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Enhancing Interleukin-6 and Interleukin-11 receptor cleavage

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ABSTRACT

Proteolytic cleavage of the membrane-bound Interleukin-6 receptor (IL-6R) by the metalloprotease ADAM17 releases an agonistic soluble form of the IL-6R (sIL-6R), which is responsible for the pro-inflammatory trans-signaling branch of the cytokine's activities. This proteolytic step, which is also called ectodomain shedding, is critically regulated by the cleavage site within the IL-6R stalk, because mutations or small deletions within this region are known to render the IL-6R irresponsive towards proteolysis. In the present study, we employed cleavage site profiling data of ADAM17 to generate an IL-6R with increased cleavage susceptibility. Using site-directed mutagenesis, we showed that the non-prime sites P3 and P2 and the prime site P1′ were critical for this increase in proteolysis, whereas other positions within the cleavage site were of minor importance. Insertion of this optimized cleavage site into the stalk of the Interleukin-11 receptor (IL-11R) was not sufficient to enable ADAM17-mediated proteolysis, but transfer of different parts of the IL-6R stalk enabled shedding by ADAM17. These findings shed light on the cleavage site specificities of ADAM17 using a native substrate and reveal further differences in the proteolysis of IL-6R and IL-11R.

1. Introduction

Proteolysis is an important post-translational modification which is, in contrast to most other protein modifications, completely irreversible. Research during the last two decades has shown that limited proteolysis of transmembrane proteins, resulting in the generation of soluble ectodomains with distinct biological functions, is critically involved in numerous physiologically as well as pathophysiologically processes (Murphy et al., 2008; Peschon et al., 1998). Among the around 560 proteolytic enzymes in the human genome, especially the metalloprotease ADAM17 has gained attention, and until today more than 70 substrates have been described (Scheller et al., 2011). Genetic deletion of ADAM17 results in embryonic lethality in mice (Peschon et al., 1998; Black et al., 1997), whereas at least three human individuals with loss-of-function mutations within ADAM17 are known (Blaydon et al., 2011; Tsukerman et al., 2015).

The pleiotropic cytokine Interleukin-6 (IL-6) is critically involved in health and disease (Wolf et al., 2014; Garbers et al., 2015). To activate its target cells, IL-6 binds first to its non-signaling IL-6 α-receptor (IL-6R), which leads to the recruitment of a homodimer of the ubiquitously expressed β-receptor glycoprotein (gp)130 and the activation of intracellular signaling cascades, among them the Janus kinase/Signal Transducer and Activator of Transcription (Jak/STAT) pathway (Garbers et al., 2015; Garbers and Scheller, 2013). Besides this classic signaling pathway, IL-6 can also bind to soluble forms of the IL-6R (sIL-6R) and signal in trans, because the agonistic IL-6/sIL-6R complex can activate all cells due to the broad expression profile of gp130 (Chalaris et al., 2011; Garbers et al., 2012). The sIL-6R is either generated by differential splicing of the *IL6R* mRNA, causing the excision of the exon encoding the transmembrane region, or proteolytic cleavage of the membrane-bound IL-6R (Chalaris et al., 2011). Enzymes that have been shown to be able to cleave the IL-6R are the metalloproteases ADAM10 and ADAM17 and the neutrophil-derived serine protease cathepsin G (Matthews et al., 2003; Garbers et al., 2011; Müllberg et al., 1993; Bank et al., 1999; Lokau et al., 2016; Riethmueller et al., 2016; Baran et al., 2013). The IL-11R is not a good substrate for ADAM17, but can be cleaved by ADAM10, and biologically active sIL-11R/IL-11 complexes can also perform trans-signaling via gp130 homodimers (Lokau et al., 2016).

Cleavage of the IL-6R occurs within the so-called stalk region, a 55 amino-acid residues long flexible stretch that connects the three extracellular domains, which contain the cytokine-binding module within the two fibronectin-type III domains, with the transmembrane and intracellular regions. We have previously shown that the length of the stalk is critical for the biological function

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of the receptor (Baran et al., 2013), but can be replaced by other amino-acid sequences of comparable length (Nitz et al., 2015). The cleavage site used by ADAM17 has been mapped between Gln-357 and Asp-358 close to the plasma membrane (Müllberg et al., 1994), although results from peptide cleavage assays and molecular modelling favor cleavage between Pro-355 and Val-356 (Riethmueller et al., 2016; Goth et al., 2015). Irrespective of the actual cleavage site, small deletions have been shown to be sufficient to completely abrogate shedding, underlining the importance of the cleavage site (Riethmueller et al., 2016; Baran et al., 2013; Müllberg et al., 1994). A coding non-synonymous single nucleotide polymorphism (rs2228145), however, which causes the exchange of Asp-358 to Ala-358, confers increased conversion rates by ADAM17 in vitro (Garbers et al., 2014), and increases sIL-6R serum levels in individuals homozygous for rs2228145 in vivo (Rafiq et al., 2007). Defining consensus cleavage sites for ADAM proteases has been difficult (Caescu et al., 2009), but cleavage site profiling data indeed show that an alanine is preferred by ADAM17 compared to an aspartic acid residue at the P1′ position within the cleavage site (Tucher et al., 2014).

In the present study, we use cleavage site profiling data to generate an "optimized" ADAM17 cleavage site within the IL-6R. By incorporating preferred amino-acid residues at the five prime and five non-prime positions instead of the original cleavage site, we construct IL-6R variants with increased proteolysis by ADAM17. Site-directed mutagenesis revealed critical positions within the cleavage site for this effect, and we further show that incorporation of the optimized cleavage site into the IL-11R is not sufficient to induce shedding by ADAM17. However, stepwise addition of parts of the IL-6R stalk region increased proteolysis of the IL-11R by ADAM17, suggesting additional regulatory elements besides the actual cleavage site.

2. Materials and methods

2.1. Cells and reagents

HEK293 cells were obtained from DSMZ GmbH (Braunschweig, Germany. Cells were grown under standard conditions in DMEM high glucose culture medium (Sigma Aldrich, Steinheim, Germany), which was supplemented with 10% fetal bovine serum, penicillin (60 mg/l) and streptomycin (100 mg/l) at 37 °C and 5% CO_2 in a standard incubator with a water-saturated atmosphere. The anti-hIL-6R mAb 4–11 was described previously (Chalaris et al., 2007). The anti-β-actin mAb (sc-47778) was obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA), the anti-GAPDH and the anti-myc antibodies were purchased from Cell Signaling Technology (Danvers, MA, USA), and the peroxidase conjugated secondary antibodies were from Pierce (Thermo Scientific, Perbio, Bonn, Germany). The Alexa Fluor488-conjugated goat anti-mouse IgG used for flow cytometry (see below) was from Thermo Scientific. PMA (phorbol-12-myristate-13-acetate) was purchased from Sigma–Aldrich (Deisenhofen, Germany). The two metalloprotease inhibitors GI (selective for ADAM10) and GW (selective for ADAM10 and ADAM17) were synthesized by Iris Biotech (Marktredwitz, Germany) (Hundhausen et al., 2003; Ludwig et al., 2005).

2.2. Construction of expression plasmids

The expression plasmid pcDNA3.1-hIL-6R encoding the complete cDNA of the human IL-6R has been described previously (Garbers et al., 2011). The myc-tagged IL-6R variant was constructed by inserting the nucleotides encoding EQKLISEEDL after the signal peptide into the pcDNA3.1-hIL-6R plasmid via splicing by overlapping extension (SOE) PCR. All IL-6R variants with mutations within the cleavage site were also constructed by SOE-PCR and inserted into pcDNA3.1-hIL-6R. The expression plasmid pcDNA3.1-myc-hIL-11R encoding the complete cDNA of the human IL-11R with a N-terminal myc-tag has been described previously (Lokau et al., 2016). The IL-11R variants IL-11R opt1 and opt2 containing the optimized ADAM17 cleavage site were constructed via splicing by overlapping extension PCR and inserted into pcDNA3.1-myc-hIL-11R. The IL-11R variant containing the whole stalk region of the human IL-6R has been described previously (Lokau et al., 2016; Nitz et al., 2015). The other IL-11R variants containing various elements of the IL-6R stalk region were constructed similarly. All generated constructs were verified via Sanger sequencing (GATC Biotech AG, Constance, Germany).

2.3. Ectodomain shedding assays in HEK293 cells

Transient transfection of HEK293 cells with different IL-6R and IL-11R variants and analysis of IL-6R and IL-11R ectodomain shedding by ADAM17 was performed as described elsewhere (Garbers et al., 2011; Lokau et al., 2016; Baran et al., 2013).

2.4. Precipitation of soluble cytokine receptors and Western blotting

Precipitation of soluble IL-6R and soluble IL-11R from cell culture supernatant and analysis via Western blot has been described in detail elsewhere (Lokau et al., 2016; Riethmueller et al., 2016).

2.5. Flow cytometry

For detection of cell surface expression of the IL-11R variants, $2–3 \times 10^5$ transiently transfected HEK293 cells were washed with FACS buffer (PBS containing 0.5% BSA) and incubated in 50 μl FACS buffer with 1:100 diluted anti-myc mAb for 60 min on ice. After washing, cells were incubated again in 50 μl FACS buffer containing Alexa Fluor488-conjugated anti-mouse mAb (dilution 1:100, from Thermo Scientific). After 60 min of staining in the dark, cells were washed twice, suspended in 200 μl FACS buffer and analyzed by flow cytometry on a BD FACS Canto II (Becton-Dickinson, Heidelberg, Germany) using FlowJo flow cytometry analysis software (FlowJo LLC, Ashland, OR, USA).

2.6. Enzyme-linked immunosorbent assay

The ELISA which detects full-length and soluble human IL-6R was performed as described previously (Baran et al., 2013). Supernatants and cells lysates were analyzed in appropriate dilutions and their concentrations calculated accordingly. As an indicator for shedding efficiency, the ratio between sIL-6R and IL-6R was calculated.

2.7. Presentation of experimental data

Data are expressed as mean values ± s.e.m. unless indicated otherwise. Proteolysis of IL-6R variants was assessed via ELISA, and the data shown are derived from three independent experiments. Relative mean fluorescence intensity (MFI) was calculated by subtraction of negative control. For all other experiments, one experiment from at least three with similar outcome is shown.