulted in a very weak or no detectable release of the CD44s ectodomain (Fig.1a). Furthermore, we compared the molecular weights of the shedded ectodomains from cotransfected CHO cells and the melanoma cell line Bro by western blot analysis. The CHO cell-derived ectodomain of CD44s displayed the same mobility like that of the melanoma cells (Fig. 1b) implicating that the CD44s ectodomain shed by cotransfected CHO cells has the same glycosylation pattern and the same restriction site as the melanoma cell derived-CD44s ectodomain.

Figure 1: (A) Detection of soluble CD44s in supernatants of transfected CHO cells. CHO cells were transfected with ADAM10 (A10), CD44s or both proteins (A10+CD44); empty vector transfected (mock) and non transfected CHO cells served as negative, HT144 and Bro cell lines (CD44s high shedding cell lines) as positive controls. Supernatants were harvested after 24h culture in serum-free medium and ELISA was performed to measure CD44s content. Data are mean (±SD) of three to five different experiments. **p<0.05 (Mann-Whitney-test). (B) Soluble CD44s from transfected CHO cells display the similar molecular weight and glycosylation pattern compared to soluble CD44s from Bro melanoma cells. Immunoblot of soluble CD44s derived from supernatants from transfected CHO cells and Bro melanoma cell line was performed.
**CD44s ectodomain is cleaved by ADAM10 at Ser^{249}**

Next, we determined the exact cleavage sites of the CD44s ectodomain in Bro melanoma cells and in CD44/ADAM10 cotransfected CHO cells. The shed ectodomain was purified from the supernatants, proteolyzed with AspN. Thereafter, the obtained fragments were deglycosylated with PNGaseF and further analysed by nano-HPLC / MALDI-TOF/TOF mass spectrometry. To determine the CD44s restriction site the very c-terminal fragment of the digested ectodomain had to be identified. Here, we could identify a 2418 Da fragment derived from cotransfected CHO cells as well as from the melanoma cell line Bro that comprises the amino acids (AA) Asp^{226} to Ser^{249} (Fig. 2). Because Ser^{249} is flanked by Glu^{250} in the CD44s ectodomain this cleavage site does not result from the AspN digestion. Thus we provide evidence that ADAM10 cleave CD44s at Ser^{249}.

**Figure 2:** Identification of the C-terminal peptide of the shed CD44s ectodomain. MALDI-TOF/TOF fragment ion mass spectrum of the C-terminal peptide of the shed CD44s ectodomain derived from Bro cells. After AspN digestion and deglycosylation the C-terminal peptide of the shed CD44s ectodomain was identified based on MS/MS data (B). The c-terminal peptide is highlighted in the C-terminal sequence of full length CD44s antigen (A).
**Mca-Dpa-TNFαAV and Mca-Dpa-TNFαAVΔSQ cleavage by recombinant human ADAM10**

To confirm that ADAM10 is capable to cleave CD44s at Ser249 in vitro peptide digests were performed. Fluorogenic peptides according to the sequence of TNFα, a known substrate of ADAM10, were synthesized. The Mca-Dpa-TNFαAV peptide consists of a restriction site between an Ala and Val residue, which is flanked n-terminally by the fluorescent Mca group and c-terminally by the quenching Dpa group. After peptide cleavage an increased fluorescent signal was observed due to the loss of internal quenching and measured in real-time using a spectrofluorometer. For validation of ADAM10s aptitude for cleaving a substrate at Ser249 we modified the TNFα restriction site Mca-Dpa-TNFαAV to the CD44s restriction site Mca-Dpa-TNFαAVΔSQ. As control a scrambled sequence in Mca-Dpa-TNFαAVΔNI was used. The hydrolysis of 10uM Mca-Dpa-TNFαAV and Mca-Dpa-TNFαAVΔSQ by 160nM rhADAM10 led to a three-fold increase of the fluorescent signal compared to the scrambled control Mca-Dpa-TNFαAVΔNI over a period of 85 minutes (Fig 3). The reaction curve was nearly linear over the time and without the enzyme no fluorescent signal was observed. These data implicate that ADAM10 is able to cleave a substrate at CD44s restriction site with the same efficiency as a substrate comprising the TNFα restriction site.

![Graph](image)

**Figure 3:** Recombinant human ADAM10 cleaves a peptide at the CD44s restriction site. Fluorogenic peptides with a flanking sequence according to the ADAM10-cleavage site in huTNFα and consisting of either the TNFα cleavage motif (Mca-Dpa-TNFαAV) or the CD44s cleavage site (Mca-Dpa-TNFαAVΔSQ) were digested by rhADAM10. As negative control a peptide was used consisting of Asn-Ile at the cleavage site. All data are mean (±SD) of three different experiments. *p<0.05 (unpaired t-test)
**Four putative binding sites in ADAM10s disintegrin and cysteine-rich domain bind CD44s**

The disintegrin and cysteine-rich domains of ADAMs have been reported to be essential for substrate recognition and binding. (reviewed by [24]) To identify the putative binding sites of ADAM10 we performed a peptide library scan spanning the sequence of ADAM10 from Gln\(^{457}\) – Glu\(^{672}\), which include in part the metalloprotease domain and the complete disintegrin and cysteine-rich domains. Each peptide corresponds to 12 AA of the ADAM10 sequence with a forward shift of two AA. Purified CD44s bound to four clusters of peptides (Fig. 4A), which correspond to the ADAM10 motives Phe\(^{485}\) – Ser\(^{528}\), Ala\(^{541}\) – Gln\(^{560}\), Lys\(^{600}\) – Cys\(^{632}\) and Leu\(^{647}\) – Leu\(^{666}\). The first two motives are located in the disintegrin domain of ADAM10 while the other two represent the binding capacity of the cysteine-rich domain. As control the same a peptide array was performed without solCD44s incubation resulting in no signal detection (Fig. 4B). In consideration of the peptide overlap on the array, we narrowed down the motives to four minimal binding motives comprising the AA displayed in Fig. 4C.

![Figure 4](image-url)

**Figure 4:** CD44s ectodomain binds to distinct stretches of an ADAM10 peptide library array. (A) An array of overlapping 12mer peptides according to the...
disintegrin and cysteine-rich domain of ADAM10 was synthesized with a forward shift of two amino acids (AA). The solCD44s protein purified from melanoma cell supernatants interacts with four distinct peptide stretches (a-d). (B) The array was performed in the same way as in A without CD44s. (C) The corresponding peptide sequences and the putative minimal binding motives are depicted.

**ADAM10-derived peptide can impair CD44s shedding from Bro and HT144 melanoma cell lines**

Next we aimed to test whether the binding of the four peptides representing putative binding motives of ADAM10 has a functional impact on CD44s shedding in melanoma cells. The four derived peptides (Tab.1) were added to the cell cultures of the high shedding melanoma cell lines Bro and HT144 in a 10 µM concentration. While the peptides comprising the motifs one to three had no effect, the very c-terminal binding motif (pep4) led to a significant decrease in CD44s ectodomain liberation (Fig 5A,B) in both cell lines. The inhibitory effect of pep4 occurs in a dose dependent manner (Fig 5C). This data indicate that at least one of the four peptides is able to compete with the interaction of ADAM10 and membrane bound CD44s.

![Figure 5: Peptides comprising the putative binding sites of ADAM10 affect CD44s ectodomain shedding in melanoma cells.](image-url)

The high shedding melanoma cell lines Bro (A) and HT144 (B) were cultured in FCS free medium consisting of 7.5 µM of a
peptide which correlates with one of the new identified minimal binding sites of ADAM10 or a mixture of all of them (peptide1-4, Tab.1). After 24 hours the cell free supernatant was collected to measure the concentration of the shed CD44s ectodomain. The mean and standard deviation of seven independent experiments is represented. P values (= 0.0131 for Bro cells and 0.0279 for HT144) were calculated using paired t-test. (C) The inhibition of CD44 shedding by peptide 4 is dose-dependent in both MM cell lines. n=3-7

Discussion
Ectodomain shedding plays a fundamental role in many cellular functions during development, inflammation and cancer [25-27]. The process of extracellular domain cleavage of membrane spanning proteins is often performed by metalloproteinases such as ADAM9, ADAM10 and ADAM17. These ADAMs have been described to be involved in shedding of various proteins including cytokines, growth factors, receptors, adhesion proteins thereby influencing cell-cell adhesion, signalling, differentiation and proliferation [28-30]. The involvement of ADAMs in human melanoma development, progression and metastasis has been described in several studies [18,31,32]. In a previous study, our group identified ADAM10 as the key protease for the constitutive ectodomain shedding of CD44s in human melanoma cell lines [18]. Blocking ADAM10 activity or expression inhibited the CD44s shedding and boosted the proliferation of melanoma cells which was demonstrated to be Ca²⁺ dependent [33]. These findings consider ADAM10 over-expression or activation as a potential therapeutic option for the treatment of primary MM. On the other hand, Lee and colleagues demonstrated that high levels of ADAM10 and shedding of the substrate L1-CAM are correlated with an induction of MM cell proliferation and migration in melanoma metastases [31]. Therefore, specific regulatory mechanisms of intended ADAM10-mediated effects have to be identified based on a deeper understanding of ADAM10 function and mechanisms in primary MM.

Here we aimed to show whether CD44s is a direct substrate of ADAM10 and to identify the domains of ADAM10, which are responsible for CD44s substrate binding and cleavage. We demonstrate for the first time, that ADAM10 directly interacts with CD44s and cleaves it at Ser²⁴⁹. Additionally, we identified four putative binding sites for CD44s in the disintegrin and cysteine-rich domain of ADAM10.

First, we examined the ability of ADAM10 to cleave CD44s. Cotransfection of ADAM10 and CD44s into CHO cells resulted in the release of the CD44s ectodomain at levels similar to the melanoma cell lines HT144 and Bro. Since no solCD44s was detected in CHO cells transfected with ADAM10 or CD44s alone, a direct interaction between ADAM10 and CD44s with a direct cleavage of CD44s by ADAM10 was conceivable. To verify this, pull down assays using either CD44 or ADAM10 as bait were performed. However, these experiments were not successful. This could be due to the supposed very short lift interaction of a protease and its substrate. Furthermore, a native surrounding membrane structure might be essential for the proposed interaction of ADAM10 and CD44 and the release during cell lysis could resolve this interaction. Thus, we performed a bimolecular fluorescence complementation
approach (BiFC) in which positive signals accumulate over the time in contrast to a pull down assay. We show that fluorescence levels of CHO cells co-transfected with CD44 and ADAM10 were comparable to a positive control (Adiponectin-receptor-domain interaction) (Figure S1). The identification of the direct CD44s/ADAM10 interaction in vitro is not thoroughly transferable into an in vivo situation since for example the molecular ratio or the compartment expression could be incisively altered in vitro. Additionally, other proteins could be associated to CD44s or ADAM10 in vivo and regulate the shedding process of CD44s in melanoma cells. In summary, the co-transfection experiments with the subsequent purification of the shedded CD44s ectomain and the identification of the native cleavage site regardless of the source suggest that ADAM10 is able to shed CD44s directly. The identified cleavage site at Ser$^{249}$ is in agreement with a previous study, where Nakamura and colleagues identified Ser$^{249}$ beside other cleavage sites Gly$^{192}$ and Gly$^{233}$ in human melanoma cell line A375. In the study of Nakamura, the metalloproteinase MT1-MMP had been suggested to be responsible for the cleavage sites Gly$^{192}$ and Gly$^{233}$ [21]. However, the responsible sheddase for the cleavage site Ser$^{249}$ remained elusive in their study. Here, together with our previous work [18] we provide conclusive evidence for CD44s cleavage by ADAM10 at Ser$^{249}$ in human melanoma cells. To verify the restriction site of CD44, an in vitro digestion of the purified full length CD44 protein by recombinant ADAM10 was carried out. Unfortunately, in our hands an in vitro digestion was not successful. We assume that this might be due to an altered protein folding of CD44 after isolation from cell membrane. Since ADAM10 cleaves native CD44 close to the membrane it is possible that a perturbed folding disturbs the process of shedding irreversibly. Since a previous report described that the substrate specificities of ADAM10 and ADAM17 depend in part on the sequence of the cleavage site and the surrounding residues [34] we tried to verify the cleavage site at Ser$^{249}$. Using a biochemical peptide cleavage assay we could show that the recombinant, functional ADAM10 ectodomain is able to cleave a peptide at the CD44s restriction site comparable to the TNFα restriction site. This is in accordance with Caescu et al. [34] who found that a Serin residue in the P1 and a Glutamine residue in the P1´ position is a motif for an ADAM10 substrate. In addition to the cleavage motif in the active centre of the enzyme the disintegrine and cysteine-rich domains of ADAMs have been reported to be responsible for the substrate recognition [35-37]. A peptide library spanning the sequence of the disintegrine and cysteine-rich domain of ADAM10 (ADAM10D+C) revealed four putative motifs in the ADAM10D+C region. The first two motifs are located in the disintegrin domain and comprise the residues Cys$^{485}$-Gly$^{500}$ (ADAM10dCys$^{485}$-Gly$^{500}$) and Cys$^{515}$-Glu$^{522}$ (ADAM10dCys$^{515}$-Glu$^{522}$) whereas the motives three and four in the cysteine-rich domain include the residues Ser$^{616}$-Thr$^{625}$ (ADAM10c Ser$^{616}$-Thr$^{625}$) and Leu$^{654}$-Ser$^{663}$ (ADAM10c Leu$^{654}$-Ser$^{663}$). In 2005, Janes et al., could show that the substrate recognition of ephrin-A5 is mediated by an acidic pocket in the cysteine-rich domain [38]. Although putative binding motives identified in our experiments are apart from this mentioned acidic binding pocket, at this state we do not exclude that ADAM10 binds CD44s by this way too. A peptide library scan considers only linear epitopes but not possible three dimensional structures. However, in human melanoma cells the binding motif
ADAM10cLeu$^{654}$-Ser$^{663}$ seems to play a considerable role in the recognition and/or binding of CD44s since a peptide composed of the ADAM10cLeu$^{654}$-Ser$^{663}$ probably competes with the according binding motif of ADAM10 resulting in a significant down regulation of shedded solCD44s in melanoma cells. Peptides consisting of the other putative binding motifs identified by us did not affect the solCD44s release. It is possible that the different binding motifs possess a high redundancy in substrate recognition and binding.

This functional impact of an ADAM10-derived peptide on CD44s-shedding might be due to competitive binding to the CD44s target region and supports experimental findings from the peptide array. To the best of our knowledge, this is the first study showing a sequence motif of ADAM10 which significantly influences the CD44s shedding capacity of ADAM10. Further in vivo experiments have to be performed to verify that this sequence influences biological effects that are related to CD44s shedding like proliferation, differentiation and metastasis of malignant melanoma.

**Figure S1:** *BiFC signal detection in CD44s and ADAM10 co-transfected CHO cells*

CHO cells were co-transfected with cDNAs for huCD44s, bovADAM10 and Adiponectin-Receptor1 (AdipoR1) coupled to either the c-terminal domain (VC) or the n-terminal domain of the Venus protein (VN) as described elsewhere (Kosel et al., 2010). Interaction of these two co-transfected proteins results in BiFC signals from interacting domains VC and VN detectable 36 hours after transfection. (A) CHO co-transfected cells with CD44s-VC and ADAM10-VN revealed distinct fluorescence signals. (B) Similar signals were detectable in CHO cells transfected with AdipoR1-VN and AdipR1-VC which are able to form a homo-dimer (Kosel et al., 2010). (C) co-transfection of ADAM10-VN and AdipoR1-VC did not result in any signals. These data indicate a specific interaction of ADAM10 and CD44s. One representative example out of n=5 is shown. Kosel D, Heiker JT, Juhl C, Wottawah CM, Blüher M, Mörl K, Beck-Sickinger AG. Dimerization of adiponectin receptor 1 is inhibited by adiponectin. 2010 J Cell Sci.;123:1320-8.

**Conclusion**

This study demonstrates that the proteinase ADAM10 cleaves the ectodomain of CD44s in a direct interaction manner in human melanoma cells. Furthermore, Ser$^{249}$ was identified as the cleavage site in ADAM10-mediated shedding. Moreover, the
ADAM10-derived peptide sequence cLeu<sup>654</sup>-Ser<sup>663</sup> was shown to significantly reduce CD44s shedding by melanoma cells suggesting that this sequence is an important binding motif of ADAM10 during CD44s-ectodomain shedding.

Materials and Methods

Materials

The following antibodies were used: mouse anti-ADAM10 (163003, R&D Systems, Minneapolis, USA), mouse anti-CD44 (SFF-304, BenderMedsystems, Vienna, Austria) and polyclonal rabbit anti-mouse peroxidase IgG (Sigma, Hamburg, Germany).

All Peptides used in this study were synthesised following the standard Fmoc (N-(9-fluorenyl)methoxycarbonyl) solid phase peptide synthesis strategy and are displayed in Table 1.

Table 1: ADAM10-derived peptides used for blocking experiments of CD44s shedding in melanoma cell lines

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Minimal Binding Motif</th>
<th>Peptide Sequence</th>
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<tr>
<td>pep1</td>
<td>ADAM10dCys&lt;sup&gt;485&lt;/sup&gt;-Gly&lt;sup&gt;500&lt;/sup&gt;</td>
<td>CKANQPEGRKCKLKPG</td>
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<tr>
<td>pep2</td>
<td>ADAM10dCys&lt;sup&gt;515&lt;/sup&gt;-Gly&lt;sup&gt;522&lt;/sup&gt;</td>
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<td>pep3</td>
<td>ADAM10cSer&lt;sup&gt;616&lt;/sup&gt;-Thr&lt;sup&gt;625&lt;/sup&gt;</td>
<td>SRHFSGRTIT</td>
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<tr>
<td>pep4</td>
<td>ADAM10cLeu&lt;sup&gt;654&lt;/sup&gt;-Ser&lt;sup&gt;663&lt;/sup&gt;</td>
<td>LARLKKAIIFS</td>
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Cell Culture

The melanoma cell lines Bro (kindly provided by Dr. J. Eberle, Berlin, [39], HT144 (kindly provided by Dr. van Muijen, [40] and CHO K1 cells (LGC Promochem Wesel, Germany) were cultured in RPMI or DMEM medium (Biochrom, Berlin, Germany), respectively, supplemented with 10% fetal calf serum (FCS, PAA, Pasching, Austria) and maintained at 37°C in a humidified 5% CO<sub>2</sub> atmosphere. For transfection, CHO cells were grown in six well plates until they reached approximately 50% confluence. Subsequently, transfected CHO cells were cultured in DMEM/FCS medium additionally supplemented with G418 (500 µg/ml) or Blasticidin S (8 µg/ml, InvivoGen, San Diego, California). All cultures were passaged by detaching the cells with trypsin/EDTA solution (Biochrom, Berlin, Germany).

Isolation of solCD44s

Soluble CD44s was collected from cell culture supernatants after 48h of serum starvation. Supernatants were cleared thereafter by centrifugation and filtration. Further purification was achieved by anion exchange chromatography (aIEX) followed by hydrophobic interaction chromatography (HIC). Both aIEX and HIC were performed using an Äkta Purifier system (GE Healthcare, Munich, Germany). The purified solCD44s was dialysed and concentrated using Vivaspin® 15 Centrifugal Concentrators (Vivaproducts, Littleton, MA, USA). The purity of
solCD44s was monitored by Coomassie stained SDS PAGE. Amounts of solCD44s were quantified using ELISA for CD44 detection (BenderMedsystems, Vienna, Austria).

**In-solution digestion and LC/MS/MS analysis**
Samples of solCD44s derived from transfected CHO cells or cell line Bro were digested overnight using endoproteinase AspN (Roche, Mannheim, Germany) according to the manufacturer’s protocols. To facilitate mass spectrometric identification, peptides were deglycosylated with PNGase F (Roche). Samples were analysed by nano-HPLC/MALDI-TOF/TOF mass spectrometry (Ultimate 3000 nano-HPLC system, Dionex and Ultraflex III, Bruker Daltonik, Leipzig, Germany) or nano-HPLC/nano-ESI - LTQ-Orbitrap - MS/MS (Ultimate nano-HPLC system, Dionex and LTQ-Orbitrap XL, ThermoFisher Scientific, Schwerte, Germany) for solCD44s from Bro cells or CHO cells, respectively. Experimental setups for LC/MS/MS analysis were used as described elsewhere [41]. Data were searched against an in-house database using Mascot (Mascotserver 2.2, Matrixscience, London, England) and peptides were identified based on MS/MS data.

**Peptide digestion**
The Mca-Dpa-TNFαAV, Mca-Dpa-TNFαAVΔSQ and Mca-Dpa-TNFαAVΔNI peptides, consisting of the TNFα restriction site (AV), the CD44s restriction site (SQ) or no restriction site (NI) were cleaved with recombinant human ADAM10 (rhADAM10, R&D Systems) according to the manufacturers protocol. Briefly, 10 µg of each peptide were dissolved in 95 µl reaction buffer (25 mM Tris, 2,5µM ZnCl2, 0,005% Brij 35, pH 9) and supplied in a 96 well Special Optic Plate (Corning B.V., Amsterdam, Netherlands). Subsequently, 500 ng rhADAM10 in 5µl reaction buffer was added. The cleavage of the peptides was measured at 37 °C all 5 minutes over a period of at least 85 minutes in a Synergy HT Multi-detection reader (Bio-tek, Bad Friedrichshall, Germany). The emission and excitation filters used were 320/20 and 400/30.

**CHO cell transfection and analysis of CD44s-shedding**
Human recombinant full-length sCD44s was cloned from a cDNA of Bro cells into pcDNA6.1 expression vector (Clontech, Heidelberg, Germany). Bovine recombinant ADAM10 in piresNeo expression vector was a kind gift from Dr. E. Parkin (Lancaster, UK; [42]. For transfection, 0,25µg plasmid was transfected into CHO cells using LipofectamineTM 2000 (Invitrogen, Darmstadt, Germany) according to the manufacturer’s instructions. Twenty-four hours after transfection the growth medium was replaced and transfected cells were selected over a period of 10 days by application of G418/ml or Blasticidin S medium. To measure the shedding capacity of the transfected CHO cells, 2 x 10⁵ cells/well were seeded in six well plates. After 12h cells were washed twice with PBS and the medium changed to 1ml minimal medium without FCS. The concentration of solCD44s was determined in supernatants collected after 24h by ELISA.
**Peptide array**
The peptide array was synthesised according to the standard SPOT synthesis protocol as described elsewhere [43]. The following peptide array scan was performed according to Frank and Dübel [44]. In brief, membranes were washed once in methanol and three times in 20mM HEPES buffer (HB, pH 7.4) and incubated in blocking buffer (BB, 5% dry milk in HB) overnight. Subsequently, membranes were incubated with either 10µg/ml solCD44s or 10µg/ml bovine serum albumin (BSA, Sigma, Munich, Germany) in BB for three hours. After washing the membranes three times with HB, they were then incubated with anti-CD44 antibody (4µg/ml in BB) for additional 3h. Repeated washing steps followed before incubating the membranes with HRP-conjugated goat-anti-mouse detection antibody in BB. After 2h of incubation the detection antibody was washed out three times with HB and the bound solCD44s visualized with ECL solution (Pierce, Rockford, USA) in a chemiluminescence imager (MultiImage Light Cabinet, Alpha Innotech, San Leandro, USA).

**Modulation of CD44s shedding by peptides covering putative ADAM10-CD44s-interaction motives**
6 x 10⁴ cells of Bro or HT144 cells were seeded into a 24 well tissue culture plastic plate. After 10h, cells were washed twice with PBS and the medium was replaced by conditioned medium consisting of the peptides covering the ADAM10 minimal binding sites (Table 1) or DMSO as control. Peptides were used at a final concentration of 7.5 µM in serum free medium. After 24h the shed solCD44s in the supernatant was determined using a CD44 ELISA.

**Western Blot**
For western blot analysis of solCD44s, cell free supernatant of either Bro or transfected CHO cells were collected and concentrated twenty-fold using Vivaspin® 15 Centrifugal Concentrators (Vivaproducts). Equal amounts of the supernatant concentrates were subjected to 10% Tris-Glycin SDS-PAGE under reducing conditions. Proteins were transferred onto PVDF membranes (Hybond-P; GE-Healthcare). Blocking was performed for 1 hour with 5% non-fat dried milk powder in PBS. The membranes were incubated with mouse anti-CD44 monoclonal antibody (SFF304, 1µg/ml in blocking buffer) followed by HRP conjugated secondary antibody and chemiluminescence substrate (ECL, Pierce, Rockford, USA) incubation. Chemiluminescent signals were detected on a luminescent image analyser (INTAS, Münster, Germany).

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ADAM10 is the Responsible Sheddase of the CD44 Ectodomain

Reference List


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