Regulated Intramembrane Proteolysis of the Interleukin-1 Receptor II by α-, β-, and γ-Secretase*

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Ectodomain shedding and intramembrane proteolysis of the amyloid precursor protein (APP) by α-, β- and γ-secretase are involved in the pathogenesis of Alzheimer disease (AD). Increased proteolytic processing and secretion of another membrane protein, the interleukin-1 receptor II (IL-1R2), have also been linked to the pathogenesis of AD. IL-1R2 is a decoy receptor that may limit detrimental effects of IL-1 in the brain. At present, the proteolytic processing of IL-1R2 remains little understood. Here we show that IL-1R2 can be proteolytically processed in a manner similar to APP. IL-1R2 expressed in human embryonic kidney 293 cells first undergoes ectodomain shedding in an α-secretase-like manner, resulting in secretion of the IL-1R2 ectodomain and the generation of an IL-1R2 C-terminal fragment. This fragment undergoes further intramembrane proteolysis by γ-secretase, leading to the generation of the soluble intracellular domain of IL-1R2. Intramembrane cleavage of IL-1R2 was abolished by a highly specific inhibitor of γ-secretase and was absent in mouse embryonic fibroblasts deficient in γ-secretase activity. Surprisingly, the β-secretase BACE1 and its homolog BACE2 increased IL-1R2 secretion resulting in C-terminal fragments nearly identical to the ones generated by the α-secretase-like cleavage. This suggests that both proteases may act as alternative α-secretase-like proteases. Importantly, BACE1 and BACE2 did not cleave several other membrane proteins, demonstrating that both proteases do not contribute to general membrane protein turnover but only cleave specific proteins. This study reveals a similar proteolytic processing of IL-1R2 and APP and may provide an explanation for the increased IL-1R2 secretion observed in AD.

Regulated intramembrane proteolysis (RIP)† is a two-step proteolytic cleavage mechanism required for signal transduction and the degradation of membrane proteins (reviewed in Refs. 1, 2). The first cleavage is referred to as ectodomain shedding and occurs within the ectodomain at a peptide bond very close to the transmembrane domain. This results in the secretion of most of the ectodomain (reviewed in Refs. 3, 4) and the generation of a membrane-bound stub, which can undergo a second cleavage within its transmembrane domain, called intramembrane proteolysis (reviewed in Ref. 5). Numerous type I membrane proteins, including Notch, CD44, and the amyloid precursor protein (APP), undergo RIP. The ectodomain shedding is carried out by members of the ADAM (a disintegrin and metalloprotease) family and additionally by matrix metalloproteases and to a lower extent by the aspartyl proteases BACE1 and BACE2 (β-site APP-cleaving enzymes 1 and 2) (for a review see Ref. 6). The subsequent intramembrane proteolysis is catalyzed by the γ-secretase protease complex, consisting of the essential proteins PS1 and PS2 (presenilin 1 or 2), nicastrin, Pen-2, and Aph-1 (for a review see Ref. 7). The presenilins are assumed to constitute the active site of γ-secretase by providing two aspartic acid residues that are critical for γ-secretase activity (8). Nicastrin functions as a receptor for γ-secretase substrates (9). As a result of γ-secretase cleavage, the C-terminal fragments (CTFs) of type I membrane proteins are processed to two smaller fragments. A small peptide is secreted into the extracellular space, whereas the intracellular domain is released into the cytosol. For some of these proteins, such as the cell surface receptor Notch and the cell adhesion proteins N- and E-cadherin, the liberated intracellular domain may participate in signal transduction through different mechanisms (2), whereas for other proteins, such as APP, the intracellular domain may be degraded without a prior role in signaling (10).

One of the proteins, for which RIP has been studied in much detail, is APP. In contrast to several other proteins undergoing RIP, the ectodomain cleavage of APP is not only catalyzed by one but by three different proteases, which cleave at distinct peptide bonds. Shedding of APP mainly occurs by an ADAM metalloprotease, which is also referred to as α-secretase and cleaves within the Aβ sequence (reviewed in Ref. 11). Addition-

* The abbreviations used are: RIP, regulated intramembrane proteolysis; APP, amyloid precursor protein; AD, Alzheimer disease; IL-1R2, interleukin-1 receptor II; ADAM, a disintegrin and metalloprotease; CTF, C-terminal fragment; sCTF, short CTF; ICF, long CTF; BACE, β-site APP-cleaving enzyme; HA, hemagglutinin; PBS, phosphate-buffered saline; NTF, N-terminal fragment; TGF, transforming growth factor; TNF, tumor necrosis factor; MS, mass spectrometry; MALDI-TOF, matrix-assisted laser desorption ionization time-of-flight; PMA, phorbol 12-myristate 13-acetate; CHAPSO, 3-(3-cholamidopropyl)dimethylammonio)-1-propanesulfonic acid; SFV, Semliki Forest virus; GPI, glycosylphosphatidylinositol; AP, alkaline phosphatase; ICD, intracellular domain; IL-1, interleukin-1; PS1, presenilin 1.
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Experimental Procedures

Reagents—The following antibodies were used: anti-HA epitope antibody HA.11 (Covance); anti-FLAGM2 antibody, anti-FLAGM2-agarose, anti-HA-9658 antibody, anti-BACE1-NT antibody, and HA-agarose (Sigma); horseradish peroxidase-conjugated goat anti-mouse and anti-rabbit secondary antibody (DAKO); Alexa 488-coupled anti-rabbit and Alexa 594 anti-mouse antibodies (Molecular Probes); anti-BACE1 and BACE2 (ProSci, against their C termini). Antibodies 6687 (against APP C terminus) (27) and 3027 (against PS1 loop) (28) were described previously. Antibody 22C11 (anti APP ectodomain) was provided by Konrad Beyreuther. Nicastrin antibody N1660 (Sigma), monoclonal presenilin NTF (29), Pen-2 1638 (30), and Aph-1 433G (31) antibodies were described before. PMA was obtained from Sigma. The metalloprotease inhibitor TAPI-1 was purchased from Peptides International. Dodecyl maltoside was purchased from Calbiochem. CHAPSO was purchased from Biomol, and the BACE inhibitor C3 was from Calbiochem (β-secretase inhibitor IV). BACE2-Fc fusion protein was kindly provided by Regina Fluhrer.

Plasmid Construction—Generation of vector peak12 expressing BACE1, BACE2, and HA-tagged alkaline phosphatase (AP) (HA-AP, soluble AP) has been described (32). The cDNAs of all shedding substrates are of human origin. Plasmid peak12/PSGL-1 3tag was cloned into peak12/HA-Xba-FLAG and encodes the signal peptide of CD5, followed by an HA epitope and the coding sequence of PSGL-1 with an AU1 tag in the extracellular domain and a FLAG tag in the cytoplasmic domain. The PSGL-1 sequence can be cut out using an XbaI and a NotI restriction site. Peak12/HA-Xba-FLAG was generated by PCR and encodes an HA tag between the HindIII and the XbaI site and a FLAG tag between the XbaI and the NotI site. To obtain peak12/CD5-HA-Xba-FLAG, the HindIII/XbaI fragment of peak12/PSGL-1 3tag was cloned into peak12/HA-Xba-FLAG and encodes the CD5 signal peptide followed by an HA and a FLAG tag. The plasmids encoding full-length IL-1R2 or its deletion mutant (lacking part of the ectodomain) (peak12/IL-1R2, peak12/Δ334-IL-1R2 and peak12/Δ322-IL-1R2) carry the CD5 signal peptide (PMGSLQPLATLYLLGMLVASVLQ), an N-terminal HA tag (YPYDVPDYA followed by the linker sequence SGGGGSGLE or SGGGGSDL for the Δ334 mutant), an N-terminal FLAG tag, and a C-terminal FLAG tag and were prepared by PCR using appropriate primers. Peak12/Δ329-IL-1R2 was generated in the same way but has no N-terminal HA tag. The PCR fragments (lacking the native signal peptide sequence of IL-1R2) were cloned into the XbaI site of peak12/CD5-HA-Xba-FLAG. Thus, the first amino acid of the IL-1R2 sequence is Alanine 23 of full-length IL-1R2 (numbering corresponding to protein accession number NP_004624 in the NCBI data base). Amino acid numbers of the IL-1R2 deletion mutants (Δ322, Δ329, and Δ334) indicate that the deletions stop before the given amino acid number, which refers to its position within the HA-IL-1R2-FLAG full-length sequence (counting without the CD5 signal peptide). Vector peak12/MMP-IL-1R2 was used for retroviral generation and was obtained by cloning the Hin-
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dIII/NotI fragment of peak12/IL-1R2 into the HindIII/NotI sites of the peak12/MMP-KilA vector (33). An additional IRES-GFP cassette from peak12/MMP-TK-IRES-GFP (obtained from Felix Randow; IRES is of encephalomyocarditis virus origin) was cloned into the NotI site of peak12/MMP-IL-1R2 to yield peak12/MMP-IL-1R2-IRES-GFP. Vectors pMDtet.G and pMD.gagpol were described previously (34). The coding sequences of CD14 (GPI-anchored protein), CD16 (GPI-anchored protein), and P-selectin (all three lacking the native signal peptide sequence) were amplified by PCR, digested with Xbal and NotI, and cloned into plasmid peak12/PSGL-1 3tag, which was cut with Xbal and NotI. The resulting plasmids encode the CD5 signal peptide followed by an HA epitope tag and the corresponding protein sequence. As templates, pCDM12/CD14 and CD16 (obtained from Brian Seed) and pCMV/P-selectin (obtained from Denisa Wagner) were used. Peak12/pro-TGFα-HA encodes human pro-TGFα with an HA tag inserted between amino acids His-43 and Phe-44, which is four amino acids C-terminal to the Ala-Val propeptide cleavage site. Thus, after signal peptide and propeptide processing, the mature pro-TGFα and the soluble TGFα retain the HA tag. Peak12/HA-pro-TGFα-FLAG contains an additional FLAG tag at the C terminus of pro-TGFα and was generated by cloning the HindIII/Xbal-digested PCR fragment of HA-tagged pro-TGFα into the HindIII and Xbal sites of peak12/CD5-HA-XBA-FLAG. TGFα and TNFα cDNAs were from ATCC. Peak12/FLAG-TNFα-HA was created by cloning a PCR fragment of FLAG-TNFα containing a 5’-HindIII site and a 3’-Xbal site that was then inserted into HindIII/Xbal peak vector, which has an HA tag between the Xbal and the NotI site. The N-terminal, cytoplasmic FLAG tag was added to TNFα by PCR and suitable primers and was cloned into the HindIII/NotI sites of the peak12 vector, resulting in peak12/FLAG-TNFα. For expression in neurons and glial cells IL-1R2, TGFα and CD16 were cut out from the corresponding peak12 plasmids using HindIII and NotI, blunt-ended, and ligated into the Smal site of the Semliki Forest virus (SFV) type 1. The identity of all constructs obtained by PCR was confirmed by DNA sequencing.

Cell Culture, Transfections, Western Blot—Human embryonic kidney 293-EBNA cells (HEK293) were cultured in Dulbecco’s modified Eagle’s medium (Invitrogen) containing 10% fetal bovine serum (Hyclone) as described (35). Cells stably expressing wild-type PS1 or its catalytically inactive mutant PS1 D385N are HEK293 cells without the EBNA gene (36). These cells were cultured as above with an additional 200 g/ml Zeocin (Invitrogen). 418 was added to culture murine embryonic fibroblasts PS1/2−/− knock-out cells. Transfections were carried out using Lipofectamine 2000 (Invitrogen). One day after transfection, the medium was replaced with fresh medium. After overnight incubation, conditioned medium and cell lysate (in 50 mM Tris, pH 7.5, 150 mM NaCl, 1% Nonidet P-40) were collected.

To analyze the effect of PMA and TAPI-1 on shedding, cells were treated as described previously (32). For the detection of secreted and cellular APP by immunoblot, the protein concentration in the cell lysate was measured, and corresponding aliquots of lysate or conditioned medium were directly loaded onto an electrophoresis gel. For transient transfections of IL-1R2, TGFα, CD14, and CD16 either together with BACE1 or BACE2, alkaline phosphatase (AP; plasmid HA-AP) was cotransfected as a transfection efficiency control. AP activity was measured as described previously (32, 37). Aliquots of the conditioned medium were treated for 30 min at 65 °C to heat-inactivate the endogenous alkaline phosphatase activity. Corresponding aliquots of lysate or conditioned medium were loaded onto the gel. Immunoblot detection was carried out using the indicated antibodies.

Infection of Primary Neurons and Glial Cells with SFV—Cortical neurons and glial cells were prepared from E14 mouse embryos from BACE1-deficient and BACE1, BACE2 double-deficient mice as described (38, 39). The BACE knock-outs were verified by Northern and Western blot detection and by functional analysis demonstrating that APP cleavage by BACE was virtually eliminated in the neurons (39).

Preparation of recombinant SFV stocks has been described previously (40). Virus was diluted 1:100 in conditioned culture medium and added to 4-day-old neurons. Two hours post-infection, cells were labeled with 100 μCi/ml [35S]methionine/cysteine for 6 h and lysed in immunoprecipitation buffer (1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS in TBS buffer). IL-1R2, TGFα, and CD16 full-length and CTFs were immunoprecipitated using anti-FLAG antibody. Immunoprecipitated material was separated by SDS-PAGE, and dried gels were exposed to a PhosphorImager (Amersham Biosciences).

Retroviral Transduction—To produce retroviral supernatants (replication-deficient Moloney murine leukemia virus), plasmids pMDtet.G and pMD.gagpol and either peak12/MMP-GFP or peak12/MMP-HA-IL-1R2-FLAG-IRES-GFP were transfected into HEK293 cells by calcium phosphate precipitation. Medium was changed after 24 h, collected after 48 h, and filtered through a sterile filter. The retroviral transductions were carried out using Polybrene (Sigma).

In Vitro Generation of IL-1R2-ICD—293E cells transiently expressing full-length IL-1R2 were incubated for 4 h with PMA prior to membrane preparation. Membrane preparations were generated as described (41) and were resuspended in citrate buffer (pH 6.4, 5 mM EDTA, inhibitor mixture from Roche Applied Science) with or without 1 μM DAPT and then incubated either at 4 or 37 °C for 2 h. After incubation the supernatant and membranes were separated via ultracentrifugation. HEK293 cells (without EBNA) stably expressing wild-type PS1 or PS1 D385N and additionally transiently expressing peak12/D329-IL-1R2-FLAG were directly subjected to membrane preparation without prior PMA incubation and then treated as above.

Coimmunoprecipitation of γ-Secretase Complex with IL-1R2 Substrate—HA-IL-1R2-FLAG was retrovirally transduced into HEK293 cells stably expressing either PS1 WT or the catalytic inactive mutant PS1 D385N. For immunoprecipitation, one 10-cm dish of each cell line was lysed in standard STE (150 mM NaCl, 50 mM Tris, 2 mM EDTA) + 1% CHAPSO buffer followed by a clarifying spin at 13,000 rpm with a Heraeus cryocentrifuge and a further purification step at 55,000 rpm in a Beckman ultracentrifuge with a TLA-55 rotor. Prior to immunoprecipitation of CTFs with FLAG M2 affinity agarose, the lysates were immunoprecipitated with HA affinity agarose for 2 h leading to
a depletion of IL-1R2 full-length protein in the lysate. Subsequently, after a 2-h incubation with the lysate, FLAG M2-agarose was washed two times each with 0.1% CHAPSO wash buffer and STE buffer and afterward eluted with 100 μg of FLAG peptide and subjected to SDS-PAGE. Immunoblot detection was carried out for IL-1R2 CTF and the γ-secretase complex components nicastrin, presenilin 1 NTF, Aph-1, and Pen-2.

**Mass Spectrometry of IL-1R2 Cleavage Sites**—For analysis of α-, β-, and γ-cleavage of secreted IL-1R2 peptides, HEK293 cells were transfected with peak12/IL-1R2, peak12/Δ334-IL1R2, or peak12/Δ322-IL1R2. 48 h after transfection, fresh medium was incubated for 4 h and subsequently put on ice. In case of peak12-Δ322-IL1R2, medium was supplemented with 1 mM DAPT during incubation to prevent turnover by γ-secretase. Protease inhibitor mixture (Sigma) was added at a dilution of 1:100. Medium was then subjected to a clarifying spin by centrifugation. Afterward medium was subjected to immunoprecipitation with HA-agarose beads for 4 h in the case of Δ334-IL1R2 construct and for 2 h in the case of Δ322-IL1R2 construct. Bound peptides were eluted either with a mixture of 0.3% trifluoroacetic acid, 50% acetonitrile, H2O saturated with α-cyanomatrix, or in the case of the IL-1R2 ectodomain with HA peptide (50 μg) in 300 mM NaCl for MALDI-TOF analysis (Voyager DESTR, Applied Biosystems) and with 0.1% formic acid, 50% methanol, H2O for nanoelectrospray ionization mass spectrometry analysis (Q-STAR Applied Biosystems). MALDI-TOF spectra were recorded in the linear mode and analyzed with Data Explorer™ (Applied Biosystems).

For determination of protein identity by mass fingerprinting, the precipitated IL-1R2 was digested with trypsin at 37 °C overnight and analyzed by MALDI-TOF. The obtained peptide fragments were compared with the NCBInr data base using Mascot™ (Matrix Science). The MALDI-TOF mass spectrometer was either internally calibrated using the masses from tryptic autodigest products or externally using a standard peptide mixture (Sequazyme calibration mixture III, Applied Biosystems). The sequence of the tryptic peptide corresponding to the N terminus of IL-1R2 was determined by direct nanospray infusion of the peptides derived from the tryptic digest and an MS/MS analysis of the doubly charged peptide with an m/z value of 1036.47 by tandem mass spectrometry using a hybrid quadrupole-time of flight mass spectrometer (Q-STAR, Applied Biosystems). MS/MS spectra were analyzed using BioAnalyst™.

**Deglycosylation of IL-1R2 Ectodomain**—The immunoprecipitated IL-1R2 ectodomain was eluted with HA peptide and subjected to deglycosylation with N-glycosidase F from the native deglycosylation kit (Calbiochem) at 37 °C overnight.

**BACE2 In Vitro Assay with Synthetic Juxtamembrane Region Peptide of IL-1R2**—50 pmol of synthetic peptide spanning from Val-322 to Ser-341 were digested in 50 mM Na+ acetate buffer, pH 4.4, at different temperatures (e.g. 4 or 37 °C) overnight. For the enzymatic digest, purified BACE2-Fc fusion protein was added to the buffer peptide mixture. For specific inhibition of BACE2, 2 μM C3 were added to the incubation mixture. Digested peptides were purified with C18 ZipTip according to the manufacturer’s protocol and measured with an ABI Destr in positive reflector mode in a range from 750 to 3000 Da.

Immunofluorescence of BACE1 and IL-1R2—COS cells were plated on glass coverslips in 24-well dishes. The next day cells were cotransfected with IL-1R2 and BACE1. 48 h after transfection coverslips were washed two times with ice-cold PBS, fixed for 20 min in 4% paraformaldehyde/sucrose, and afterward washed again two times with PBS. To clear cells from remaining paraformaldehyde, the cells were washed with 100 mM NH4Cl for 3 min. Finally the cells were permeabilized with 1% Triton X-100 for 1 min and again washed two times with ice-cold PBS. After permeabilization cells were double-stained with a polyclonal BACE1 NT antibody and a monoclonal HA antibody (both 1:1000). Cells were washed again and afterward incubated with Alexa 488- (BACE1) and Alexa 594 (IL-1R2)-coupled secondary antibody. Cells were then washed in PBS/water and fixed on a glass specimen with Moviol. Specimens were then investigated under a Zeiss confocal microscope.

**RESULTS**

**Shedding of IL-1R2 by a Metalloprotease**—To study the proteolytic processing of IL-1R2, an HA epitope tag was added to the extracellular N terminus after the signal peptide sequence and a FLAG tag to the cytosolic C terminus of the IL-1R2 sequence (Fig. 1A). IL-1R2 was transiently expressed in human embryonic kidney 293-EBNA cells (HEK293). Conditioned medium and cell lysates were separated by gel electrophoresis and analyzed by immunoblot. Compared with mock-transfected cells, the IL-1R2-expressing cells revealed secretion of a 55-kDa fragment (Fig. 1B), which is consistent with the size of IL-1R2 secreted from the human keratinocyte cell line HaCaT (42). This fragment was detected using the anti-HA tag antibody (Fig. 1B) but not the FLAG tag antibody (not shown). Thus, this fragment lacks the C terminus and corresponds to the secreted IL-1R2 ectodomain, which is generated by ectodomain shedding of full-length IL-1R2. Full-length IL-1R2 in the cell lysate was detected with both antibodies (HA and FLAG) at around 65 kDa in the cell lysate (Fig. 1B, shown for the FLAG tag antibody). Upon lower exposure the protein band is a doublet of bands (not shown), which are likely to correspond to immature and to mature, fully glycosylated IL-1R2. Additionally, the FLAG antibody detected smaller IL-1R2 fragments in the cell lysate at around 8, 9, and 16 kDa (Fig. 1B). Because of their size and because they were not detected with the HA antibody, they are likely to be the CTFs, which remain after secretion of the IL-1R2 ectodomain. The 8-kDa fragment (short CTF, sCTF) was present in larger amounts than the longer one of 9 kDa (ICTF). The 16-kDa fragment may be a dimer of the 8-kDa fragment and is mainly visible when larger amounts of the 8-kDa fragment are present (Fig. 1B, PMA-treated samples). The epitope-tagged IL-1R2 was shed in the same manner as described previously for the corresponding untagged wild-type protein (24–26) because the shedding was stimulated by the phorbol ester PMA and inhibited by the metalloprotease inhibitor TAPI-1 (Fig. 1B). PMA and TAPI-1 did not only alter IL-1R2 secretion, but also increased (PMA) or decreased (TAPI-1) the generation of the IL-1R2 CTF (Fig. 1B), indicating that IL-1R2 undergoes proteolytic processing in a metalloprotease-dependent manner at a site close to its transmembrane domain.
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**A**

\[
\begin{array}{c}
\text{NH}_2 \quad \text{IL-1R2} \quad \text{M} \\
\text{Lumen} \quad \text{HA} \quad \text{FLAG} \quad \text{Cytosol}
\end{array}
\]

**B**

\[
\begin{array}{c}
\text{CON} \quad \text{PMA} \quad \text{CON TAPI1} \quad \text{MOCK} \\
\text{sec} \quad \text{sec} \quad \text{sec} \quad \text{sec} \\
\text{Lys} \quad \text{17} \quad \text{16} \quad \text{55} \quad \text{75}
\end{array}
\]

**FIGURE 1.** Shedding of IL-1R2 occurs in a metalloprotease-dependent manner. **A,** schematic drawing of epitope-tagged IL-1R2, which consists of the CDS signal peptide (gray) followed by an HA epitope tag (dotted), the IL-1R2 coding sequence without its signal peptide (open box), and a C-terminal FLAG tag (black). N and C termini as well as luminal and cytosolic domains are indicated. **M,** membrane. **B,** HEK293 cells were transiently transfected with IL-1R2 or with the peak12 control vector (MOCK). Experiments for IL-1R2 transfected cells are shown as duplicates. For the stimulation of ectodomain shedding, cells were treated for 4 h with ethanol as a control (CON) or with the phorbol ester PMA (1 μM). For the inhibition of shedding, cells were pre-treated for 45 min and after medium change for another 4 h with Me₂SO as a control or with the metalloprotease inhibitor TAPI-1 (25 μM). **Upper two panels** show different exposure (exp.) times of the same immunoblot, which visualizes secreted IL-1R2 in the conditioned medium (CM). **Lower panel,** full-length (fl) protein and the C-terminal fragments were detected in the cell lysate (Lys) with the anti-FLAG tag antibody. As a control, treatment with compounds did not affect β-actin levels (bottom panel). The β-actin blot is from a different but equivalent experiment compared with the IL-1R2 immunoblots. Shown are representative blots from three independent experiments.

**IL-1R2 Is a Novel Substrate for γ-Secretase**—Because different type I membrane proteins, including APP and Notch, are first processed by a metalloprotease and then undergo intramembrane proteolysis by γ-secretase, we next tested whether IL-1R2 is also processed within its transmembrane domain. If IL-1R2 is indeed a novel substrate for γ-secretase, its CTF should accumulate in cells lacking active γ-secretase and should no longer be converted to the intracellular domain (ICD) fragment. To test this possibility, three different experimental conditions of γ-secretase inhibition were tested using the following: (a) the well characterized γ-secretase inhibitor DAPT (43), (b) the dominant-negative and catalytically inactive PS1 D385N mutant (8), and (c) PS1/PS2-deficient mouse embryonic fibroblasts. First, HEK293 cells expressing IL-1R2 were treated overnight with or without DAPT. Full-length and CTFs of IL-1R2 were detected in the cell lysate by immunoblot using an antibody against the C-terminal FLAG tag. Compared with control treated cells, DAPT strongly increased the amount of the IL-1R2 sCTF without increasing full-length IL-1R2 (Fig. 2A). This finding is consistent with γ-secretase cleaving IL-1R2. Interestingly, DAPT did not increase the amount of the ICD, suggesting that ICF is either converted to the sCTF and thus does not accumulate or that the ICF is not significantly processed by γ-secretase. Second, IL-1R2 was transiently transfected into wild-type kidney 293 cells (not the 293-EBNA cell variant) stably expressing wild-type presenilin 1 (PS1) or the dominant-negative PS1 D385N mutant (36). In agreement with a γ-secretase cleavage of IL-1R2, PS1 D385N strongly increased the amount of short IL-1R2 sCTF compared with wild-type PS1-expressing cells (Fig. 2B). As in Fig. 2A, the ICF did not accumulate significantly but was generally more clearly detected than in the other figures, where the slightly different HEK293-EBNA cell variant (Fig. 2A) and the fibroblasts (Fig. 2C) were used. Third, compared with wild-type mouse embryonic fibroblasts, retrotransduction of IL-1R2 into fibroblasts deficient in PS1 and PS2 resulted in accumulation of the IL-1R2 CTF (Fig. 2D). This finding is consistent with IL-1R2 CTF and the γ-secretase complex being tested in a coimmunoprecipitation experiment. To this aim IL-1R2 was expressed in HEK293 cells stably expressing the γ-secretase subunit presenilin 1 in the wild-type (WT) or in the catalytically inactive D385N form. Immunoprecipitation of the IL-1R2 C-terminal fragment led to coprecipitation of all four γ-secretase complex subunits nicastrin, presenilin 1, Aph-1, and Pen-2 (Fig. 2D). As expected, PS1 was only detected in wild-type but not in PS1/PS2-deficient cells (Fig. 2C, bottom panel shows the PS1 CTF). Additionally, a possible interaction between the IL-1R2 CTF and the γ-secretase complex was tested in a coimmunoprecipitation experiment. As a result of γ-secretase cleavage, the IL-1R2 CTF should be converted to the ICD fragment. As above, generation of this fragment should be inhibited when γ-secretase cleavage is blocked. Similar to what is known for the APP ICD (44), the IL-1R2 ICD may be short lived and could not be seen in the cultured cells under steady state conditions (see above Figs. 1 and 2). Thus, an established in vitro γ-secretase cleavage assay (41) was used for the detection of the IL-1R2 ICD. IL-1R2 was transiently transfected into HEK293 cells. The cells were additionally treated with PMA, which results in increased ectodomain shedding of IL-1R2 and in an increased amount of IL-1R2 CTFs (Fig. 1B), which can serve as substrates for subsequent γ-cleavage. Membranes from these cells containing full-length IL-1R2 and CTFs were incubated for 2 h at 37 °C. This resulted in the generation of the ICD of IL-1R2, which was released from the membrane and found in the supernatant after ultracentrifugation (Fig. 3A, bottom panel). Addition of the γ-secretase inhibitor DAPT or incubation at 4 °C prevented ICD generation (Fig. 3A). As an additional control, a further in vitro cleavage assay was carried out, in which an N-terminally truncated form of IL-1R2 was expressed. This mutant protein lacks most
Inhibition of γ-secretase leads to an accumulation of IL-1R2 C-terminal fragments. A, HEK293 cells were transiently transfected with IL-1R2 or with peak12 control vector (MOCK) as in Fig. 1. Cells were treated overnight with Me2SO as a control or with the highly specific γ-secretase inhibitor DAPT (1 μM). Experiments for IL-1R2 were carried out in duplicate. The anti-FLAG tag antibody was used to detect full-length (fl) proteins and their C-terminal fragments in the immunoblot. The short CTF of IL-1R2 in the control treated cells is visible upon longer exposure of the blot (middle panel). As a control, treatment with DAPT did not affect β-actin levels (bottom panel). B, HEK293 cells stably expressing wild-type PS1 (PS1 Wt) or a dominant-negative PS1 mutant (PS1 D385N) were transiently transfected with control vector or with IL-1R2 as indicated above the blot. Full-length (fl) IL-1R2 and its CTFs (sCTF and ICTF) were detected using the anti-FLAG tag antibody. In these cells, the ICTF is more clearly detected than in the cell lines in the other panels.

Identification of the γ-secretase Cleavage Site by Mass Spectrometry—Besides ICD generation, γ-secretase cleavage is expected to generate a soluble N-terminal peptide derived from IL-1R2, which we refer to as IL-1R2β-peptide in analogy to the APP-derived Aβ peptide and the Notch-derived Nβ peptide (47). To determine the cleavage site of γ-secretase within the IL-1R2 transmembrane domain, Δ334-IL-1R2 was expressed in HEK293 cells. Compared with Δ329-IL-1R2, this mutant has an HA tag at the N terminus (Fig. 4A), which was used to immunoprecipitate the IL-1R2β-peptide from the conditioned medium. MALDI-TOF mass spectrometry analysis revealed three major peptide species and several minor peptides (Fig. 4B). All peptides were generated in a γ-secretase-dependent manner, because the γ-secretase inhibitor DAPT blocked their generation. As expected, none of the peptides was visible in control transfected cells. The peptide peak at 3678 Da corresponds to a cleavage site between Ser-353 and Leu-354 in the IL-1R2 transmembrane domain, just outside of the IL-1R2 CTF. The shortest peptide with a mass of 2507 Da corresponds to a cleavage site at the very N terminus or just outside of the IL-1R2 transmembrane domain. The shortest peptide with a mass of 2507 Da may contribute to the IL-1R2 CTFs generated in the control and transduced cells. The lower panel shows a longer exposure of the blot above. C, mouse embryonic fibroblasts from wild-type mice (PS1+/+) or from PS1 and PS2 double-deficient mice (PS1−/−) were transduced with retroviruses encoding epitope-tagged IL-1R2. Nontransduced cells served as controls for the specificity of the IL-1R2 bands. Secreted (sec) IL-1R2 in the supernatant (upper panel) as well as full-length (fl) IL-1R2 (2nd panel from top) and its C-terminal fragments (3rd and 4th panels from top, showing different exposure times) were detected by immunoblotting with antibodies against the indicated epitope tags. The PS1 CTF was only detected in wild-type (PS1+/+) but not in PS1-deficient (PS1−/−) cells (bottom panel). Shown are representative blots from three independent experiments. D, HEK293 cells stably expressing either PS1 WT or PS1 D385N were transduced with the same construct as in C. IL-1R2 CTFs were immunoprecipitated from the lysate and analyzed for IL-1R2 CTF as well as for coprecipitated γ-secretase components nicastrin, PS1NTF, Aph-1, and Pen-2.
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**FIGURE 3.** In vitro γ-secretase assay generates IL-1R2 intracellular domain. A, HEK293 cells were transiently transfected with the IL-1R2 expression vector or with control (CON) vector. Cells were treated for 4 h with the phorbol ester PMA (1 μM). Membrane preparations of the cells were incubated for 2 h at 4 or 37 °C (indicated above the blots) with or without the γ-secretase inhibitor DAPT (1 μM). Supernatants (S100) and pellets (P100) of a 100,000 × g ultracentrifuge spin were analyzed with an antibody against the C-terminal FLAG-epitope tag. Full-length (fl) IL-1R2 and CTFs were found in the membrane-bound pellet fraction, whereas the intracellular domain was mainly seen in the soluble fraction (ICDs). Low amounts of ICD were also detected in the membrane fraction (ICDm). *Middle panel* shows a longer exposure of *upper panel*. B, schematic drawing of epitope-tagged full-length (fl) IL-1R2 and the deletion mutant lacking a large part of the ectodomain. Constructs are not drawn to scale. The IL-1R2 expression construct consists of an N-terminal HA epitope tag (black lines; not present in Δ329-IL-1R2), and a C-terminal FLAG tag (black). Construct number 329 indicates the starting amino acid relative to the HA-IL-1R2-FLAG sequence. C, HEK293 cells stably expressing wild-type PS1 (PS1 Wt) or the dominant-negative PS1 Δ385N mutant (PS1 Δ385N) were transiently transfected with Δ329-IL-1R2 or with control vector (CON). Membrane preparations were incubated for 3 h at the same temperatures and inhibitor conditions as in A. *Upper panel* shows that Δ329-IL-1R2 is found in the membrane-bound pellet fraction after ultracentrifugation (P100), whereas the soluble ICD resulting from γ-secretase cleavage is found in the supernatant (S100). Detection was carried out using the FLAG antibody. Shown are representative blots from three independent experiments.

brane domain. A potential additional e-cleavage at the C-terminal end of the transmembrane domain was not investigated.

**BACE1 and BACE2 Increase Secretion of IL-1R2**—Because of the similarities of proteolytic processing between IL-1R2 and APP, we next tested whether IL-1R2 may also be a substrate for BACE1 or BACE2. To this aim, IL-1R2 and either control vector or BACE1 or BACE2 were transiently transfected into HEK293 cells. As a control, we verified that expression of both proteases increased secretion of APP (data not shown). Full-length IL-1R2 and its proteolytic fragments were detected using antibodies against the N-terminal HA epitope tag or against the C-terminal FLAG tag. Expression of BACE1 and BACE2 increased IL-1R2 secretion and the generation of the corresponding IL-1R2 CTFs in the cell lysate (Fig. 5A, short and a long exposure are shown for the CTF blot). This was paralleled by a decrease in the amount of full-length IL-1R2 in the cell lysate, suggesting that IL-1R2 can be cleaved by BACE1 and BACE2. Interestingly, although expression of BACE1 and BACE2 increased the proteolytic processing of IL-1R2, it did not change the apparent molecular weight of the cleavage products (secreted IL-1R2 and CTFs) (Fig. 5A). Because this is the same size as the fragments, which were generated in the PMA-inducible metalloprotease-dependent manner (Fig. 1B), this result suggests that the BACE1 and BACE2 cleavage sites in IL-1R2 are located very close to the metalloprotease cleavage site. Next, we tested whether IL-1R2 processing was reduced in murine primary hippocampal neurons lacking BACE1 or in glial cells deficient in both BACE1 and BACE2. However, no change in the amount of secreted IL-1R2 or in the amount of the IL-1R2 CTF was detected between the wild-type cells and the knock-out cells (data not shown). Potentially, in the absence of BACE1 or BACE2 IL-1R2 may undergo increased cleavage by a metalloprotease. The use of the metalloprotease inhibitor TAPI-1 did not significantly alter IL-1R2 processing in the BACE-deficient cells (not shown), presumably because the inhibitor is not potent enough to fully block CTF generation under wild-type conditions (see Fig. 1B). As a consequence of the BACE deficiency, no changes in IL-1R2 processing may be observed, because BACE1/BACE2 and the metalloprotease lead to IL-1R2 cleavage products of the same apparent molecular weight. This is different from APP and PSGL-1, which are both cleaved by BACE1 in such a distance from the metalloprotease cleavage site that the CTFs resulting from these cleavage events can be clearly separated by gel electrophoresis. As a consequence the BACE1-induced CTF, but not the metalloprotease-induced CTF of APP and PSGL-1, was absent in BACE1-deficient cells as we and others have shown previously (32, 48).

To exclude the possibility that transfection of BACE1 or BACE2 increased IL-1R2 shedding simply by activating the α-secretase-like metalloprotease cleavage, we tested the following: (a) whether the metalloprotease inhibitor TAPI-1 is able to reduce the BACE1-induced increase in IL-1R2 shedding, (b) whether BACE1 and IL-1R2 colocalize in transfected cells, and (c) whether BACE2 is able to cleave in vitro a synthetic peptide encompassing the juxtamembrane domain of IL-1R2. First, HEK293 cells were transfected with IL-1R2 and either BACE1 or empty vector as a control. Treatment with the metalloprotease inhibitor TAPI-1 largely inhibited IL-1R2 shedding under control conditions (Fig. 5B, see also Fig. 1B) but did not significantly reduce the increased IL-1R2 shedding in the BACE1-transfected cells. Addition of the specific BACE inhibitor C3 (49, 50) was able to reduce the increased IL-1R2 shedding in the BACE1-transfected cells. This shows that BACE1 transfection did not indirectly increase IL-1R2 shedding by stimulating the α-secretase-like cleavage. Second, immunofluorescence microscopy was used to test for a colocalization between BACE1 and IL-1R2 in transfected COS cells, which are large
and adherent and thus allow good visualization of the cellular localization of both proteins. Colocalization was observed at or close to the plasma membrane, in vesicular structures, and at perinuclear sites (Fig. 5C), which agrees with the intracellular localization of BACE1 observed in other studies (48, 51, 52). Third, an Fc fusion protein of BACE2 was purified and incubated at 37 °C in vitro with a synthetic peptide encompassing the juxtamembrane domain of IL-1R2. This domain was previously shown to be required for IL-1R2 secretion (42). Generation of the cleavage products was monitored by mass spectrometry (Fig. 5D). The intact peptide incubated without BACE2 showed the expected mass of 2219.7 Da (Fig. 5D). Incubation with BACE2 resulted in the formation of two smaller fragments with masses of 916.7 and 1321 Da, revealing a cleavage between Phe-329 and Gln-330 (Fig. 5D). The fragment peaks were not observed when BACE2 was incubated in the absence of the peptide, showing the specificity of the peptide fragment peaks. Importantly, the specific BACE protease inhibitor C3 (Fig. 5D) as well as incubation at 4 °C (not shown) completely inhibited the peptide cleavage. Together, these experiments indicate that IL-1R2 can be directly cleaved by BACE1 and BACE2.

Mass Spectrometric Determination of the BACE1 and BACE2 Cleavage Sites in Cultured Cells—Next, we tested whether the BACE protease cleavage site in IL-1R2 observed in vitro was also found in IL-1R2-expressing HEK293 cells. First, the mass of the secreted IL-1R2 ectodomain was measured to determine its C-terminal amino acid. The IL-1R2 ectodomain was immunoprecipitated from PMA-treated HEK293 cells. PMA treatment increases IL-1R2 secretion (Fig. 1B) and allows the purification of sufficient amounts of the IL-1R2 ectodomain for linear mode MALDI-TOF mass spectrometric analysis. This revealed a broad peak at around 50 kDa (Fig. 6A), which fits well with the apparent molecular mass of around 55 kDa in the immunoblot (Fig. 1B). Because IL-1R2 contains five potential N-glycosylation sites within its ectodomain, the immunoprecipitated ectodomain was treated with N-glycosidase F to remove the N-linked sugars. This resulted in a sharper mass peak at 35 kDa (Fig. 6A). This peak was specific to IL-1R2 because it was not present in the medium of control transfected cells, which did not express IL-1R2. Additionally, a tryptic digest of this 35-kDa fragment and a subsequent analysis of the N-terminal peptide by tandem mass spectrometry revealed the N terminus to start with the tyrosine residue of the N-terminal HA tag (not shown), which remains after signal peptide cleavage. If no other post-translational modifications are present, the 35-kDa peak is consistent with the C terminus of the IL-1R2 ectodomain ending in amino acid proline 309 at a distance of 35 amino acids from the membrane (Fig. 6B, marked with an arrow; measured mass 35,053.67 Da; calculated mass 35057.58 Da).

![Figure 4](image-url) Determination of γ-secretase cleavage site by mass spectrometry. A, schematic drawing of the sequence of the Δ334-IL-1R2 construct. *, **, and *** indicate the C termini of the peptides identified in B. Additional but minor peptide peaks were observed, which can be assigned to C-terminally truncated forms of peptides * and **. Sequence of the transmembrane domain is boxed. For a comparison, the transmembrane sequence of APP and the corresponding γ- and ε-secretase cleavage sites are indicated. B, HEK293 cells were transiently transfected with Δ334-IL-1R2 (upper two panels) or control vector (MOCK, lower two panels) and additionally treated with Me2SO as a control or with the γ-secretase inhibitor DAPT. Supernatant was immunoprecipitated with HA-agarose beads. Peptide masses were determined by mass spectrometry using MALDI-TOF. C, list of identified peptides and their deviation from the theoretical mass calculated with GPMAW (Lighthouse).
FIGURE 5. **BACE1 and BACE2 increase processing of IL-1R2.**

A, HEK293 cells were transiently transfected with control vector (MOCK) or with the epitope-tagged IL-1R2 (a short and a long exposure of the CTF blot are shown) as indicated above the blots. Cells were cotransfected as indicated with empty vector as a control (CON), BACE1 or BACE2 in duplicate experiments. Conditioned media (CM) and cell lysates (Lys) were separated by gel electrophoresis. Proteins were detected with the indicated antibodies. Vertical lines on the blot indicate that the samples were run on the same gel but not directly next to each other. B, HEK293 cells were transfected as in A. Cells were pretreated for 45 min and after medium change for another 4 h either treated with Me2SO (DMSO) as control or the metalloprotease inhibitor TAPI-1 (25 μM) or with TAPI-1 (25 μM) plus the BACE inhibitor C3 (2 μM). Two different exposure times of the sIL-1R2 and the sCTF blots are shown. Note that TAPI-1 reduces IL-1R2 secretion in the control cells (left 6 lanes) but not in the BACE1-transfected cells. The BACE inhibitor C3 can slightly further reduce IL-1R2 secretion when coincubated with TAPI-1 in control cells. As a control, treatment with compounds did not affect β-actin levels (bottom panel). Vertical lines on the blot indicate that the samples were run on different gels. C, COS cells were transiently transfected with IL-1R2 and BACE1 and stained for IL-1R2 (red) and BACE1 (green). Confocal fluorescence microscopy reveals plasma membrane, vesicular, and perinuclear colocalization of IL-1R2 and BACE1. D, an IL-1R2 juxtamembrane peptide encompassing amino acids 322–341 was incubated with a BACE2-Fc fusion protein in vitro in an acidic sodium acetate buffer under different conditions: 1, incubation of peptide alone; 2, peptide plus BACE2-Fc; 3, peptide plus BACE2-Fc plus BACE inhibitor C3; 4, BACE2-Fc alone. Peptide and fragments were purified and analyzed by mass spectrometry. Peptide sequence and identified cleavage sites are indicated below the spectra.
Da). This site is several amino acids more N-terminal than the cleavage site determined in the in vitro BACE2 assay (Fig. 5D). Potentially, the initial cleavage occurs at Phe-329 and is then followed by carboxypeptidase cleavage (see “Discussion”). Next, to reduce the potential carboxypeptidase trimming of the ectodomain, the serum concentration was reduced from 10 to 1%, and the incubation time of the conditioned medium was reduced from overnight incubation to 4 h. Additionally, a truncated IL-1R2 construct (Δ322-IL-1R2) was used. This protein lacks most of the IL-1R2 ectodomain but retains the juxtamembrane cleavage region, which is required for secretion (42), and was used in the in vitro assay and also encodes the transmembrane and cytoplasmic domains of IL-1R2 (Fig. 6, C and D). Thus, Δ322-IL-1R2 is similar to N-terminally truncated APP constructs, which, like full-length APP, are cleaved by α- and β-secretase. Δ322-IL-1R2 and either control vector BACE1 or BACE2 were cotransfected into HEK293 cells. A strong increase in the formation of IL-1R2 CTFs was observed in the BACE1- and BACE2-expressing cells compared with the control cells (Fig. 6C). These CTFs had the identical apparent molecular weight as the CTFs generated from full-length IL-1R2 (not shown), revealing that cleavage of the truncated Δ322-IL-1R2 occurs in the same manner as for full-length IL-1R2. The secreted short ectodomain of Δ322-IL-1R2 was immunoprecipitated from the conditioned medium and analyzed by MALDI-TOF mass spectrometry. Cells expressing Δ322-IL-1R2 (but not transfected with BACE1 or BACE2) secreted a peptide with a mass of 3112.2 Da, which was not present in control transfected cells not expressing Δ322-IL-1R2. Because these cells were not transfected with BACE1 or BACE2, this peptide is likely to result from the metalloprotease cleavage and corresponds to a cleavage between Arg-333 and Thr-334. Additional minor peaks were observed at lower masses and correspond to C-terminally truncated peptides. They may arise through alternative proteolytic cleavages or because of the remaining low level carboxypeptidase activity. Transfection of BACE1 and BACE2 led to a strong increase in the generation of a peptide with a mass of 2614 Da, which corresponds to a cleavage between Phe-329 and Gln-330. This site is identical to the one determined in the in vitro BACE2 cleavage assay. The metalloprotease generated fragment (3112 Da) was only visible at low amounts. This analysis indicates that the

![Image](https://example.com/image1.png)

**FIGURE 6. Determination of the BACE1 and BACE2 cleavage sites in HEK293 cells.** A, HA-agarose beads were used to immunoprecipitate secreted IL-1R2 from the conditioned medium of PMA-treated IL-1R2-expressing HEK293 cells and either left untreated (upper panel) or deglycosylated with N-glycosidase F (middle panel). MALDI-TOF mass spectrometric analysis was used to determine the mass of the precipitated fragment. No peptides were detected in the medium of mock-transfected control cells (lower panel). B, sequence of epitope-tagged IL-1R2. The N-terminal HA tag plus a linker sequence as well as the C-terminal linker sequence plus the FLAG tag are underlined. The transmembrane domain is boxed. The putative C terminus of the secreted ectodomain is marked by an arrow. The putative cleavage sites by the metalloprotease and BACE1 and BACE2 as determined in D are indicated with arrowheads. C, HEK293 were transfected with empty vector (MOCK) or cotransfected with Δ322-IL-1R2 and either empty vector or BACE1 or BACE2. Full-length (fl) Δ322-IL-1R2 and CTFs (two different exposure times) are seen in immunoblot using an antibody against the C-terminal FLAG tag. D, immunoprecipitated supernatants of 293E cells expressing Δ322-IL-1R2 either with empty vector or BACE1 or BACE2 were subjected to MALDI-TOF MS. Measured and calculated masses are compared in the table below the spectra. The resulting cleavage sites are indicated in the schematic figure of Δ322-IL-1R2 and compared with cleavage sites of α- and β-secretase in case of APP. Asterisks indicate the β- and α-secretase-like cleavage sites in Δ322-IL-1R2.
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metalloprotease cleavage site and the BACE1/BACE2 cleavage sites in IL-1R2 are indeed very close to each other, at a distance of four amino acids.

BACE1 and BACE2 Selectively Enhance Secretion of a Subset of Shedding Substrates—To rule out the likelihood that transfected BACE1 and BACE2 simply increased the cleavage of any shedding substrate, including IL-1R2, five additional membrane proteins were tested for their potential cleavage by both proteases. Pro-TGFα (converted to soluble TGFα upon shedding) and the cell adhesion protein P-selectin are type I membrane proteins, the lipopolysaccharide receptor CD14, and the Fcγ receptor CD16 are GPI-anchored proteins, and TNFα is a type II membrane protein. Like APP and IL-1R2, all proteins undergo ectodomain shedding in a metalloprotease-dependent manner and can be found in soluble form in physiological body fluids (53, 54). The proteins were tagged with an HA epitope tag in their extracellular domain. The three type I and II membrane proteins (pro-TGFα, P-selectin, and TNFα) were additionally tagged with a FLAG tag in their intracellular cytoplasmic domain. Transient transfection of these constructs together with control vector, BACE1, or BACE2 was carried out as above for APP and IL-1R2. The full-length and secreted forms of all proteins were detected in the cell lysate and the conditioned medium, respectively, at their expected apparent molecular weight (Fig. 7, A–E). BACE1 and BACE2 increased the secretion and reduced the cellular levels of pro-TGFα (Fig. 7A) and of CD16 (Fig. 7B) but not of P-selectin, TNFα, and CD14 (Fig. 7, C–E), revealing that both proteases do not cleave all membrane proteins undergoing ectodomain shedding. Moreover, for pro-TGFα an increase in the CTFs was detected in the cell lysate (Fig. 7A), which is consistent with a proteolytic cleavage very close to the membrane domain. Similar to IL-1R2, the BACE1- and BACE2-induced CTFs of pro-TGFα had a very similar apparent molecular weight as the CTFs observed under control conditions (Fig. 7A). Because CD16 is a GPI-anchored protein, no CTFs were observed in the lysate. Expression of transfected BACE1 and BACE2 was verified by immunoblot (not shown).

DISCUSSION

Several type I membrane proteins undergo RIP, which is a proteolytic cascade initiated by metalloprotease-mediated ectodomain shedding and followed by γ-secretase-dependent intramembrane proteolysis. Among these proteins, only APP is additionally subject to ectodomain shedding by the β-secretase BACE1 and its homolog BACE2. Thus, APP serves as a good reference for the study of the proteolytic processing of type I membrane proteins. Processing of APP is a key event in the pathogenesis of AD. Proteolytic processing of another membrane protein, the IL-1R2 has also been linked to AD. In particular, increased amounts of the secreted IL-1R2 ectodomain have been detected in the cerebrospinal fluid of patients with an early stage but not a late stage of AD compared with age-matched controls (22, 23). In agreement with previous studies (24–26), we found that shedding of IL-1R2 shows the typical features of a protein undergoing α-secretase-like ectodomain shedding by an ADAM protease. IL-1R2 shedding could be stimulated with the phorbol ester PMA and could be blocked by the metalloprotease inhibitor TAPI-1. By using mass spectrometry we found that the α-secretase-like cleavage site is located in the membrane-proximal stalk region of IL-1R2. The ectodomain has proline 309 as a C-terminal residue, whereas the truncated Δ322-IL-1R2 has Arg-333 as a C-terminal amino acid. This could indicate that there are two different cleavage sites for the metalloprotease. However, we assume that the initial cleavage site is at Arg-333 and that the ectodomain is then trimmed, potentially by a carboxypeptidase, until proline 309. In fact, carboxypeptidases cleave inefficiently at proline residues (55) and thus may not be able to further trim the C terminus beyond proline 309. Moreover, a previous study (42) would agree well with a cleavage at amino acid 332 but not at 309. That study replaced amino acids His-324 through Ser-341 in the membrane-proximal domain of IL-1R2 by the corresponding residues of the epidermal growth factor receptor membrane-proximal domain, and they found that this mutation prevented normal secretion of the IL-1R2 ectodomain (42). Additionally, the APP α-cleavage is strongly reduced by a proline mutation close to the cleavage site (56, 57), suggesting that metalloprotease-mediated ectodomain shedding occurs in helical conformations, making it unlikely that the α-like cleavage in IL-1R2 occurs directly at proline 309. Interestingly, APP is cleaved
C-terminally to a lysine residue and IL-1R2 C-terminally to an arginine, suggesting a preference of the α-secretase-like cleavages for a positively charged residue.

The CTF, which is generated upon ectodomain shedding of full-length IL-1R2, can be further cleaved by γ-secretase. Using different experimental approaches, including a γ-secretase inhibitor and cells deficient in γ-secretase activity, we demonstrate that IL-1R2 is a novel γ-secretase substrate. This fits well with the general idea that the CTFs of type I membrane proteins, which undergo ectodomain shedding, are subsequently further cleaved by γ-secretase, leading to the release of their ICD. γ-Secretase cleavage of CTFs and generation of the ICD may serve different purposes, including signal transduction and membrane protein degradation (2). Similar to APP, the physiological role of γ-cleavage of IL-1R2 remains unknown. It may be a degradation pathway for the IL-1R2 CTF. Alternatively, it may allow the IL-1R2 ICD to participate in a new kind of signal transduction, similar to Notch.

An additional outcome of our study is that transfection of the β-secretase BACE1 and its homolog BACE2 can stimulate secretion of IL-1R2 and generation of its CTFs. As determined by mass spectrometry, the cleavage occurs C-terminally to a phenylalanine, which agrees well with the known cleavage specificity of BACE1 and BACE2. Both proteases preferentially cleave after hydrophobic residues, such as leucine in the Swedish mutant form of APP, in PSGL-1, and ST6Gal I or after methionine in wild-type APP (32, 58–60).

If IL-1R2 is also cleaved at endogenous expression levels of BACE1 and BACE2 and thus constitutes a novel substrate for both proteases, its cleavage should be reduced in BACE1 or -2 knock-out cells, which, however, was not the case. This is in contrast to other established substrates for both proteases, such as APP (39, 61–63), PSGL-1 (32), the sialyltransferase ST6Gal I (64), β-subunits of voltage-gated sodium channels (65), and neuregulin-1 (50, 66). This finding could be interpreted in two ways, one being that IL-1R2 is not a physiological substrate for BACE1 and -2 and is only cleaved upon overexpression of both proteases. Although we cannot fully rule out this possibility, we consider it unlikely because of the points discussed below. First, we found that BACE1 and -2 do not simply cleave all membrane proteins tested. For example, TNFα, P-selectin, and CD14 (this study) do not undergo increased shedding upon transfection of BACE1 and -2. Likewise, we previously reported that both proteases did not increase shedding of L-selectin and TNF receptor 2 (32). Thus, BACE1 and -2 clearly are specific with regard to the proteins that are cleaved and secreted in response to expression of both proteases. Second, BACE1 is expressed in all tissues, which express IL-1R2. Therefore, IL-1R2 cleavage may also occur at endogenous expression levels of BACE1. Third, we found that BACE1 and -2 induced cleavage of IL-1R2 at a peptide bond very close (four amino acids difference) to the cleavage site of the α-secretase like metalloprotease. This situation is very similar to APP, where the BACE2 cleavage site is at a distance of three to four residues from the α-secretase cleavage site, such that the APP CTFs generated by both proteases (C83 through α-cleavage and C79 through BACE2 cleavage) are not distinguishable by gel electrophoresis (67, 68). As a consequence, the amount of C83/C79 CTFs in BACE2 knock-out cells is not significantly different from wild-type cells (39), because C83 is still generated and presumably to an enhanced extent. Likewise, we expect that the potential decrease in IL-1R2 processing in BACE1- and BACE2-deficient cells would be compensated for by an increased metalloprotease cleavage, such that no net change in total IL-1R2 processing would be observed. If a similar situation of nearby cleavage sites by different proteases is found in other membrane proteins, it may be a particular challenge to unequivocally identify a given protein as a novel substrate for BACE proteases. In fact, a similar result was observed for the type I membrane protein TGFα and the GPI-anchored protein CD16. Similar to IL-1R2, processing of both proteins was strongly enhanced upon transfection of BACE1 and -2, but their processing was not significantly altered in BACE1-deficient or BACE1 and BACE2 double-deficient cells (not shown). Both TGFα and CD16 are subject to an α-secretase-like cleavage in their membrane-proximal domain, which generates fragments of the same apparent molecular weight as the ones generated by transfected BACE1 and BACE2. Thus, like IL-1R2, both TGFα and CD16 may be cleaved by BACE1 and -2 at a site very close to or identical to the metalloprotease cleavage site. Potentially, BACE1 and BACE2 may have a more general role as alternative α-secretase-like proteases and be involved in the shedding of additional type I, type II, and GPI-anchored membrane proteins known to undergo ectodomain shedding. This hypothesis may also provide a molecular explanation for the relatively mild phenotype observed in BACE1-deficient (61–63) and in BACE1 and BACE2 double-deficient mice (39). With regard to the large number of ADAM proteases, which have at least partially redundant functions (4), BACE1 and -2 seem to be expressed at low levels. If both proteases act as alternative α-secretases, their loss of expression could be compensated for by the ADAM proteases and would not necessarily lead to a similarly strong phenotype as it is observed for some of the ADAM protease knock-out mice (69, 70).

Regardless of whether or not IL-1R2 is cleaved by the endogenous BACE1, our finding that increased BACE1 expression enhances IL-1R2 shedding may provide a molecular explanation for a neuropathological change observed in AD brain. BACE1 expression is up-regulated in AD patients (71, 72) and thus may potentially be the cause of the enhanced amount of soluble IL-1R2 found in AD patients (22, 23). The increase in IL-1R2 shedding has been suggested to be a reaction of the brain to increased IL-1 concentrations found in AD brain (22). Given that IL-1 has been linked in multiple ways to AD (18), the increase in soluble IL-1R2 may be a cellular response aimed at binding the excess IL-1 and preventing detrimental effects of too much IL-1 in the brain.

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