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Cleavage of the Interleukin-11 receptor induces processing of its C-terminal fragments by the gamma-secretase and the proteasome

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ABSTRACT

The cytokine Interleukin-11 (IL-11) signals through the membrane-bound IL-11 receptor (IL-11R), which is expressed in a cell-type specific manner. We have recently shown that the metalloprotease ADAM10 can cleave the IL-11R. The liberated soluble IL-11R (sIL-11R) ectodomain can bind its ligand, and the resulting IL-11/sIL-11R complex can activate cells that do not express the IL-11R (*trans*-signaling). In this study, we show that the remaining C-terminal fragment (CTF1) after ADAM10-mediated cleavage is subsequently cleaved within the membrane by the gamma-secretase complex, and that the resulting shorter CTF2 is further degraded by the proteasome. In contrast to other transmembrane receptors, e.g. Notch, we find no evidence that the IL-11R CTF can translocate into the nucleus to act as a transcription factor, suggesting that regulated intramembrane proteolysis of the IL-11R CTF acts as a mechanism to clear the plasma membrane from remaining protein fragments after cleavage of its ectodomain.

1. Introduction

Interleukin-11 (IL-11) is a member of the interleukin-6 (IL-6) family of cytokines [1]. IL-11 initially binds to a membrane-tethered IL-11R, and this IL-11/IL-11R complex leads to the recruitment of a homodimer of the ubiquitously expressed receptor gp130 [1,2]. This pathway has been termed classic signaling and can solely occur in cells that express IL-11R, allowing cell-type specificity due to a distinct IL-11R expression pattern [2,3]. Homodimerization of gp130 enables the activation of intracellular signaling cascades, notably the Jak/STAT pathway [4].

We have recently shown that IL-11 can also activate cells via a *trans*-signaling pathway [5,6]. Here, the membrane-bound IL-11R is cleaved by the metalloprotease ADAM10, and the liberated soluble IL-11R (sIL-11R) ectodomain can bind IL-11 with similar affinity as the membrane-bound variant, forming an agonistic IL-11/sIL-11R complex. IL-11 *trans*-signaling can be efficiently blocked by sgp130Fc [5], a protein that consists of the extracellular portion of gp130 dimerized by the Fc part of a human IgG antibody and is currently in phase II clinical trials [7]. With the help of sgp130Fc, we could recently show *in vivo* that fertility of female mice requires IL-11 classic, but not *trans*-signaling [8]. Furthermore, IL-11 *trans*-signaling was dispensable for tumorigenesis in a mouse model of gastric tumor formation [9].

The cleavage of transmembrane proteins by proteases like ADAM10 (so-called α-cleavage) can induce further proteolytic processing of the remaining parts of the initial substrate. This process, also known as regulated intramembrane proteolysis [10], is often performed by the γ-secretase complex. The γ-secretase belongs to the aspartic proteases and consists of the four subunits presenilin, Pen-2, Aph-1 and nicastrin [11]. Intramembrane cleavage results in the liberation of an intracellular region, which can act as a transcription factor and influence gene expression, as it has been shown for the Notch receptor [12]. For several other substrates, including the Interleukin-6 receptor [13], no functional role for the so-called C-terminal fragments (CTFs), could be determined, leading others to speculate that the γ-secretase could serve as a proteasome of the plasma membrane [14].

In the present study, we analyzed whether the IL-11R is a substrate for the γ-secretase complex. We show that ADAM10-mediated cleavage of the IL-11R results in the formation of a CTF that is further processed by the γ-secretase and finally degraded by the proteasome.

2. Materials and methods

Cells lines – Ba/F3-gp130 and Ba/F3-gp130-IL-11R cells have been described previously [15]. Retroviral transduction of Ba/F3-

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gp130 cell lines was performed as described [16]. HEK293 cells were obtained from DSMZ GmbH (Braunschweig, Germany). All cells were grown in DMEM high glucose culture medium (Gibco/Thermo Fisher Scientific, Waltham, MA, USA), supplemented with 10% fetal bovine serum, penicillin (60 mg/l) and streptomycin (100 mg/l) and cultured in a standard incubator with a water-saturated atmosphere at 37 °C and 5% CO_2. Ba/F3-gp130 cells were cultured in the presence of 10 ng/ml Hyper-IL-6 [17]. Ba/F3-gp130 cell lines expressing IL-11R variants were cultured with 10 ng/ml IL-11 instead [15]. Murine embryonic fibroblasts (MEFs) from wildtype mice have been described previously [18]. For stable transduction of MEF cells, Phoenix-Eco cells (U. Klingmüller, DKFZ, Heidelberg, Germany) were transfected with pMOWS plasmid encoding for IL-11R. MEF cells were transduced with retrovirus-containing supernatant as described previously [19]. Two days after transduction, stable clones were selected with 2 μg/ml Puromycin.

Antibodies, proteins and chemicals – The following antibodies were used: STAT3 (124H6), pSTAT3 (pY705), Myc (71D10), and GAPDH (14C10) from Cell Signaling Technology (Frankfurt/M., Germany), IL-11R (N-20), IL-11R C-term (K-20), and actin (C-4) from Santa Cruz (Santa Cruz, CA, USA), and GFP (clones 7.1 and 13.1) from Roche Applied Science (Penzberg, Germany). As secondary antibodies, IRDye 800CW Goat anti-Mouse and IRDye 680RD Donkey anti-Rabbit from LICOR Biosciences (Lincoln, NE, USA), and AlexaFluor647-conjugated anti-rabbit and AlexaFluor488-conjugated anti-goat antibody from Thermo Fisher Scientific (Waltham, MA, USA) were used. Hyper-IL-6 was expressed and purified as described previously [17,20]. Human IL-11 was expressed and purified as described previously [5]. Ionomycin, DAPT, Marimastat, AEBSF, E64, Pepstatin A, compound E, leptomycin B, MG132, NH_4Cl, BafilomycinA1, Epoxomicin were purchased from Sigma-Aldrich, St. Louis, MO, USA.

Expression plasmids – A pcDNA3.1 expression plasmid encoding the human IL-11R with an N-terminal myc-tag was described previously [15]. Based on this, a C-terminal fusion construct with eGFP (pcDNA3.1-IL-11R-GFP) as well as a C-terminal deletion variant lacking the intracellular region (pcDNA3.1-IL-11RΔICD) were constructed via splicing-by-overlapping-extensions PCR. For retroviral transduction, the open reading frames were subcloned into the pMOWS vector.

Flow cytometry – Cell surface IL-11R was analyzed with anti-myc and AlexaFluor647-conjugated anti-rabbit antibodies as described previously [6].

Confocal microscopy – MEF cells stably expressing the IL-11R were seeded onto coverslips and treated with Leptomycin B for 16 h. For staining, cells were washed with PBS and fixed with 4% PFA for 20 min. Then, cells were again washed with PBS and incubated with 0.12% glycine for 10 min. After another washing step, cells were permeabilised with 0.1% TritonX-100. To minimize unspecific staining, cells were blocked with 10%FCS in 0.1% TritonX-100 in PBS. Staining with the anti-IL-11R C-Term antibody (K-20) and AlexaFluor488-conjugated anti-goat antibody was performed in blocking solution in a humidified chamber for 1 h each, with five washing steps between primary and secondary antibody. Coverslips were mounted onto microscopy slides using ProLong Gold Antifade reagent with DAPI (Thermo Fisher Scientific, Waltham, MA, USA). Images were acquired with an Olympus FV1000 confocal laser scanning microscope.

Stimulation and lysis of cells – Analysis of IL-11R ectodomain shedding has been described previously [5]. For detection of IL-11R fragments, HEK293 cells were transiently transfected with expression plasmids encoding for IL-11R variants. 36 h after transfection, cells were treated as indicated in the individual experiments for 16 h. Then, cells were harvested and lysed in lysis buffer (50 mM Tris-HCl pH 7.5, 150 mM NaCl, 1% Triton-X 100 and Complete protease inhibitor cocktail (Roche Applied Science, Penzberg, Germany)). The protein concentration of lysates was determined using the BCA protein assay kit (Thermo Fisher Scientific, Waltham, MA, USA). For analysis of STAT3 phosphorylation, Ba/F3-gp130-derived cell lines were starved for 3 h and then stimulated with cytokines as indicated. Cells were harvested by centrifugation and directly boiled in Laemmli buffer.

Western blotting – Equal amounts of cell lysate were separated by SDS-PAGE and blotted onto nitrocellulose membranes. These were blocked with Odyssey blocking buffer (LI-COR Biosciences, Lincoln, NE, USA) for 1 h at room temperature. Then, membranes were treated with primary antibody at 4 °C overnight and, after washing, with IRDye-conjugated secondary antibodies for 1 h in the dark at room temperature. Signals were analyzed with an Odyssey Fc and the ImageStudio software (LI-COR Biosciences, Lincoln, NE, USA).

Cell viability assays – Cell viability assays using the CellTiter-Blue Cell Viability Assay kit (Promega, Mannheim, Germany) was performed as described previously [15].

Presentation of experimental data – Data are shown as the mean ± SD from three independent experiments unless indicated otherwise. For Western blots, flow cytometry data and cell viability assays, one experiment from at least three with similar outcome is shown. Quantification of Western blots was done as described previously [5].

3. Results

3.1. An IL-11R-GFP fusion protein is biologically active and allows the detection of a C-terminal fragment (CTF)

We have previously shown that the IL-11R can be proteolytically cleaved by the metalloprotease ADAM10, which results in the liberation of a biologically active sIL-11R ectodomain [5,6]. Because a large number of transmembrane proteins undergo further cleavage events after this initial α-cleavage [21], we sought to investigate whether this is also the case for the IL-11R, and whether this is relevant for the biological function of the IL-11R. Due to the fact that the remaining stub of the IL-11R after ADAM10-mediated cleavage would have a size of around 5.1 kDa und thus would be very small and difficult to analyze, we generated a C-terminal GFP-fusion protein, which would give rise to a C-terminal fragment of about 32 kDa (Fig. 1A).

In order to validate that the C-terminal modification did not alter the biological function of the IL-11R, we generated stably transduced Ba/F3-gp130 cell lines expressing either IL-11R or IL-11R-GFP, which revealed no difference in either cell-surface abundance, phosphorylation of the transcription factor STAT3 or IL-11-dependent cell proliferation (Fig. 1B–D). Furthermore, both IL-11R variants were equally well expressed at the surface of transiently transfected HEK293 cells (Fig. 1E), and ionomycin-induced ADAM10-mediated shedding of IL-11R-GFP gave rise to a sIL-11R, demonstrating that also proteolysis of the IL-11R was not influenced by the C-terminal fusion with GFP (Fig. 1F). Interestingly, when we analyzed lysates of HEK293 cells expressing IL-11R-GFP via Western blot, we could identify a strong signal for a C-terminal fragment (CFT1) with the predicted size of about 32 kDa, which was reactive to an anti-GFP antibody as well as to an antibody that was raised against the intracellular, C-terminal region of the IL-11R (Fig. 1G).

3.2. The γ-secretase is important for CTF1 processing and CTF2 generation

After the α-cleavage, many type-I transmembrane proteins are

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