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Interleukin-6–interleukin-11 receptor chimeras reveal ionomycin-induced proteolysis beyond ADAM10

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Interleukin-6 (IL-6) and interleukin-11 (IL-11) are two important pleiotropic cytokines, both of which signal through a homodimer of the β-receptor gp130. Specificity is gained through the unique, nonsignaling α-receptors IL-6R and IL-11R. Soluble variants of IL-6R and IL-11R also exist. Both membrane-bound receptors can be cleaved by the metalloprotease ADAM10. Here, we use ten different chimeric receptors consisting of different parts of IL-6R and IL-11R and analyze their susceptibility toward cleavage by ADAM10. As expected, all chimeras are substrates of ADAM10. However, we observed that cleavage of chimeric receptors containing the stalk region of the IL-11R could be blocked by the protease inhibitor GI (selective for ADAM10), but not by the protease inhibitor GW (selective for both ADAM10 and ADAM17), suggesting that another protease besides ADAM10 is involved in cleavage of these chimeras.

Keywords: gp130interleukin-11 receptor; interleukin-6 receptor; proteolysis

Interleukin-6 (IL-6) and interleukin-11 (IL-11) are both members of the IL-6 family of cytokines [1]. IL-6 possesses both anti- and pro-inflammatory properties, and especially the latter ones make it an important target for therapy [2,3]. Healthy humans have very low or even undetectable levels of IL-6 in their circulation, which rise dramatically in practically all inflammatory diseases. Antibodies blocking the biological activity of IL-6 are approved for different malignancies, including rheumatoid arthritis, giant cell arteritis, Castleman disease, and cytokine release syndrome [2], and some clinical studies show that IL-6 blockade is also beneficial in patients with Covid-19 infection [4–6].

In contrast to IL-6, IL-11 is less well characterized and its important roles in development, inflammation, and cancer development have just started to be unraveled in recent years. IL-11 was for a long time described as an anti-inflammatory cytokine and was therefore used in the clinic to treat patients with thrombocytopenia after chemotherapy [7]. Nowadays, it is known that tumors especially in colon [8] and stomach [9,10] rely on constant IL-11 signaling, and while no compounds targeting IL-11 have been approved for clinical use yet, studies in which IL-11 is blocked [8,11] look promising.

IL-6 and IL-11 activate similar intracellular signaling cascades like Janus kinase (Jak)/Signal Transducer and Activator of Transcription (STAT) signaling through a homodimer of the ubiquitously expressed β-receptor gp130 [1]. Both cytokines possess unique nonsignaling α-receptors, termed IL-6 receptor (IL-6R) and IL-11R, respectively, to which the cytokines initially bind. Because gp130 is expressed ubiquitously, the expression pattern of IL-6R and IL-11R determines which cells in the human body can react to which cytokine.

Importantly, also soluble variants of IL-6R (sIL-6R) and IL-11R (sIL-11R) exist. These soluble receptors

Abbreviations
ADAM, a disintegrin and metalloprotease; GI, protease inhibitor GI254023X; GW, protease inhibitor GW280264X; IL, interleukin; Jak, Janus kinase; MMP, matrix metalloprotease; STAT, Signal Transducer and Activator of Transcription.

bind their ligands with similar affinity as their membrane-tethered counterparts, and the resulting complexes consisting of cytokine and soluble cytokine receptor are biologically active agonists that can bind to and activate gp130 homodimers and thus in principle activate all cells of the human body. In contrast to signaling *via* membrane-bound receptors, which is the so-called classic signaling pathway, trans-signaling refers to signaling of IL-6 and IL-11 initiated *via* soluble cytokine receptors.

Different mechanisms exist how soluble cytokine receptors can be generated [12]. The sIL-6R is either generated by alternative splicing of its mRNA [13] or *via* proteolytic cleavage of the membrane-bound IL-6R by the metalloproteases ADAM10 and ADAM17 [14–18]. We were able to show that the majority of the sIL-6R is indeed generated by proteolysis and only about ~ 15% of the sIL-6R in the human circulation originate from alternative splicing [19]. Of note, alternative splicing results in ten novel amino-acid residues at the C-terminus of the sIL-6R that are not present in the full-length IL-6R or the sIL-6R generated by proteolysis [20]. This difference does not influence the biological function of the sIL-6R because IL-6 binds to the cytokine-binding module located within the D2 and D3 domains, which is independent of the sIL-6R C-terminus. ADAM10 is also the protease that cleaves the membrane-bound IL-11R [21]. Although much less is known about the sIL-11R compared to sIL-6R *in vivo*, sIL-11R is part of the human plasma proteome [22] and can be detected in the serum of healthy volunteers, albeit at lower concentrations than the sIL-6R [21]. No sIL-11R originating from alternative splicing has been described in the literature to date.

Both receptors have the same overall topology, consisting of three extracellular domains (D1–D3), a long flexible stalk that connects them to the transmembrane helix, and an intracellular region that is crucial for sorting in polarized cells [23]. We have shown previously that this modular organization can be used to create chimeric receptors in which individual or several parts are swapped between IL-6R and IL-11R [24]. Using this approach, we could transfer cytokine binding properties [24] as well as susceptibility toward cleavage by ADAM17 between the two receptors [21]. However, how these chimeras react toward cleavage by ADAM10 has not been investigated so far.

The catalytic domains of ADAM10 and ADAM17 share high homology, and the development of specific chemical compounds that are able to discriminate between them has been challenging. Nevertheless, several hydroxamate compounds with different potency against the two proteases have been developed, which bind directly to the active site of the proteases and thereby block their catalytic activity [25]. The protease inhibitor GI254023X (GI) is 100-fold more potent to inhibit ADAM10 compared to ADAM17, while protease inhibitor GW280264X (GW) blocked ADAM10 and ADAM17 with similar potency [25]. Using both inhibitors side-by-side therefore allows to determine the contribution of both proteases to the proteolytic cleavage of a given substrate.

In this study, we analyzed proteolysis of ten different IL-6R/IL-11R chimeras by ADAM10. We find that all receptors, albeit to different extents, are substrates for ADAM10. However, our experiments using different small molecule protease inhibitors suggest that some chimeric proteins are subject to proteolysis by another, so far unidentified protease.

Materials and methods

Cells and reagents

HEK293 cells were cultured in DMEM high-glucose culture medium (Sigma-Aldrich, St. Louis, MO, USA) supplemented with 10% fetal bovine serum, penicillin (60 mg·L^{-1}), and streptomycin (100 mg·L^{-1}), and kept at 37 °C and 5% CO2 in a standard incubator with a water-saturated atmosphere. Ionomycin was purchased from Sigma-Aldrich. The small molecule inhibitors GI254023X (GI) and GW280264X (GW) were synthesized by Iris Biotech (Marktredwitz, Germany) [26]. The anti-myc-tag (71D10) and anti-GAPDH (14C10) antibodies were obtained from Cell Signaling Technology (Danvas, MA, USA). The anti-IL-6R antibody 4-11 was produced in house.

Construction of expression plasmids

The pcDNA3.1 expression plasmids for myc-tagged hIL-11R, IL-6R and chimeras thereof used in this study have been described previously [21].

Analysis of ectodomain shedding

Cytokine receptor shedding upon ionomycin stimulation was analyzed as described previously [21]. In brief, HEK293 cells were transiently transfected with expression plasmids encoding the cytokine receptors. Forty-eight hours later, cells were washed with PBS, pretreated with 3 μM GI or GW for 30 min as indicated and stimulated with 1 μM ionomycin for 60 min. Cells and supernatants were harvested and subjected to western blot analysis. For this, proteins from the supernatants were precipitated by adding an equal volume of 20% trichloracetic acid, incubating the samples on ice for 20 min and subsequent centrifugation at 18 000 ***g*** for 20 min at 4 °C. The precipitates were washed

with ice-cold acetone and dried after a second centrifugation at 18 000 ***g*** for 20 min at 4 °C. The pellets were then boiled in 2.5x reducing Laemmli buffer. Cells were lysed [50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Triton-X-100, complete protease inhibitor cocktail (Roche Applied Science, Mannheim, Germany)] and the lysates were also boiled in Laemmli buffer.

Western blotting

Cell lysates and supernatant precipitates were separated by SDS-PAGE and subsequently blotted onto PVDF membranes. The membranes were blocked with 1% BSA in TBS for 1 h at room temperature and afterward incubated with primary antibodies at 4 °C overnight. After washing, membranes were treated with peroxidase-conjugated secondary antibodies for 1 h at room temperature and then, after another washing step, signals were detected using either the ECL Chemocam Imager (Intas Science Imaging, Göttingen, Gemany) or the ChemiDoc MP Imager (Biorad, Hercules, CA, USA) and the EMD Millipore™ Immobilon™ Western Chemiluminescent HRP Substrate (Merck Millipore, Darmstadt, Germany). If necessary, the membrane was stripped using the Restore Western Blot Stripping Buffer (Thermo Fisher Scientific, Waltham, MA, USA). After stripping, the membrane was washed and again blocked before another antibody was applied.

Densitometric analysis

Densitometric quantification of western blots was performed with the ImageStudio Lite software (LI-COR Biosciences, Lincoln, NE, USA). The ratio of receptor signal and the GAPDH signal in the cell lysate was calculated to determine cellular receptor levels. Then, the ratio of the signal intensity from the receptor in the supernatant and the cellular receptor level was calculated and normalized to the unstimulated control. Data are presented as mean ± SEM from three independent experiments.

Statistical analysis

Statistical analysis was carried out with GraphPad prism 8 (GraphPad Software, San Diego, CA, USA). Data were analyzed by one-way ANOVA followed by Tukey's multiple comparisons test.

Results

IL-6R and IL-11R are both cleaved by ADAM10

We have previously shown that the IL-11R is a substrate for ADAM10 [21], and we and others have also demonstrated that the IL-6R can be cleaved by this protease [15–19] (Fig. 1A). Both cytokine receptors have a similar topology consisting of three extracellular domains (D1–D3), an unstructured stalk region that connects them to the transmembrane helix, and an intracellular region (Fig. 1B). In order to analyze ADAM10-mediated cleavage and to verify our previous results, we transiently transfected HEK293 cells with expression plasmids encoding either human IL-6R or IL-11R and stimulated them with either the ionophore ionomycin, which is an established activator of ADAM10, or the solvent DMSO as control. This resulted in the generation of sIL-6R (Fig. 1C) and sIL-11R (Fig. 1D), respectively, as shown by western blot of proteins precipitated from the cell culture supernatant. We verified the activation of ADAM10 by pretreatment of the cells with the two metalloprotease inhibitors GI (selective for ADAM10) and GW (selective for both ADAM10 and ADAM17) [25]. Both inhibitors were indeed able to reduce sIL-6R and sIL-11R generation, showing that ADAM10 was activated by ionomycin treatment and thus verifying our previous results, although GW appeared to be less able to block sIL-11R generation (Fig. 1C,D and [15–19,21]).

Swapping of stalk, transmembrane, and intracellular regions between IL-6R and IL-11R

We have created several chimeric variants of IL-6R and IL-11R in order to demonstrate their modular organization [24] and to analyze their susceptibility toward cleavage by ADAM17 [21]. However, an analysis how these chimeric proteins behave regarding ADAM10-mediated cleavage is lacking. We first analyzed proteolysis of IL-11R-S/T/I, which contains the three extracellular domains of the IL-11R combined with stalk, transmembrane, and intracellular regions of the IL-6R (Fig. 2A). As described above, we transiently transfected HEK293 cells with an expression plasmid encoding this variant and stimulated the cells with ionomycin or DMSO and pretreated with GI, GW, or DMSO. As shown in Fig. 2B, we detected robust sIL-11R generated upon ionomycin stimulation, which was reduced to the amount of unstimulated cells when pretreated with GI or GW. We performed a similar experiment with IL-6R-S/T/I, which contains the three extracellular domains of the IL-6R and stalk, transmembrane, and intracellular regions of the IL-11R (Fig. 2A). IL-6R-S/T/I was also cleaved by ADAM10 as judged by GI- and GW-mediated inhibition of ionomycin-induced proteolysis (Fig. 2C). We concluded from these experiments that, because the general topology of the two receptors is the same [24]

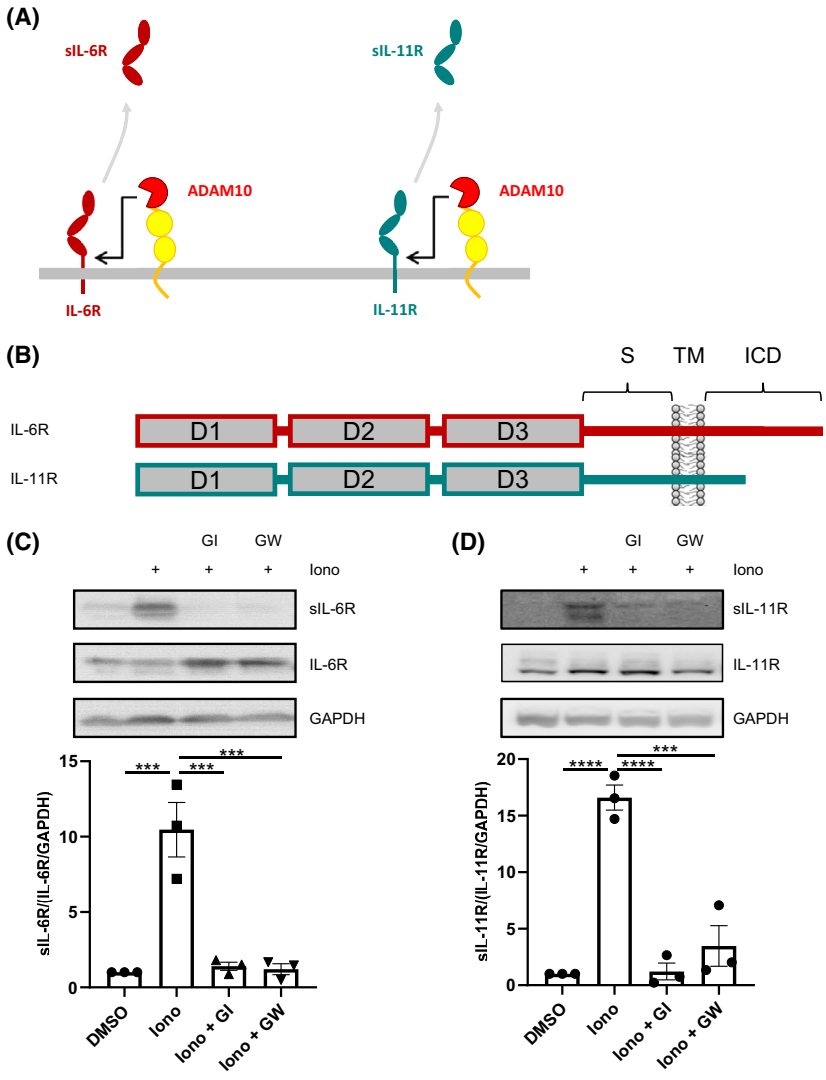

Fig. 1. IL-6R and IL-11R are both cleaved by ADAM10. (A) Schematic representation of IL-6R and IL-11R cleavage by the metalloprotease ADAM10 and the release of soluble IL-6R and IL-11R into the supernatant. (B) Schematic overview of the IL-6R and IL-11R. The three extracellular domains D1, D2, and D3 are followed by a stalk region (S), a transmembrane part (TM), and an intracellular region (ICD). (C) HEK293 cells transiently transfected with an expression plasmid encoding IL-6R were stimulated with 1 μM ionomycin or DMSO as control for 60 min. Where indicated, cells were treated with 3 μM of the protease inhibitors GI or GW 30 min prior to stimulation. sIL-6R precipitated from the supernatant and IL-6R in the cell lysates were detected by western blot. GAPDH was visualized to ensure equal loading. One representative experiment and the quantification of three independent experiments with similar outcome are shown (mean ± SEM, ***$P < 0.001$). (D) The experiment was performed as described for panel C, but HEK293 cells were transiently transfected with an expression plasmid encoding IL-11R. One representative experiment and the quantification of three independent experiments with similar outcome are shown (mean ± SEM, ***$P < 0.001$, ****$P < 0.0001$).

and both are substrates of ADAM10, the exchange of domains does not influence proteolysis by ADAM10 per se.

Exchange of individual regions of the IL-11R reveals importance of the stalk region

Having exchanged stalk (S), transmembrane (TM), and intracellular (ICD) regions simultaneously, we next sought to analyze how swapping of individual regions would affect proteolysis by ADAM10 and therefore tested IL-11R-S, IL-11R-TM, and IL-11R-ICD (Fig. 3A). IL-11R-S was cleaved upon ionomycin stimulation, which was blocked to a similar degree by GI and GW, showing that this IL-11R variant is a bona fide ADAM10 substrate (Fig. 3B). IL-11R-TM was also efficiently cleaved upon stimulation with ionomycin, and this cleavage was completely inhibited by GI and GW pretreatment (Fig. 3C). In contrast, although IL-11R-ICD was cleaved upon ionomycin stimulation, this cleavage could only be blocked completely by GI (Fig. 3D). Interestingly, pretreatment with GW was less effective and allowed some sIL-11R generation, as the cells pretreated with GW released more sIL-11R than the DMSO-treated cells (Fig. 3D). These results suggest that another protease that is activated by calcium influx into the cell participates in the cleavage of the IL-11R-ICD variant. Furthermore, they suggest that the stalk region of the IL-11R is required for this proteolytic cleavage, because exchange of this region in IL-11R-S completely prevents cleavage by this GW-insensitive, ionomycin-activated protease (Fig. 3B). In contrast, exchange of the intracellular region of the IL-11R with their IL-6R

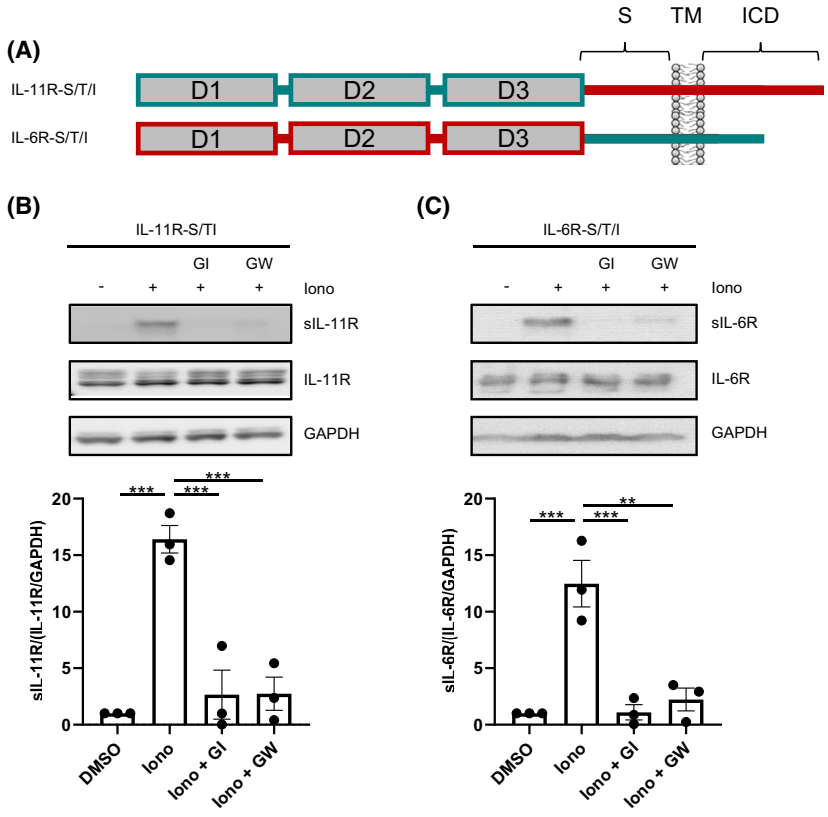

Fig. 2. Swapping of stalk, transmembrane, and intracellular regions between IL-6R and IL-11R. (A) Schematic overview of the IL-6R/IL-11R chimeras used in the experiments. The three extracellular domains D1, D2, and D3 are followed by a stalk region (S), a transmembrane part (TM), and an intracellular region (ICD). (B, C) HEK293 cells transiently transfected with an expression plasmid encoding (B) IL-11R-S/T/I or (C) IL-6R-S/T/I were stimulated with 1 μM of ionomycin or DMSO as control for 60 min. Where indicated, cells were treated with 3 μM of the protease inhibitors GI or GW 30 min prior to stimulation. Soluble receptors precipitated from the supernatant and receptors in the cell lysates were detected by western blot. GAPDH was visualized to ensure equal loading. One representative experiment and the quantification of three independent experiments with similar outcome are shown (mean ± SEM, $^{**}P < 0.01$, $^{***}P < 0.001$).

counterpart did not cause this effect (Fig. 3D). At this point, we also cannot rule out a contribution of the transmembrane helix, because the IL-11R-TM variant is also inhibited by GI and GW pretreatment (Fig. 3C).

Exchange of the stalk region of the IL-6R reveals cleavage by another protease

We next analyzed the corresponding IL-6R variants IL-6R-S, IL-6R-TM, and IL-6R-ICD in which individual regions of the IL-6R have been replaced by their IL-11R counterparts (Fig. 4A). Intriguingly, ADAM10-mediated proteolysis of the variant IL-6R-S could be observed after stimulation with ionomycin, as it was completely prevented by pretreatment by GI (Fig. 4B). However, proteolysis was only partly inhibited by GW, as it showed no statistically significant difference from cells treated with ionomycin only, and showed significantly higher sIL-6R levels compared to GI treatment (Fig. 4B). These results indicate that the introduction of the IL-11R stalk region allows cleavage by a so far unidentified protease (Fig. 4B). In contrast, while the IL-6R-TM and IL-6R-ICD variants were also cleaved after stimulation by ionomycin, both GI and GW inhibited sIL-6R generation, showing that only ADAM10 was able to cleave these two IL-6R chimeras (Fig. 4C,D). From these results, we concluded that the exchange of the stalk region and not of the transmembrane or the intracellular regions triggers release of the sIL-6R after ionomycin stimulation by another protease in addition to ADAM10.

Insertion of the ADAM17 cleavage site into the IL-11R alters ionomycin-induced proteolysis

We have previously shown that the exchange of a stretch of 10 amino acid residues within the stalk region in close proximity to the plasma membrane is sufficient to transfer susceptibility toward cleavage by ADAM17 between IL-6R and IL-11R [21]. We termed these two variants IL-11R-10AS, which rendered the IL-11R susceptible for ADAM17-mediated cleavage, and IL-6R-10AS, which was not efficiently cleaved by ADAM17 in contrast to IL-6R wild type (Fig. 5A and [21]). In order to analyze whether these mutations influence proteolysis by ADAM10, we performed similar experiments as for the other cytokine receptor chimeras. As shown in Fig. 5B, IL-11R-10AS was cleaved by ADAM10 after ionomycin stimulation. However,

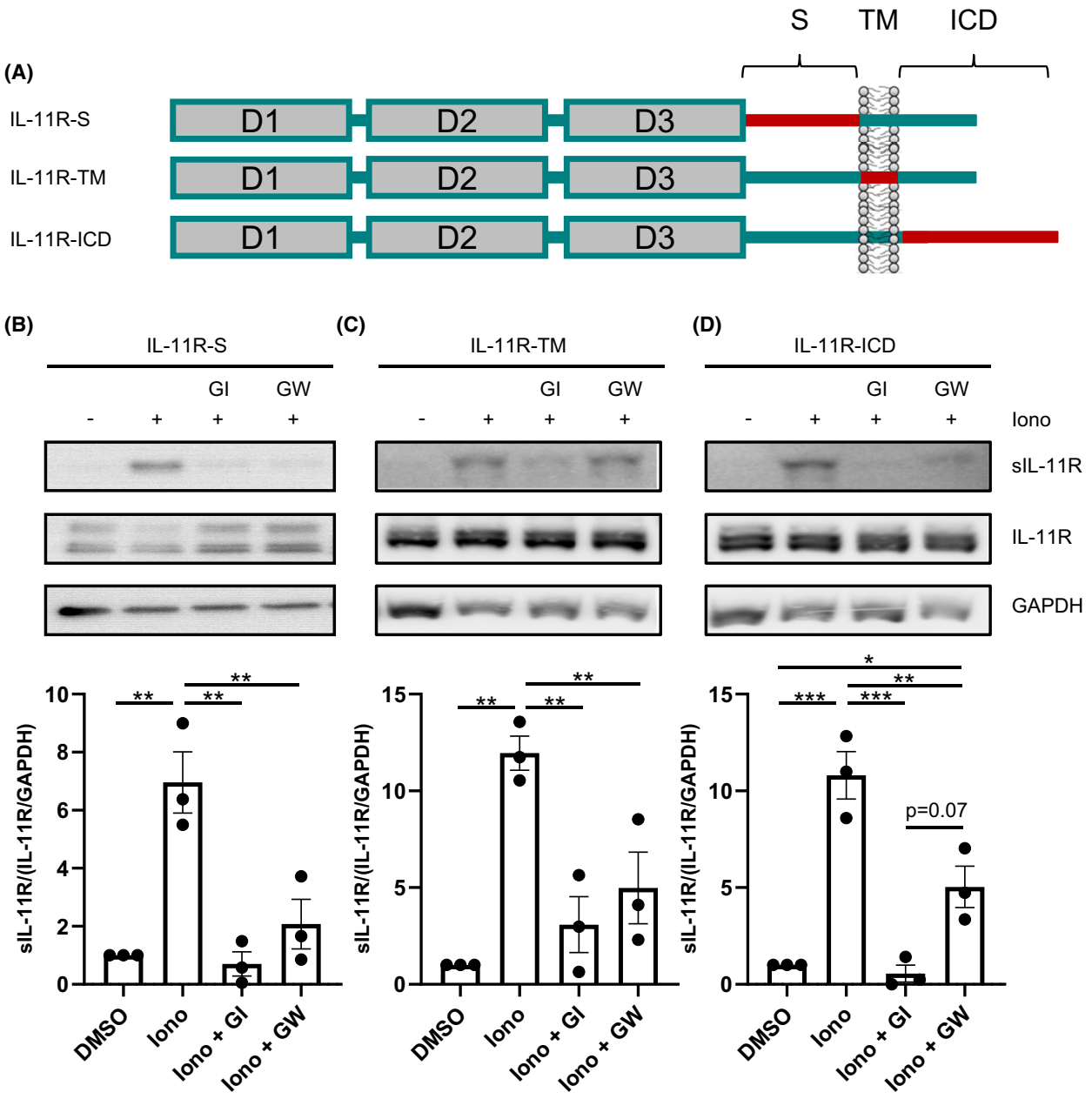

Fig. 3. Exchange of individual regions of the IL-11R reveals contribution of the intracellular region. (A) Schematic overview of the IL-6R/IL-11R chimeras used in the experiments. The three extracellular domains D1, D2, and D3 are followed by a stalk region (S), a transmembrane part (TM), and an intracellular region (ICD). (B–D) HEK293 cells transiently transfected with an expression plasmid encoding (B) IL-11R-S, (C) IL-11R-TM, or (D) IL-11R-ICD were stimulated with 1 μM ionomycin or DMSO as control for 60 min. Where indicated, cells were treated with 3 μM of the protease inhibitors GI or GW 30 min prior to stimulation. Soluble IL-11R precipitated from the supernatant and IL-11R in the cell lysates were detected by western blot. GAPDH was visualized to ensure equal loading. One representative experiment and the quantification of three independent experiments with similar outcome are shown (mean ± SEM, $*P < 0.05$, $**P < 0.01$, $***P < 0.001$).

while GI was able to reduce sIL-11R cleavage, we observed also for this variant that GW was not capable of reducing ionomycin-induced cleavage. In contrast, ionomycin-induced cleavage of IL-6R-10AS was blocked by both inhibitors, underlining that ADAM10 was the sole protease cleaving this IL-6R variant (Fig. 5C).

Finally, we sought to determine whether higher concentrations of GW would be able to inhibit the cleavage of IL-11R-10AS. Therefore, we repeated our

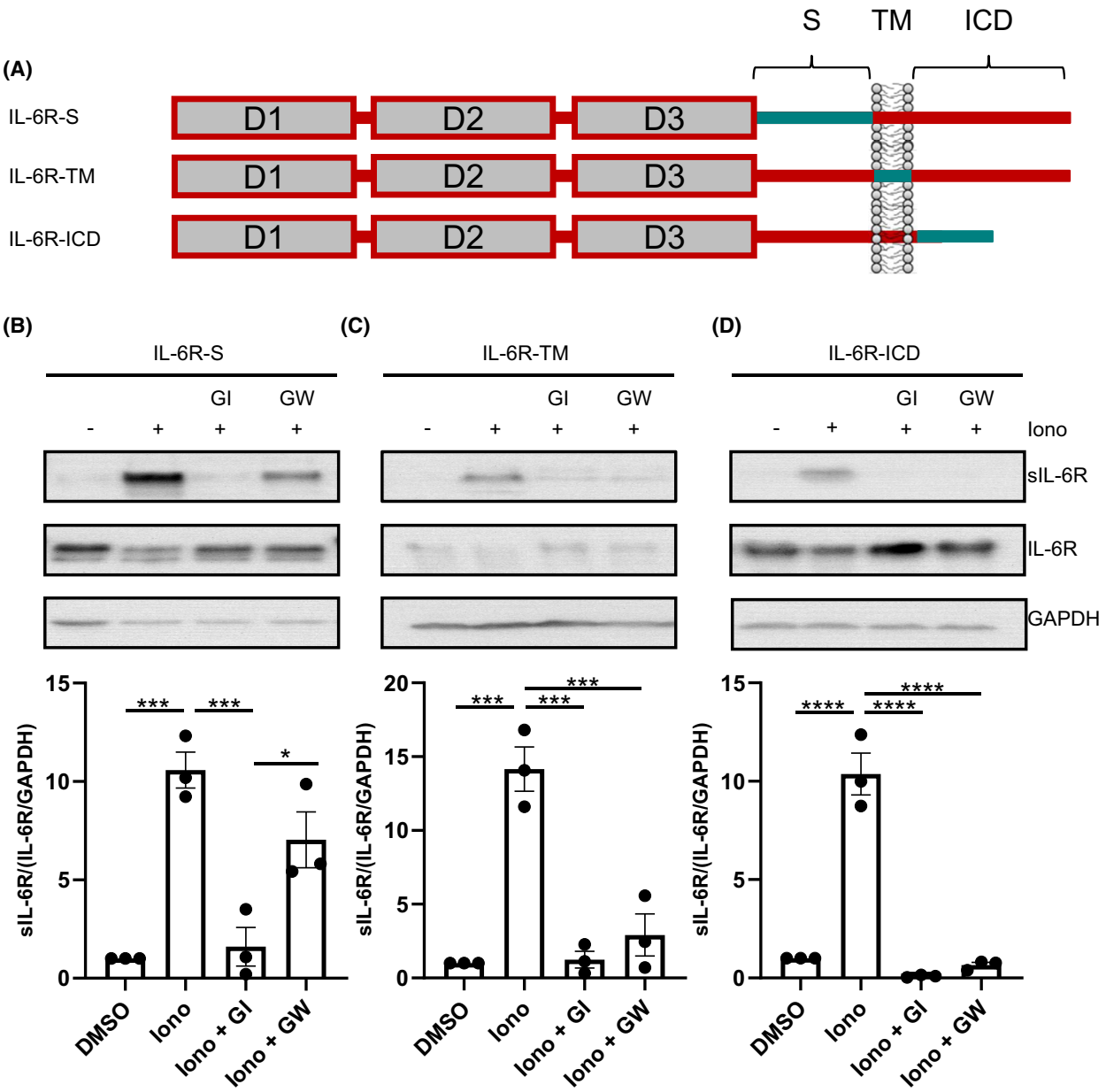

Fig. 4. Exchange of the stalk region of the IL-6R reveals cleavage by another protease insensitive to inhibition by GW. (A) Schematic overview of the IL-6R/IL-11R chimeras used in the experiments. The three extracellular domains D1, D2, and D3 are followed by a stalk region (S), a transmembrane part (TM), and an intracellular region (ICD). (B–D) HEK293 cells transiently transfected with an expression plasmid encoding (B) IL-6R-S, (C) IL-6R-TM, or (D) IL-6R-ICD were stimulated with 1 μM of ionomycin or DMSO as control for 60 min. Where indicated, cells were treated with 3 μM of the protease inhibitors GI or GW 30 min prior to stimulation. Soluble IL-6R precipitated from the supernatant and IL-6R in the cell lysates were detected by western blot. GAPDH was visualized to ensure equal loading. One representative experiment and the quantification of three independent experiments with similar outcome are shown (mean ± SEM, *$P < 0.05$, ***$P < 0.001$, ****$P < 0.0001$).

experiment, but used higher concentrations of GI and GW. As shown in Fig. 5D, at a concentration of 3 μM GI significantly better inhibited ionomycin-induced proteolysis compared to 3 μM GW, which held also true at a concentration of 10 μM each. At 30 μM, however, ionomycin-induced proteolysis of the IL-11R-10AS variant was equally well blocked by both inhibitors (Fig. 5D). We concluded from this that the IL-11R stalk region can be processed by a GW-resistant (or at least less sensitive) protease.

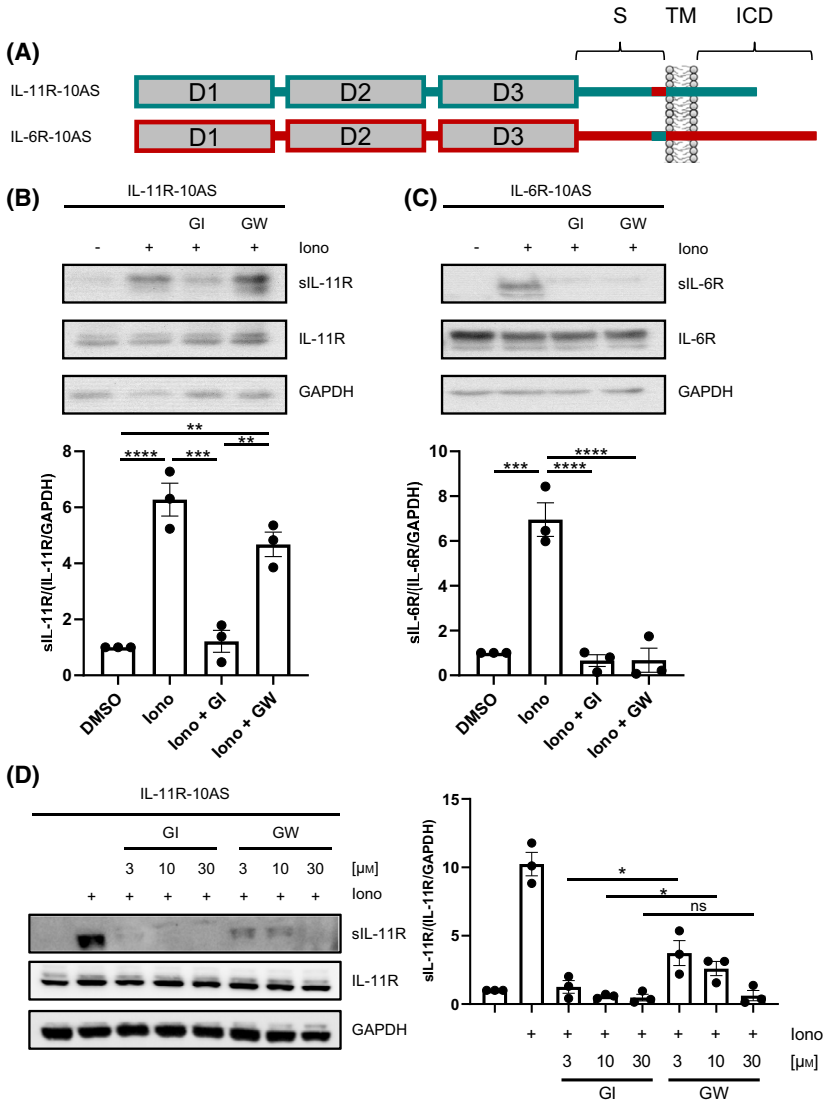

Fig. 5. Insertion of the ADAM17 cleavage site into the IL-11R alters ionomycin-induced proteolysis. (A) Schematic overview of the IL-6R/IL-11R chimeras used in the experiments. The three extracellular domains D1, D2, and D3 are followed by a stalk region (S), a transmembrane part (TM), and an intracellular region (ICD). (B, C) HEK293 cells transiently transfected with an expression plasmids encoding (B) IL-11R-10AS, or (C) IL-6R-10AS were stimulated with 1 μM of ionomycin or DMSO as control for 60 min. Where indicated, cells were treated with 3 μM of the protease inhibitors GI or GW 30 min prior to stimulation. Soluble receptors precipitated from the supernatant and receptors in the cell lysates were detected by western blot. GAPDH was visualized to ensure equal loading. (D) The experiment was performed as described for panel B, but the cells were pretreated with 3, 10, or 30 μM of the protease inhibitors GI and GW as indicated. One representative experiment and the quantification of three independent experiments with similar outcome are shown (mean ± SEM, $*P < 0.05$, $**P < 0.01$, $***P < 0.001$, $****P < 0.0001$, ns, not significant).

In conclusion, we identify several chimeric cytokine receptor constructs that are differently proteolysed compared to the wild-type receptors. A likely explanation is the involvement of a so far unidentified protease. As all of these constructs contain the stalk region of the IL-11R, it suggests that a cleavage site for this protease does not exist in the stalk region of the IL-6R, but only in the IL-11R stalk region.

Discussion

Proteolysis of IL-6R and IL-11R is the major molecular mechanism that controls whether these cytokines activate their target cells *via* their membrane-bound receptors (classic signaling) or signal *via* soluble receptors (trans-signaling) [27,28]. Understanding how proteases recognize their substrates and how cytokine receptor proteolysis is regulated is not only scientifically important, but can also be used to design and develop therapeutics that target cytokines and soluble cytokine receptors [2,12].

Both IL-6R and IL-11R are substrates of the metalloprotease ADAM10, which can be activated *in vitro* by the ionophore ionomycin. We have created several chimeric variants of IL-6R and IL-11R in order to demonstrate their modular organization [24] and to analyze their susceptibility toward cleavage by ADAM17 [21]. Importantly, all these chimeric receptors are biologically active and are generated and transported to the cell surface at comparable amounts. Furthermore, our data show that all ten chimeras are substrates of ADAM10 and that the amount of soluble cytokine receptor generated by proteolytic cleavage does not differ dramatically between the ten chimeric

receptors. Given that cholesterol- and sphingolipid-rich microdomains of the plasma membrane, also called lipid rafts, are known to modulate ADAM10-mediated proteolysis of membrane-bound proteins, for example, CD44 [29], the amyloid precursor protein [30] or the IL-6R [15], it is surprising that the exchange of the transmembrane region has a rather mild influence regarding proteolysis, as it might alter the positioning of the receptor within these microdomains.

We have shown previously that calcium influx into the cell by other means, for example, by activation of the purinergic P2X7 receptor *via* ATP, also activates ADAM10-mediated cleavage of the IL-6R [16]. The involvement of ADAM10 in these and many other experiments, also by other groups, was verified by the use of the small chemical inhibitors GI and GW, which both block ADAM10 [25,26]. We were surprised to learn that the ionomycin-induced proteolysis of some chimeric receptors was completely blocked by GI, but not by GW. This phenomenon cannot be attributed to ADAM10-mediated cleavage alone, but suggested that at least one additional, so far unknown, protease is involved in the cleavage of these receptors. It is likely that the effect of the other protease(s) is usually masked by ADAM10-mediated cleavage of the receptors, and can only be seen when ADAM10 is inhibited. We and others have previously reported ionomycin-induced cleavage in ADAM10-deficient murine embryonic fibroblasts and HEK293 cells, which was usually attributed to compensatory shedding by ADAM17 [16,18,21,31]. In light of our findings described in this study, it might be worthwhile to reconsider these results. Furthermore, we cannot completely rule out that other biologic mechanisms beyond the involvement of another protease contribute to the observed phenomenon.

Using our swapping approach, we could show that the stalk region of the IL-11R is the key determinant of the susceptibility toward this cleavage pattern. Introduction of the IL-11R stalk into the IL-6R (IL-6R-S, Fig. 4A) was sufficient to reveal GW-insensitive cleavage, which was not the case when the transmembrane or the intracellular part was exchanged (IL-6R-TM and IL-6R-ICD, Fig. 4C,D). This suggests that the protease cleaves within the stalk region of the IL-11R, and that the stalk region of the IL-6R lacks a sufficient cleavage site, because introduction of the IL-6R stalk into the IL-11R (IL-11R-S, Fig. 3B) shows no cleavage. Interestingly, we see stronger effects in receptor variants in which only individual domains are exchanged, but only nonsignificant effects in wild-type IL-11R (Fig. 1D) and IL-6R-S/T/I (Fig. 2C). The reason for this is currently unclear and warrants further investigation.

We have also no further information on the nature of this protease, but given the fact that GI is able to suppress its biological activity, it is very likely to assume that it is also a metalloprotease. Furthermore, our data show that higher concentrations of GW are able to suppress proteolysis, arguing for a protease, which is better inhibited by GI than GW. Although GI and GW are extensively used as specific inhibitors of ADAM10 and ADAM17, initial studies revealed also inhibition of several members of the matrix metalloprotease (MMP) family, including MMP1, MMP3, MMP9, and MMP13 [25]. Interestingly, MMP9 and MMP13 are fourfold better blocked by GI than GW, making these metalloprotease possible candidates for the observed effects. However, most metalloproteases have not been screened for inhibition by the two hydroxamate compounds, and therefore other proteases might be involved as well. Other possible explanations, for example, that some of the receptor mutants are able to displace the inhibitor from the active site of the protease, are less likely, given that the hydroxamate inhibitors directly bind the zinc cofactor within the active site [25]. Further research is warranted to identify the responsible protease or any other mechanism that contributes to the observed effect.

Author Contributions

CG conceived and coordinated the study and wrote the paper. JL performed and analyzed the experiments and contributed to writing of the paper. Both authors analyzed the results and approved the final version of the manuscript.

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Data accessibility

The data that support the findings of this study are available from the corresponding author [christoph.garbers@med.ovgu.de] upon reasonable request.

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