A disintegrin and metalloprotease 17 Dynamic Interaction Sequence, the Sweet Tooth for the Human Interleukin 6 Receptor*

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**Background:** A disintegrin and metalloprotease 17 (ADAM17) releases many proinflammatory mediators.

**Results:** The conserved ADAM seventeen dynamic interaction sequence (CANDIS) mediates effective substrate binding and is controlled by the disulfide-regulated conformation of the preceding membrane-proximal domain (MPD).

**Conclusion:** CANDIS, together with the MPD, represents a novel key regulatory element.

**Significance:** We investigate the molecular details of a novel kind of regulation.

A disintegrin and metalloprotease 17 (ADAM17) is a major sheddase involved in the regulation of a wide range of biological processes. Key substrates of ADAM17 are the IL-6 receptor (IL-6R) and TNF-α. The extracellular region of ADAM17 consists of a prodomain, a catalytic domain, a disintegrin domain, and a membrane-proximal domain as well as a small stalk region. This study demonstrates that this juxtamembrane segment is highly conserved, α-helical, and involved in IL-6R binding. This process is regulated by the structure of the preceding membrane-proximal domain, which acts as molecular switch of ADAM17 activity operated by a protein-disulfide isomerase. Hence, we have termed the conserved stalk region “Conserved ADAM seventeen dynamic interaction sequence” (CANDIS). Finally, we identified the region in IL-6R that binds to CANDIS. In contrast to the type I transmembrane proteins, the IL-6R, and IL-1RII, CANDIS does not bind the type II transmembrane protein TNF-α, demonstrating fundamental differences in the respective shedding by ADAM17.

The type I transmembrane protein a disintegrin and metalloprotease 17 (ADAM17) is a key protease that is involved in the regulation of a wide variety of biological processes. The cleavage of cell surface proteins by ADAM17, the so-called ectodomain shedding, constitutes a rapid and irreversible regulatory switch. On one hand, ADAM17 is involved in physiological and protective processes in development, regeneration, and immune responses, which is illustrated by the perinatal lethality of ADAM17-deficient mice (1) as well as by the compromised regenerative capabilities of hypomorphic ADAM17 mice (2). On the other hand, ADAM17 is associated with uncontrolled inflammation and cancer progression, as evidenced by studies using conditional knockout mice (3–5). The broad range of biological processes that are influenced by ADAM17 is reflected by the wide variety of more than 75 substrates that are shed by this enzyme. These substrates belong to diverse groups like growth factors, adhesion molecules, proinflammatory cytokines, as well as cytokine receptors (5) and include proteins like vascular cell adhesion molecule 1 (6), L-selectin (7), TNF-α (8, 9), TNF-α receptor 1 (TNFR1) (10), the interleukin 1 receptor (IL-1R) II (10), and the interleukin 6 receptor (IL-6R) (11).

The shedding process not only provides a possibility for down-regulation of cell surface proteins but also for initiating or inhibiting autocrine or paracrine signaling via soluble proteins like agonistic cytokines or (ant)agonistic soluble cytokine receptors. For example, shedding of proTNFα leads to the generation of the actual proinflammatory, soluble cytokine. Cleavage of the TNFRI decreases the TNF-α sensitivity of cells expressing the respective receptor, such as monocytes, macrophages, and hepatocytes (12–14). In addition, the soluble TNFR1 ectodomain acts as an antagonist of TNF-α signaling mediated by its membrane-bound form. Both effects result in immunosuppressive and antiapoptotic properties of TNFRI shedding (13, 14). In clear contrast, shedding of IL-6R leads to the generation of an agonist of IL-6-mediated signaling, the soluble IL-6R (sIL-6R). In contrast to the membrane-bound IL-6R, which is associated with regenerative processes, the sIL-6R bears mainly proinflammatory properties (15–17).

ADAM17 and ADAM10 are atypical members of the ADAM family because their extracellular region comprises the following domains: a prodomain, a catalytic domain, a disintegrin domain, and a membrane-proximal domain (MPD) as well as a...
small stalk region (18–20). In contrast, typical members of the ADAM family comprise a cysteine-rich and EGF-like domain instead of the MPD (20). According to its type I transmembrane protein topology, the extracellular part of ADAM17 ends in a single transmembrane region and a cytoplasmic tail (20).

The mechanisms of regulation of ADAM17 shedding activity are yet to be fully elucidated (21, 22). Of the non-catalytically active extracellular domains, the disintegrin domain is known to interact with integrins, resulting in steric hindrance and inactivation of ADAM17 (23–26). Stimulation leads to the dissociation of the inactive complex, resulting in a catalytically active protease (24, 25).

In addition, the disintegrin domain of ADAM17 has been suggested to act as a scaffold. In this structural model, the extracellular part adopts a C-shaped structure, placing the MPD in close proximity to the catalytic domain (18, 27). This model implies a key role for the MPD in the regulation of ADAM17 activity. Accordingly, the MPD is involved in substrate recognition, multimerization, and regulation of ADAM17 (10, 18, 19, 28–34). The MPD exists in two isoforms and acts as a molecular switch in ADAM17. One isoform is open and elongated, corresponds to the active enzyme, and allows the molecule to be flexible. The second isoform is rigid and compact and corresponds to the inactive form (19). The two isoforms differ in their disulfide bond pattern, and the protein–disulfide isomerase (PDI) controls ADAM17 activity by converting the flexible active MPD into the rigid inactive one (19). Thereby, two disulfide bonds undergo a specific isomerization. This process changes the affected disulfide pattern from a sequential arrangement (Cys600-Cys635 and Cys630-Cys641), which is present in the elongated structure, to an overlapping pattern (Cys600-Cys635 and Cys630-Cys641), which exists in the compact, structured MPD and is associated with the inactive enzyme (19).

A small stalk region between MPD and transmembrane region is highly conserved, even between humans and Drosophila melanogaster. Here we show that binding of the IL-6R, but not TNF-α, which are both key ADAM17 substrates, is mediated by this small α-helical juxtamembrane segment, which we termed “conserved ADAM seventeen dynamic interaction sequence” (CANDIS). Furthermore, we demonstrate that the substrate binding property of CANDIS is modulated by the MPD, which is switched by PDI.

**EXPERIMENTAL PROCEDURES**

*In Silico Methods*—The sequential alignment was performed with ClustalW2. For secondary structure prediction, the method of Rost and Sander (35) as well as “HELIQUEST” (36) were used.

*Modeling*—The model structure of the CANDIS helix was built using the WHATIF software (37).

*CD Spectroscopy*—Circular dichroism measurements were carried out with a Jasco-J-720-CD spectropolarimeter (Japan Spectroscopic Corp., Easton, MD) as described in Ref. 38. All peptides were solved in 50 mM sodium phosphate buffer (pH 7.4), and the measurements were performed at 20 °C in a 0.01-cm quartz cuvette.

**Coupling of Antibodies and Peptides to N-Hydroxysuccinimimid-activated Sepharose**—Murine anti-PC antibody (HPC4), murine nonsense antibody, or peptides were coupled to N-hydroxysuccinimimid-activated Sepharose 4 Fast Flow (GE Healthcare) according to the instructions of the manufacturer. For peptide-coated beads, the following peptides were used: CANDIS (peptide b), RVQDVIERFWDIDQLS_GSGSGK; pA10, VDADGPLARLKKAIFSP_GSGSGK; and control peptide (cp), DQWRVQFEDFIDLFRS_GSGSGK. For empty beads, the coupling procedure was performed only with PBS. All peptides were purchased from Biosyntan GmbH (Berlin, Germany) or Peptides & Elephants GmbH (Potsdam, Germany).

**Pulldown Experiments with Peptide-coated Beads**—Cells transiently expressing human IL-6R, human IL-6R variants, or MPD17plusCANDIS-GPI were harvested and lysed in 1 ml of lysis buffer (50 mM Tris (pH 7.5), 200 mM NaCl, 2 mM EDTA, 1% Triton X-100, 5% glycerol, and complete protease inhibitor mixture without EDTA (Roche Applied Science)). Peptide-coated beads, CANDIS, pA10, cp, and empty beads were pre-washed with lysis buffer plus 5% BSA. For the pulldown experiments, the lysate was divided into 200-μl aliquots, and 800 μl of lysis buffer was added to each aliquot. This mixture was incubated with 30 μl of bead suspension (CANDIS-, cp-, or pA10-coated beads or empty beads) for 30 min on ice. After incubation, five washing steps with lysis buffer were performed.

**Cell Culture and Transfection**—ADAM17ex/ex mouse embryonic fibroblasts (MEFs) (2) and HEK293 cells were cultured in high-glucose DMEM with 10% FCS, streptomycin (100 mg/ml), and penicillin (60 mg/liter) (all from PAA Laboratories, Cölbe, Germany) in a humidified incubator (37 °C, 5% CO2).

For pulldown and coimmunoprecipitation experiments, 4 × 106 HEK293 cells were seeded in 10-cm culture dishes. After 24 h, a premixed solution of 8 μg of DNA and 20 μl of polyethyleneimine (1 mg/ml) in 500 μl of DMEM was added for transient transfection. Cells were harvested 48 h after transfection.

For ADAM17 activity assays, 4 × 105 ADAM17ex/ex MEF cells were seeded in 6-well plates. After 24 h, transfection was performed with 4 μg of DNA using TurboFect (Thermo Fisher Scientific Biosciences/Fermentas, St. Leon-Rot, Germany) according to the instructions of the manufacturer. The activity assays were performed 24 h after transfection.

**Coimmunoprecipitation**—Before coimmunoprecipitation, beads were preincubated with 1 ml of lysis buffer supplemented with 5% BSA and 5 mM CaCl2. HEK293 cells transiently coexpressing HE-ADAM17 and TIMP3 or wild-type ADAM17 and TIMP3, respectively, were lysed in 1 ml of lysis buffer plus 5 mM CaCl2. The lysates were divided into two parts, and the same amounts of lystate were incubated with 30 μl of HPC4- or nonsense antibody-coated beads (see above) for 30 min on ice. After incubation, the beads were washed five times with lysis buffer plus 5 mM CaCl2.

**Expression Plasmids of Tagged ADAM17 Plasmids**—To N-terminally tag murine pro-TNF-α with a 3× FLAG epitope, a pcDNA4TO-FLAG (N) vector was created by designing an oligonucleotide encoding the 3× FLAG epitope (MMDYKHDGDKYKDHIDYKDDDK) and inserting it in the pcDNA4TO plasmid via the HindIII and NotI restriction sites. The cDNA
encoding the murine proTNF-α was amplified from murine spleen cDNA and subsequently cloned in the pcDNA4TO-FLAG (N) vector via the NotI and XhoI restriction sites. The human IL-1RII contains a myc tag between the signal peptide and the mature IL-1RII, as described previously (28).

Western Blotting—Western blot experiments were performed as described previously (29). For detection of the human IL-6R, its deletion variants, as well as chimeras V and X, we used the monoclonal murine antibody 4-11 (39) for visualization of the catalytic domain of ADAM17 (PDB code 1BKC), the mature IL-1RII, and Data Analysis

Conserved ADAM Seventeen Dynamic Interaction Sequence

Construction of Plasmids Coding for Human IL-6R Deletion Variant Human IL-6RΔGlu317–Gln322—The human IL-6R deletion variant IL-6RΔGlu317–Gln322 was cloned via splicing by overlapping extension PCRs and cloned with a 5′ KpnI site and a 3′ NotI site into pcDNA3.1, as described previously (40).

Construction of Plasmids Coding for IL-6R, IL-11R, and Chimeras Thereof—Expression plasmids for human IL-6R have already been described (39, 41). The coding sequence for the human IL-11R, N-terminally fused with a myc tag, was purchased from Invitrogen/GenArt and cloned into the pcDNA3.1 vector via a 5′ KpnI site and a 3′ NotI site. The creation of cytokine receptor chimeras was performed according to Garbers et al. (41). In brief, sequences of human IL-6R and human IL-11R were aligned, and borders between the individual domains were assigned. All chimeras were constructed using standard cloning procedures. To create chimera I, the stalk region of the human IL-11R (Thr317 to Ala370) was replaced by its counterpart from human IL-6R (Thr316 to Pro365). Chimera V was generated by replacing the human IL-6R stalk region (Thr316 to Pro365) by the IL-11R stalk (Thr317 to Ala370). In chimera IX, the 10 amino acid residues directly before the transmembrane domain of the IL-11R (Asp361 to Ala370) were replaced by the analogous region of the human IL-6R (Val356 to Pro365) and vice versa in chimera X. All chimeras were cloned into pcDNA3.1 using a 5′ KpnI site and a 3′ NotI site.

Human IL-6R Ectodomain Shedding Assays in HEK293 Cells and Data Analysis—Ectodomain shedding assays and their analysis for human IL-6R have been described previously in detail (40, 41). In brief, HEK293 cells were transiently transfected and stimulated for 2 h with 100 nM PMA or dimethyl sulfoxide as a control. Where indicated, cells were pretreated for 30 min with the metalloprotease inhibitor marimastat (10 μM). The supernatant was collected, cleared from debris by centrifugation, transferred into fresh tubes, and stored at −20 °C for ELISA analysis (see below). To compare shedding of the different human IL-6R constructs, the amount of soluble wild-type human IL-6R generated after PMA stimulation was set to 100%, and all other values were calculated in relation to this reference value.

ELISA—An ELISA specific for the human IL-6R was performed using the monoclonal antibody 4-11 (39) as a capture antibody and biotinylated Baf227 (R&D Systems, Wiesbaden, Germany) as a detection antibody, as described previously (39–41).

Construction of Plasmids Coding for ADAM17p10—CANDIS was exchanged in PC-tagged, full-length human ADAM17 with the corresponding peptide of human ADAM10 (Fig. 1C) by overlapping PCR using a primer with respective overhangs as in the construction of HE-ADAM17.

ADAM17 Activity Assay Using Alkaline Phosphatase-tagged Substrates and Data Analysis—ADAM17 activity was measured as described earlier (28). ADAM17ex/ex MEFs were transfected with either wild-type ADAM17, HE-ADAM17, ADAM17p10, or peGFP together with N-terminally alkaline phosphatase-tagged IL-1RII (AP-IL-1RII) (28) in a ratio of 1:5. One day after transfection, the transfection medium was replaced by high-glucose DMEM. Cells were either left untreated, stimulated with 100 nM PMA, or stimulated with PMA and treated with 50 μM of the metalloprotease inhibitor GM6001 (Merck Chemicals/Millipore, Schwalbach, Germany). Two hours later, the supernatants and cells were harvested. Cell pellets were lysed, and AP activity in the lysates and cell supernatants was measured as described earlier (28) using p-nitrophenylphosphate (Sigma-Aldrich, Taufkirchen, Germany) as a substrate. To determine shedding activity, the absorptions at 405 nm of supernatants were divided by those of the corresponding cell lysates. The ratios were normalized against the respective sample treated with PMA and marimastat. To exclude residual shedding activity of ADAM17ex/ex MEFs, the values of the mock-transfected samples were subtracted from those of wild-type, HE-ADAM17, and ADAM17p10 samples. Afterward, the shedding activity of the PMA-treated wild-type ADAM17 sample was set to 100%. Student’s t test was performed.

HEK293 cells were cotransfected with extracellular AP-tagged substrates (human IL-1RII or humane TNF-α) and an empty vector (mock) or PDI6 expression vector (Thermo Scientific) in a ratio of 1:10. The activity assay was performed as described for ADAM17ex/ex MEFs, but instead of 50 μM GM6001, 10 μM of the metalloprotease inhibitor marimastat was used, and, after normalization of the ratios, the shedding activity of the PMA-treated mock samples was set to 100%.

Visualization of the Catalytic Domain of ADAM17—To visualize the catalytic domain of ADAM17 (PDB code 1BKC), the PyMOL program (42) was used.

Exchange of Amino Acid Residues E406H and H415E in PC-tagged ADAM17—PC-tagged, full-length ADAM17 was used as template to generate the HE-ADAM17 mutein sequence by overlap extension PCR. The template was separated into two halves, with the cut at the site of the intended mutation. The 5′ half was generated using a forward primer annealing on the N-terminal end of native murine ADAM17 (GGGGTACCAT-GAGGCGGCGTCTCC) and a reverse primer bearing the first mutation (CTGCGCTCCAAATTTATGTCCTCproposal:TAATTTGGAGCAGAAGAAGACCCTGATGGGCTAGCAGA-

TTTGTAACCAGGTCAGCTTCC). The second half was generated using a forward primer annealing on the C-terminal
end of ADAM17 (CGGGATCCGCACTCTGTCTCTTTGCT-GTCAACTCG). After purification, both products were mixed in a 1:1 ratio and amplified by the flanking N-terminal and C-terminal primers. The resulting construct bears an exchange in amino acid residues E406H and H415E and was cloned in pcDNA3.1 (Invitrogen).

Flow Cytometry Analysis—For flow cytometry analysis, ADAM17ex/ex MEF cells (2) were transfected either with wild-type ADAM17 or with HE-ADAM17. Staining and analysis were performed as described earlier (29), but monoclonal antibodies directed against the extracellular part of murine ADAM17 were used.

PDI-affected Coimmunoprecipitation—HEK293 cells transiently expressing human IL-6R, MPD17plusCANDIS-GPI, or HE-ADAM17 were harvested and lysed with 1 ml of lysis buffer plus 5 mM CaCl2. HPC4-coated beads (60 μl) were washed with lysis buffer supplemented with 5% BSA and 5 mM CaCl2 and incubated with MPD17plusCANDIS-GPI or HE-ADAM17 lysates for 30 min on ice. After incubation, the beads were washed with PBS (pH 7.4), and the suspension was divided into two parts that were either treated with 10 μM reduced PDI in PBS or with PBS only for 15 min at room temperature. The reduced PDI was prepared as described earlier (19). After another washing step with lysis buffer plus 5 mM CaCl2, both aliquots of beads were incubated with the human IL-6R lysate for 30 min on ice. After incubation, the beads were washed five times with lysis buffer plus 5 mM CaCl2.

Analysis of the Band Intensity of Western Blot Analyses—For quantifying the PDI-treated coimmunoprecipitations, the intensities of the human IL-6R bands and the ADAM17 construct bands were measured with the ImageJ program (43). The human IL-6R values were divided by the ADAM17 construct measurement values for each sample to obtain ratios. Within one experimental setting with a PDI- and a PBS-incubated sample, the ratios of both samples were divided by the ratio of the PBS-incubated sample.

RESULTS

An Unusually Conserved Region in the Stalk Region of ADAM17—ADAM17 is a multidomain, membrane-bound protease. Its extracellular part contains a prodomain, a catalytic domain, a disintegrin domain, and an MPD. The MPD is connected to the transmembrane region by a short stalk of 29 amino acid residues (Fig. 1A). A comparison of the respective amino acid sequences of ADAM17 from different species revealed that the first 14 amino acid residues of this juxtamembrane region are highly conserved (Fig. 1B). Notably, such high conservation does not exist in case of ADAM10, the closest relative of ADAM17.

Because the 14 amino acid residues directly adjacent to the MPD of ADAM17 are highly conserved and do not belong to the MPD (19), we hypothesized that this segment might be important for the functionality of ADAM17. A secondary structure prediction revealed that these amino acid residues form an α-helix. This feature suggests that this juxtamembrane segment of ADAM17 might be important for protein-protein interactions. Therefore, we named this region CANDIS.
to the wild-type sequence (peptide a), whereas the second peptide contained only the region predicted to be α-helically fused C-terminally to a flexible linker (peptide b). A corresponding peptide of ADAM10 (pA10) and a randomized sequence of CANDIS (cp) were used as controls. Both CANDIS peptides showed CD spectra typical for an α-helical conformation (Fig. 1C). In contrast, spectra of both control peptides represented random coil structures. In the case of peptide a, the α-helical content and/or stability seemed even more pronounced compared with peptide b. These findings confirmed that CANDIS formed an α-helix.

**CANDIS Binds to the IL-6R and IL-1RII but not to TNF-α**—For the human IL-6R and IL-1RII, the MPD of ADAM17 has been described to be involved in ligand recognition (28). In this particular study, the MPD was linked by a glycosylphosphatidylinositol (GPI) anchor to the plasma membrane (Fig. 2A). Because this MPD17 construct contained CANDIS, we changed its name to MPD17plusCANDIS-GPI, and our next question was whether CANDIS alone is able to recognize the human IL-6R. To determine whether the stalk region of IL-6R is needed for CANDIS binding, we generated chimera I (IL-11R with the stalk of the IL-6R) and chimera V (vice versa, Fig. 3A). Using CANDIS and control peptides coupled to Sepharose beads, chimeras I and V were analyzed with respect to their CANDIS binding properties. The resulting Western blot analysis revealed that the stalk region of the IL-6R was essential for CANDIS binding because chimera I (consisting of IL-11R with

**FIGURE 2. CANDIS interacts with the ADAM17 substrate IL-6R.** A, schematic of a GPI-anchored membrane-proximal domain of human ADAM17 (MPD17plusCANDIS-GPI). The stalk region encompasses CANDIS as well as a PC and a myc tag (29). B, sequences of peptides that were coupled to Sepharose beads. The first peptide contains CANDIS, the second contains the corresponding sequence of ADAM10 (pA10), and the third peptide contains a randomized sequence of CANDIS (cp). C, Western blot (WB) analysis of the precipitation of the IL-6R by the peptides coupled to Sepharose beads. D, pulldown of human IL-1RII, murine TNF-α (FLAG-tagged), and human IL-6R by CANDIS or cp revealed that CANDIS interacts specifically with the IL-6R and IL-1RII but not with proTNF-α, as shown by Western blot analysis with the stated antibodies.

**Binding Site of CANDIS in the IL-6R**—Because CANDIS was identified as an IL-6R binding module of ADAM17, our next aim was to identify the CANDIS binding site in the humane IL-6R. Although IL-6R is described to be a substrate of ADAM17, IL-11R is not. Chimeras of the human IL-6R and human IL-11R were generated to identify the CANDIS binding site within the IL-6R. To determine whether the stalk region of IL-6R is needed for CANDIS binding, we generated chimera I (IL-11R with the stalk of the IL-6R) and chimera V (vice versa, Fig. 3A). Using CANDIS and control peptides coupled to Sepharose beads, chimeras I and V were analyzed with respect to their CANDIS binding properties. The resulting Western blot analysis revealed that the stalk region of the IL-6R was essential for CANDIS binding because chimera I (consisting of IL-11R with
the stalk of IL-6R was specifically pulled down by CANDIS, whereas chimera V (consisting of IL-6R with the stalk of IL-11R) was not (Fig. 3B).

Next, the requirement of the membrane-proximal region of IL-6R, containing the cleavage site of ADAM17, for CANDIS binding was analyzed. The cleavage site of ADAM17 in the human IL-6R is located C-terminally of Gln-357 (44). Chimeras IX and X were cloned (Fig. 3A). Chimera IX contained the sequence of IL-11R with the membrane-proximal region of IL-6R (exchange of Asp361 to Ala370 of IL-11R to Val356 to Pro365 of IL-6R). Accordingly, chimera X contained the IL-6R with the membrane-proximal region of the IL-11R (vice versa to chimera IX). Pulldown experiments with these chimeras revealed that the ADAM17 cleavage site and the membrane-proximal region of the IL-6R had no influence on CANDIS binding because chimera X (IL-6R with the membrane-proximal region of IL-11R) was pulled down by CANDIS, whereas chimera IX (IL-11R with the membrane-proximal region of IL-6R) was not (Fig. 3B).

To further narrow down the CANDIS interaction site of the IL-6R, we used IL-6R stalk region deletion variants (Fig. 3C), which have been described recently (40). The variant IL-6RΔΔA333–Val362 (Δ30) comprised a deletion of the ADAM17 cleavage site as well as of the C-terminal part of the stalk region. As shown in Fig. 3D, IL-6RΔΔA333–Val362 (Δ30) was specifically pulled down by CANDIS beads but not by control beads, indicating that the CANDIS binding site was not in the deleted part C-terminally adjacent to the ADAM17 cleavage site. In contrast, a deletion variant IL-6RΔGlu317–Thr352 (Δ36) lacking the N-terminal part of the stalk region did not bind to CANDIS (Fig. 3D). Taken together, these results suggested that CANDIS binds directly adjacent to domain 3 within amino acid residues Glu317 to Gln332 of the IL-6R stalk region (Fig. 3E).

To verify this finding, amino acid residues Glu317 to Gln332 were deleted in another IL-6R mutein, resulting in IL-6RΔGlu317–Gln332 (Δ16) (Fig. 4A). CANDIS pulldown experiments clearly demonstrated that this variant, in contrast to wild-type IL-6R, did not bind to CANDIS (Fig. 4B). Subsequently, we investigated whether binding of CANDIS to IL-6R had an influence on the eligibility of IL-6R as a substrate for ADAM17. For this purpose, the mutein IL-6RΔΔGlu317–Gln332 (Δ16) and wild-type IL-6R were transiently transfected into HEK293 cells that endogenously expressed ADAM17. Two days later, ADAM17 activity was stimulated by the addition of PMA, and the shed IL-6R in the supernatant was measured by ELISA. Intriguingly, shedding of the IL-6R deletion variant without the CANDIS binding site (IL-6RΔGlu317–Gln332 (Δ30)) was reduced significantly compared with the wild-type IL-6R (Fig. 4C). These data suggest that, even though shedding is not abolished
completely, binding of ADAM17 to IL-6R via CANDIS occurs in the region Glu317 to Gln332 of the receptor and is essential for efficient shedding of the IL-6R by ADAM17.

To confirm the impact of CANDIS in ADAM17 shedding activity, we exchanged CANDIS in the wild-type molecule with the corresponding peptide of ADAM10, thereby generating a chimera called ADAM17p10. Afterward, its shedding ability was analyzed. To this end, mouse embryonic fibroblasts from hypomorphic ADAM17 mice (ADAM17ex/ex MEFs) were used, which showed a reduction in ADAM17 expression of about 95% (2). Because all trials to transfect AP-tagged human IL-6R into these cells failed, human AP-tagged IL-1RII was used as substrate. Nevertheless, the data obtained clearly show that the shedding activity of the chimeric ADAM17p10, lacking CANDIS, is reduced significantly compared with wild-type ADAM17 (Fig. 4D).

**IL-6R Binding to the MPD-CANDIS Region Can Be Inhibited by PDI**—ADAM17 enzymatic activity is down-regulated by the action of cell surface-located PDI (19, 33). In this context, the MPD of ADAM17 acts as a molecular switch (19). Therefore, we investigated whether the inhibitory effect of PDI was due to blocked access of the juxtamembrane CANDIS to its substrate as a consequence of the structural change in the extracellular part of ADAM17. Because PDI activity is influenced by protease inhibitors, the first attempts to precipitate wild-type ADAM17 with human IL-6R failed because of protease activity and self-digestion. To prevent this, we constructed an ADAM17 variant (Fig. 5) that was catalytically inactive but retained an almost native architecture in its active site. Because ADAM17 is a member of the metzincin superfamily, it contains a Zn2+ ion in its active site that is coordinated by three histidine residues in the conserved HEXXHXGXHXXHD motif. The glutamic acid residue in this motive acts as a catalytic base (20). To obtain an inactive ADAM17 variant, we used an approach that was already applied to create an inactive matrix metalloprotease 9 mutein being still able to bind its inhibitor, the tissue inhibitor of metalloproteases 1 (TIMP-1) (45). To obtain a corresponding mutein of ADAM17, the active site of C-terminally PC-
tagged murine ADAM17 was altered by site-directed mutagenesis of E406H and H415E (Fig. 5A), which changed the HEXXHXXGXXH ED motif into a HHXXHXXGXXEED motif and resulted in a variant termed HE-ADAM17. The successful expression of HE-ADAM17 on the cell surface was verified by flow cytometry (Fig. 5B).

Next, the enzymatic activities of HE-ADAM17 and wild-type ADAM17 were compared. ADAM17<sup>ex/ex</sup> MEFs were cotransfected with N-terminally AP-tagged IL-1RII as an ADAM17 substrate and either wild-type ADAM17 or HE-ADAM17. One day after transfection, the shedding activity of the cells was tested by stimulation with PMA and detection of the released IL-1RII ectodomain (Fig. 5C). The HE-ADAM17 mutein did not show any shedding activity, neither unstimulated nor after PMA stimulation. In contrast, cells transfected with wild-type ADAM17 displayed a significant release of the IL-1RII ectodomain after PMA stimulation (Fig. 5C). To ensure that differences in the ADAM17 activity assay were not due to variations in the expression levels of the transfected cells, all lysates of the assay were examined by Western blot analyses in which PC-tagged ADAM17 wild-type and HE-mutein were detected by an anti-PC antibody (HPC4). DMSO, dimethyl sulfoxide. E, to test whether the overall structure of the active center was unaltered, HE-ADAM17 and wild-type ADAM17 were coexpressed with TIMP3 in HEK293 cells. After cell lysis, ADAM17 variants were precipitated via their PC tags, and coprecipitated TIMP3 was detected by Western blotting. IP, immunoprecipitation; noAB, non-relevant antibody. GM, GM6001.
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PMA-stimulated samples (IL-1RII and TNF-α) were below 0.05, they were considered statistically significant.

activity regarding IL-1RII and TNF-

either with empty pcDNA3.1 or the PDIA6 expression vector. The next day, transfected cells were either left untreated, stimulated by 100 nM PMA, or stimulated

MPD17plusCANDIS-GPI. HE-ADAM17 or MPD17plusCANDIS-GPI were expressed in HEK293 cells, bound to Sepharose beads, and either treated with PDI or left untreated (PBS). These samples were used to pull down the IL-6R from lysates of IL-6R-overexpressing HEK293 cells. The samples were analyzed by Western blotting, which clearly showed that PDI-treated HE-ADAM17 and MPD17plusCANDIS-GPI lost their ability to bind IL-6R. B, for quantification, the band densities of the IL-6R and ADAM17 constructs from A, as a representative example, were measured with ImageJ software (43) and expressed as their ratio (see “Experimental Procedures”). These ratios showed that binding of both HE-ADAM17 and MPD17plusCANDIS-GPI to the IL-6R decreased at least 5-fold after treatment with PDI and was considered to be statistically significant. Three independent experiments displayed \( p < 0.005 \), which was calculated by Students \( t \) test. C, overexpression of PDA6 suppresses shedding of IL-1RII as well as TNF-α. Extracellular AP-tagged substrates were cotransfected into HEK293 cells either with empty pcDNA3.1 or the PDA6 expression vector. The next day, transfected cells were either left untreated, stimulated by 100 nM PMA, or stimulated by 100 nM PMA in the presence of 10 \( \mu \)M metalloprotease inhibitor marimastat (Ma). Two hours later, the supernatant and cells were harvested, and shedding activity regarding IL-1RIi and TNF-α and significance of experience were calculated as described under “Experimental Procedures.” Because \( p \) values of both PMA-stimulated samples (IL-1RIi and TNF-α) were below 0.05, they were considered statistically significant.

mininally PC-tagged ADAM17 or HE-ADAM17 were cotransfected with TIMP3 and, after cell lysis, immunoprecipitated via their PC tags. The following Western blot analyses showed that both wild-type ADAM17 and HE-ADAM17 interacted with TIMP3 in a comparable manner (Fig. 5E). Because the HE-ADAM17 was inactive but still able to bind an interaction partner with its active site, this mutein was used to study the interaction of ADAM17 with the IL-6R in terms of the PDI-catalyzed inactivation of ADAM17.

To test whether PDI abrogates the recognition of the IL-6R by ADAM17, coprecipitation experiments were performed. HEK293 cells were transiently transfected with IL-6R or HE-ADAM17. HE-ADAM17-expressing cells were lysed, and HE-ADAM17 was bound to Sepharose beads via its C-terminal PC tag. These beads were either incubated with reduced PDI in PBS or in PBS without PDI as a control. Afterward, the beads were incubated with cell lysates of HEK293 cells expressing IL-6R. The amount of IL-6R bound to the bead-coupled HE-ADAM17 was analyzed by Western blotting. In contrast to the non-PDI-treated samples, PDI pretreatment prevented binding of IL-6R to HE-ADAM17 (Fig. 6, A and B). These results clearly indicate that the structural change in the extracellular part of ADAM17, mediated by PDI, abrogates IL-6R recognition.

Because the PDI-catalyzed structural change occurs in the MPD, further experiments were conducted using MPD17plusCANDIS-GPI instead of HE-ADAM17 (see Fig. 2A). This construct contained the MPD and the juxtamembrane CANDIS but no additional domains of ADAM17. As shown in Figs. 6A and 4B, binding of MPD17plusCANDIS-GPI to IL-6R was also prevented by pretreatment of MPD17plusCANDIS-GPI with PDI. These results suggest that PDI treatment, which converts the open flexible MPD to its closed, rigid structure (19), is sufficient to abrogate IL-6R recognition by CANDIS. This effect is most likely due to an abrogated access of CANDIS to IL-6R because of steric hindrances by the closed MPD. Taken together, the MPD of ADAM17 acts as a switch within the molecule. This switch abrogates IL-6R recognition by a specific juxtamembrane α-helical segment, CANDIS, and is controlled by PDI.

Because previous experiments demonstrated that exogenous PDI or overexpression of PDIA6 reduces shedding of proheparin-binding epidermal growth factor (33) and human IL-6R (19), an intriguing question was whether this is also the case with IL-1RII or TNF-α. Hence, we performed ADAM17 shedding assays using extracellular alkaline phosphatase-tagged IL-1RII and TNF-α. Interestingly, shedding of both substrates by ADAM17 was reduced significantly by overexpression of PDIA6 (Fig. 6C). These results suggest that, although TNF-α is not accessed by CANDIS, the PDI-mediated structural change in the MPD of ADAM17 is sufficient to abrogate shedding by the inactivation of ADAM17.

DISCUSSION

ADAM17 is a major sheddase of various substrates that are involved in regeneration processes and immune responses. An uncontrolled release of growth factors, cytokines, and cytokine receptors catalyzed by the enzyme has to be prevented to minimize the risk of cancer progression and autoimmune diseases. In contrast, the absence or reduction of growth factor release from the cell surface by ADAM17 diminishes regenerative processes (2). Hence, ADAM17 activity has to be controlled tightly and efficiently. Accordingly, activated ADAM17 is switched off rapidly by a PDI-mediated structural change of the MPD of ADAM17 (19, 33). Intriguingly, this off switch changes the flexible, elongated MPD structure into a compact, rigid one (19). So far, it has been completely unknown how this structural change

![FIGURE 6. PDI conversion of ADAM17 abrogates access of CANDIS to IL-6R. A, Western blot analysis of IL-6R pulled down by HE-ADAM17 or MPD17plusCANDIS-GPI. HE-ADAM17 or MPD17plusCANDIS-GPI were expressed in HEK293 cells, bound to Sepharose beads, and either treated with PDI or left untreated (PBS). These samples were used to pull down the IL-6R from lysates of IL-6R-overexpressing HEK293 cells. The samples were analyzed by Western blotting, which clearly showed that PDI-treated HE-ADAM17 and MPD17plusCANDIS-GPI lost their ability to bind IL-6R. B, for quantification, the band densities of the IL-6R and ADAM17 constructs from A, as a representative example, were measured with ImageJ software (43) and expressed as their ratio (see “Experimental Procedures”). These ratios showed that binding of both HE-ADAM17 and MPD17plusCANDIS-GPI to the IL-6R decreased at least 5-fold after treatment with PDI and was considered to be statistically significant. Three independent experiments displayed \( p < 0.005 \), which was calculated by Students \( t \) test. C, overexpression of PDA6 suppresses shedding of IL-1RII as well as TNF-α. Extracellular AP-tagged substrates were cotransfected into HEK293 cells either with empty pcDNA3.1 or the PDA6 expression vector. The next day, transfected cells were either left untreated, stimulated by 100 nM PMA, or stimulated by 100 nM PMA in the presence of 10 \( \mu \)M metalloprotease inhibitor marimastat (Ma). Two hours later, the supernatant and cells were harvested, and shedding activity regarding IL-1RII and TNF-α and significance of experience were calculated as described under “Experimental Procedures.” Because \( p \) values of both PMA-stimulated samples (IL-1RIi and TNF-α) were below 0.05, they were considered statistically significant.

![A, B, C]...
Conserved ADAM Seventeen Dynamic Interaction Sequence

modulated ADAM17 activity. In this study, we demonstrate for the first time how shedding of the IL-6R, one of the most important substrates of ADAM17, is controlled by the enzyme and which amino acids are crucial for the particular enzyme-substrate interaction in both ADAM17 and the IL-6R.

Directly adjacent to the MPD of ADAM17, we discovered a unique highly conserved region of 14 amino acid residues with an α-helical character. Although this region displays secondary structure elements, it seems to not belong to a distinct domain. The fact that the MPD folded properly in a single monomeric protein (19) underscores the finding that CANDIS is not a part of the MPD. Interestingly, a CANDIS-like region is not present in ADAM10, the closest structural relative of ADAM17. Even though the two proteases share some substrates, there are significant differences. For example, ADAM17 always needs an activation step (21, 22), whereas ADAM10 can also be constitutively active. Accordingly, the activation and regulation of both proteases are different (21, 22). In addition, the cleavage sites of ADAM10 and ADAM17 in the human IL-6R, a substrate of both proteases, are not identical (40). Furthermore, the mode of substrate recognition appears to be quite different because the CANDIS of ADAM17 binds to the human IL-6R but the corresponding region of ADAM10 does not.

Previous reports demonstrated species-specific differences with respect to IL-6R shedding. Although human IL-6R is shed efficiently by human as well as by murine ADAM17, murine IL-6R is processed to a lesser extent (41, 46). These findings support our results because the human and murine CANDIS are identical. In contrast, the CANDIS-binding region of the human IL-6R shows only 38% identity to the corresponding region in the murine IL-6R. Because the overall identity between human and murine IL-6R is also relatively low (54%), it is not surprising that they are recognized and processed in different ways.

To identify the exact CANDIS binding site in the human IL-6R, deletion variants of the receptor human IL-6R and IL-6R/IL-11R chimeras were used. Pulldown experiments with these proteins allowed us to narrow down the CANDIS-binding site in the stalk region of the IL-6R to the sequence E317RSRPPAENEVSTPMQ332. These amino acid residues are located directly adjacent to the C-terminal domain (domain 3) in the extracellular part of the IL-6R. In contrast to CANDIS, this region in the IL-6R has definitely no α-helical character. The importance of this region for binding and effective shedding by ADAM17 was proven by an IL-6R deletion variant lacking exactly this sequence (IL-6RΔGlu317-Gln332 (Δ16)). In these experiments, PMA was used to stimulate shedding because PMA does not activate IL-6R shedding by ADAM10, only by ADAM17 (21, 41). The IL-6R variant lacking the CANDIS binding site was shed significantly less than wild-type IL-6R. A comparable result was obtained performing the reciprocal experiment, in which we used an ADAM17 chimera containing the sequence of ADAM10 corresponding to CANDIS (ADAM17p10) and tested it in an IL-1RII shedding assay (Fig. 5D). The residual shedding in the absence of CANDIS suggested that the affinity of ADAM17 to substrates like human IL-6R and IL-1RII was increased strongly, but not absolutely controlled, by CANDIS. This is reasonable because no major structural changes in the IL-6RΔGlu317-Gln332 (Δ16) compared with the wild-type IL-6R are to be expected. The reduced, but not abrogated, shedding of IL-6RΔGlu317-Gln332 (Δ16) clearly shows that the substrate-binding site of CANDIS is not identical with the ADAM17 cleavage site.

After identification of CANDIS as a key binding site of ADAM17 for the IL-6R, its link to the MPD, the molecular off switch of ADAM17, was studied. ADAM17 activity is blocked by a conformational change from an elongated, open MPD to a compact, closed conformation mediated by PDI through disulfide isomerization (19). Our results suggest that this structural change indeed blocks access of the juxtamembrane CANDIS to ADAM17 substrates and, thus, blocks ADAM17 activity. Juxtamembrane segments appear to be central regulatory units within membrane proteins. Thereby, these α-helical regions are switched by structural changes and are needed for appropriate regulation of the full-length molecule. A juxtamembrane segment in the epidermal growth factor receptor has been described recently to be a crucial part in the activation of this receptor (47, 48). Although this intracellular EGF receptor element is neither functionally nor structurally related to the extracellular CANDIS, these two examples put the focus on such juxtamembrane regions as a novel and very important class of structural regulatory elements.

Taken together, we show that CANDIS is responsible for IL-6R binding of ADAM17 and that the CANDIS-binding site of the IL-6R is different from its ADAM17 cleavage site. In turn, IL-6R binding by CANDIS is abrogated by the PDI-mediated conformation switch of the MPD of ADAM17. In accordance, shedding of human IL-6R is blocked by overexpression of PDIA6 (19). Because CANDIS also binds to the IL-1RII, it is not surprising that overexpression of PDIA6 also reduces its shedding, most likely because of an enhanced inactivation rate of ADAM17. Although this effect does not seem to be as strong as in the case of IL-6R, one should keep in mind that HEK293 cells express PDIA1 as well as PDIA6. Both enzymes are well known to be located on the cell surface (33, 49–55). Hence, the effect observed in PDIA6 overexpression experiments will be an additive effect on endogenously active PDIs. In contrast, the finding that PDIA6 reduces TNF-α shedding to the same extent as that of IL-1RII was surprising. Hence, inactivation of ADAM17 by PDIs seems to be independent of the topology of the substrates and mediated because of the structural change of the MPD. This abrogates substrate accessibility by CANDIS but, potentially, also restricts access to the catalytic site. To clarify this point in detail, a next step will be to solve the structure of either full-length ADAM17 or its ectodomain in the presence or absence of PDI and, ultimately, with or without different ADAM17 substrates to reveal the true shape of the ectodomain and its access to its substrates.

CANDIS seems to be essential for binding of transmembrane type I proteins like human IL-6R and IL-1RII, but other substrates like the transmembrane-type II protein proTNF-α might be accessed by ADAM17 in different ways. This is in accordance with the finding that full-length ADAM17 is able to precipitate TNF-α (56), whereas MPD17plusCANDIS (28) and CANDIS alone are not (Fig. 2D). These data suggest that at least two different modes of interactions exist that depend on the
topology of the substrates. Because ADAM17 has more than 75 known substrates, to study the interaction modes of various classes of substrates and to categorize them will be a challenge but can provide important insights into the action and control of ADAM17 and signaling proteases in general.

Because ADAM17 generates two of the most important initiators of immune responses, namely TNF-α and the sIL-6R, ADAM17 is a key factor in the pathophysiology of autoimmune and chronic diseases (5, 17, 57). Hence, ADAM17 has a high potential as therapeutic target. Until now, there are no successful treatments with specific ADAM17 inhibitors (58). One main reason is that small molecule inhibitors lack specificity and also inhibit related proteases of the matrix metalloprotease and ADAM families (58). To overcome this difficulty, Tape et al. (34) developed an ADAM17 antibody that binds to extracellular parts of the enzyme, thereby inhibiting shedding activity. This antibody is a potential therapeutic option because it displays promising results in respect of tumor growth in an in vivo ovarian cancer model (58), which points out that antibodies might be promising therapeutic options. In line with this, the knowledge about specific interaction sites and actions of MPD and CANDIS for different types of substrates could open the field for specific therapeutic interventions by using specific blocking antibodies.

An inhibition of regeneration is most likely problematic in autoimmune diseases and chronic inflammation. Importantly, proTNF-α and IL-6R show regenerative properties in their membrane-bound forms and are converted by shedding into proinflammatory mediators (5, 17, 57). In line with these findings, treatment of chronic inflammatory diseases like rheumatoid arthritis and inflammatory bowel diseases by long term blockade of TNF-α signaling can be associated with dangerous side effects, such as an increased risk of infection, the development of autoimmune diseases, and malignancy (57). This may be due to the fact that a complete blockade of TNF-α signaling also affects the regenerative, immunoprotective signaling transmitted by TNFRII and proTNF-α and not only the proinflammatory signals mediated by TNFRI and soluble TNF-α (57). Similarly, a complete blockade of IL-6 signaling is unfavorable because membrane-bound IL-6R is known to be involved in the regenerative response and defense of bacterial infections, whereas the sIL-6R mediates the pathological inflammatory responses (15–17). Hence, it might be advantageous to selectively block the generation of sIL-6R because a selective blockade of sIL-6R showed therapeutic potential in many models of acute and chronic inflammation (16, 17). A selective blockade of sIL-6R generation, without affecting the TNF-α and membrane-bound IL-6R signaling cascades, could probably be achieved by CANDIS-binding antibodies or single Fv fragments, suggesting that these molecules might be promising and exciting tools to selectively block proinflammatory processes and still permit regenerative activities of this cytokine.

Acknowledgments—We thank Jessica Schneider and Jonas Hiesener of sIL-6R generation, without affecting the TNF-tively block the generation of sIL-6R because a selective block-

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