Interleukin-1 Receptor Type 1 Is a Substrate for γ-Secretase-dependent Regulated Intramembrane Proteolysis

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Biochemical and genetic studies have revealed that the presenilins interact with several proteins and are involved in the regulated intramembrane proteolysis of numerous type 1 membrane proteins, thereby linking presenilins to a range of cellular processes. In this study, we report the characterization of a highly conserved tumor necrosis factor receptor-associated factor-6 (TRAF6) consensus-binding site within the hydrophilic loop domain of presenilin-1 (PS-1). In coimmunoprecipitation studies we indicate that presenilin-1 interacts with TRAF6 and interleukin-1 receptor-associated kinase 2. Substitution of presenilin-1 residues Pro-374 and Glu-376 by site-directed mutagenesis greatly reduces the ability of PS1 to associate with TRAF6. By studying these interactions, we also demonstrate that the interleukin-1 receptor type 1 (IL-1R1) undergoes intramembrane proteolytic processing, mediated by presenilin-dependent γ-secretase activity. A metalloprotease-dependent proteolytic event liberates soluble IL-1R1 ectodomain and produces an ~32-kDa C-terminal domain. This IL-1R1 C-terminal domain is a substrate for subsequent γ-secretase cleavage, which generates an ~26-kDa intracellular domain. Specific pharmacological γ-secretase inhibitors, expression of dominant negative presenilin-1, or presenilin deficiency independently inhibit generation of the IL-1R1 intracellular domain. Attenuation of γ-secretase activity also impairs responsiveness to IL-1β-stimulated activation of the MAPKs and cytokine secretion. Thus, TRAF6 and interleukin receptor-associated kinase 2 are novel binding partners for PS1, and IL-1R1 is a new substrate for presenilin-dependent γ-secretase cleavage. These findings also suggest that regulated intramembrane proteolysis may be a control mechanism for IL-1R1-mediated signaling.

The mammalian interleukin 1 receptor-Toll-like receptor (IL-1R-TLR)3 superfamily is known to play important roles in innate immunity and inflammatory responses (1–4). Family members are characterized as type 1 integral membrane proteins, with a cysteine-rich extracellular domain for ligand binding and a catalytically deficient, homologous cytoplasmic Toll/IL-1R domain (TIR domain) (3). As the receptors have no intrinsic enzymatic activity, they form multimeric receptor complexes and through homophilic protein-protein interactions recruit and cooperate with a number of cytosolic protein partners to produce cellular responses. These signaling molecules include the interleukin receptor-associated kinases (IRAKs), tumor necrosis factor receptor-associated factor-6 (TRAF6), and a set of TIR domain-containing adapter proteins as follows: myeloid differentiation factor 88 (MyD88), MyD88 adapter-like protein (Mal; also known as TIRAP), TIR domain-containing adapter protein inducing interferon-β (TRIF, also termed TICAM-1), and TRIF-related adapter molecule (TRAM, also termed TIRP or TICAM-2) (3). A critical event in the activation of intracellular signaling pathways by IL-1β acting via IL-1R type 1 (IL-1R1) or bacterial lipopolysaccharide acting via Toll-like receptor 4 (TLR4) is the oligomerization and Lys-63-linked polyubiquitination (K63-pUb) of TRAF6 (5–8). This results in the amplification of a signaling pathway culminating in the activation of the mitogen-activated protein kinases (MAPK), and transcription factors NF-κB and Tpl2 (9, 10).

The responsiveness of the IL-1/Toll receptor superfamily regulates several biological functions, including appropriate adaptive and innate immunity, control of programmed cell death within the developing central nervous system, stress response, and bone metabolism (1), whereas uncontrolled responses, because of disproportionate activation of the TLR-IL-1R pathways, contribute to fatal immune disorders (2, 3). Therefore, to retain immune homeostasis and the health of an organism while avoiding severe disorders, TLR-IL-1R signaling is carefully regulated. The IL-1 ligands (IL-1α and IL-1β) have two homologous receptors, IL-1 receptor type 1 (IL-1R1) and IL-1 receptor type 2 (IL-1R2) (4). Upon IL-1α/β binding, IL-1R1 forms a complex with IL-1 receptor accessory protein (IL-1RaPc) and initiates a cytosolic signaling cascade as discussed above (4). In contrast, IL-1R2 functions as a “decoy” domain; IL, interleukin; JNK, c-Jun N-terminal kinase; SAPK, stress-activated protein kinase; ERK, extracellular signal-regulated kinase; MAPK, mitogen-activated protein kinase; MEF, mouse embryonic fibroblast; PBS, phosphate-buffered saline; ELISA, enzyme-linked immunosorbent assay; NTF, N-terminal fragment; DAPT, N-[N-(3,5-difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester; PS, presenilin; TIR, Toll/IL-1R; PS-KO, presenilin-deficient; mAb, monoclonal antibody; RIP, regulated intramembrane proteolysis.
receptor, which undergoes ectodomain shedding and competes with cell surface IL1-R1 for ligand binding (11). An IL-1 receptor antagonist exists, which binds to IL-1R1 and blocks ligand binding (12). In addition to the existence of decoy and antagonistic receptors, post-translational modifications, including glycosylation (13), ubiquitination (14, 15), phosphorylation (16), and proteolysis (17, 18), also contribute to the sensitive control and degree of receptor activation seen in vivo (2, 19, 20). Therefore, an important approach to deciphering the regulation of cytokine signaling is not only the characterization of receptor-interacting proteins and assembly of signaling pathways, but also the study of receptor post-translational modifications.

One important mechanism by which cytokine signaling is regulated is through the generation and release of soluble cytokine receptors, which can bind their corresponding ligand (cytokine) and either positively or negatively regulate their biological activity (21). Tumor necrosis factor (TNF)-α-converting enzyme (ADAM-17) is a member of the ADAM (a disintegrin and metalloproteinase domain) family of zinc metalloproteinases, which functions as a cytokine receptor sheddase for the TNF receptor and IL-1R/TLR superfamilies (21–23). Additionally, TNF-α-converting enzyme mediates a proteolytic cleavage event in the proximal extracellular domain of numerous other type 1 membrane proteins, including amyloid precursor protein, Jagged, Delta, pro-TNF-α, the TNF receptors, IL-1R2, IL-2-α receptor, IL-6 receptor, Notch, p75 neurotrophin receptor (p75NTR), and ErbB4 (18, 24–32). Some of these proteins undergo a subsequent intramembrane cleavage event within the membrane-anchored C-terminal fragment (CTF) to generate soluble intracellular domains (ICD) (27–34). This cleavage event is referred to as regulated intramembrane proteolysis (RIP) (35) and involves the activity of the presenilin-dependent γ-secretase protease, which was initially characterized because of its involvement in the cleavage of amyloid precursor protein and generation of Aβ (36–39). The γ-secretase complex is composed of at least four components, presenilin (PS1 or PS2), nicastrin, Pen-2, and Aph-1 (Aph-1αL, Aph-1αS, or Aph-1b), and all four proteins are necessary for full proteolytic activity (40). More recently, several additional factors that interact with or alter activity of the γ-secretase complex have been identified, including CD147 (41), p23/TMP21 (42), and Rer1p (43). It is believed that nicastrin functions in substrate recognition (44, 45), whereas presenilin forms the catalytic core by functioning as an aspartyl protease (37, 38). RIP has now emerged as an important mechanism for regulating cellular responsiveness, cholesterol metabolism, and immune surveillance (27, 32, 33, 36, 46–51). In some instances, RIP is part of a signal transduction cascade whereby the ICD of specific receptors translocates to the nucleus, associates with transcription factors, and regulates the transcription of target genes (29, 33, 52–56, 129, 130). In other cases, studies demonstrate that subsequent to ectodomain shedding, intramembrane proteolysis initiates degradation of the membrane spanning domains of receptors or termination of receptor-signaling functions (35, 57). Independent of γ-secretase activities, through specific protein-protein interactions the presenilins have also been implicated in several intracellular processes, including cell adhesion (58), regulation of calcium homeostasis (59), trafficking of select membrane proteins and/or intracellular vesicles (60, 61), Wnt/β-catenin signaling (62), and neuronal plasticity (36, 39). These functions have been corroborated by reports of defective signal transduction in cells lacking presenilins, where loss of presenilins affect β-catenin (63), phosphatidylinositol 3-kinase/Akt, mitogen-activated protein kinase kinase/extracellular signal-regulated kinase (ERK) (64), stress-activated protein kinase (SAPK)/c-Jun N-terminal kinase (JNK) (65), and B cell receptor signaling (50).

In this study we show that PS1 contains a TRAF6-binding domain and interacts with the IL-1R1 adapter proteins TRAF6 and IRAK2. We further demonstrate that the IL-1R1 is a novel substrate for RIP. The data presented demonstrate that IL-1R1 undergoes metalloprotease-mediated ectodomain shedding, represents a novel substrate for presenilin-dependent γ-secretase proteolysis, and antagonizing γ-secretase activity negatively regulates the IL-1R1 signaling pathway.

**EXPERIMENTAL PROCEDURES**

cDNA Expression Vectors—A cDNA encoding PS1 wild type was amplified by standard PCR techniques and subcloned into pcDNA3.1 (Invitrogen), as described previously (66). Site-directed mutagenesis was performed using a two-primer pair method outlined by QuikChange™ site-directed mutagenesis kit (Stratagene). For the generation of PS1*P374N/E376A, the mutagenesis primer pairs were as follows: 5’-GCAGATTTACCTCGGTGTTAAGACGGAGAGGCAAGGGGAGTAAAACTTGGAATTGG-3’ and 3’-CGTGCTAGAGCAGCACTCACTTGTCCGCTTCCCCCTCATTTTGAACCTAACC-5’. For the generation of PS1*D257A/D385A, pCDNA3-PS1*D257A was used as template (kindly provided by B. de Strooper, KULeuven, Belgium) and the mutagenesis primer pairs were as follows: 5’-CTTTGGATTGGAGCGACCACTTCTGAGG-3’ and 5’-GCTAGAAAATGAAGAGCTCTCCAATCCAAG-3’. The source of other plasmids was as follows: IL-1R1, IRAK2-Myc, kIRAK2-Myc, and AU1-MyD88 (M. Muzio, Mario Negri Institute, Milan, Italy) (67), rat p75NTR (E. Shooter, Stanford University School of Medicine, San Francisco, CA), and FLAG-TRAF6 (Tularik Inc, San Francisco).

Antibodies and Reagents—All reagents were obtained from Sigma unless otherwise stated. Phorbol 12-myristate 13-acetate (PMA), TNFα protease inhibitor-1 (TAPI-1), and the γ-secretase inhibitors, XIX (68), compound E, and N-[N-(3,5-difluorophenacyl)-l-alanyl]-S-phenylglycine t-butyl ester (DAPT) (69), were from Calbiochem. Recombinant human IL-1β was from Tebu-bio (Peterborough, UK). Anti-IL-1R1 ectodomain, anti-IL-1R1 C-terminal domain, anti-JNK, anti-phosphorylated JNK, anti-1kBα, and phosphorylated-1kB were from Santa Cruz Biotechnology; anti-Myc mAb, anti-β-actin mAb, and anti-FLAG mAb were from Sigma; anti-PS1 NTF (mAb 1563) and anti-IL-1R1 C-terminal domain, anti-JNK, anti-phosphorylated JNK, anti-1kBα, and phosphorylated-1kB were from Santa Cruz Biotechnology; anti-Myc mAb, anti-β-actin mAb, and anti-FLAG mAb were from Sigma; anti-PS1 NTf (mAb 1563) was from Chemicon; anti-AU1 (Covance) and anti-PS1 CTF (mAb 3.6.1) were from Scios Inc. (Sunnyvale, CA).

Cell Lines—All cell lines, HEK293T and immortalized murine embryonic fibroblasts (MEFs), were cultured under standard conditions as described previously (37, 70). Transient transfection of HEK293T cells was done with the calcium phosphate method as described (70). Wild type and PS1*P374N/E376A.
Regulated Intramembrane Proteolysis of IL-1R1

MEFs (provided by B. de Strooper, KU Leuven, Belgium) (37) were transected by Metafectene™ Pro (Biontex).

Transient Transfection and PMA-induced Receptor Proteolysis—Subconfluent cell cultures were transiently transected by calcium phosphate precipitation (HEK293T) or Metafectene™ Pro (MEFs) with 3–5 µg of each of the indicated constructs. After 24–36 h, cells were washed with ice-cold PBS and lysed on ice in lysis buffer (50 mM HEPES, 150 mM NaCl, 2 mM EDTA, 0.1–1% Nonidet P-40, 10 mM sodium orthovanadate and protease inhibitor mix (Complete™, Roche Applied Science)). Total protein concentrations were normalized using the bicinchoninic acid method (Pierce). For induced and antagonized ectodomain shedding, cells were pretreated for 2 h with PMA (200 ng/ml) or TAPI (50 µM), respectively. The γ-secretase inhibitors (50 nM compound E (71), 30 nM XIX (68), and 4 µM DAPT (69)) were used at the indicated concentration and for the indicated times.

Trichloroacetic Acid Precipitation and Detection of Soluble IL-1R1—HEK293T cells were transiently transected with 2 µg of pcDNA3 (vector) or pcDNA3-IL-1R1. Then 34 h after transfection the culture medium was replaced with hormone- and serum-free medium. For PMA-stimulated ectodomain shedding, cells were stimulated with PMA (200 ng/ml) for 2 h, and culture media were collected, and cells were lysed. Protein in the culture media was precipitated with trichloroacetic acid and resuspended in a one-third volume of 1 M Tris base and a two-third volume of SDS sample buffer containing 2-mercaptoethanol. Samples were resolved by SDS-PAGE and analyzed by immunoblot.

Coimmunoprecipitation and Western Blot Analysis—For immunoprecipitation of endogenous proteins, subconfluent cultures of HEK293T were stimulated with or without 10 ng ml⁻¹ recombinant IL-1β. Cells were then washed with ice-cold PBS and lysed in 1 ml of lysis buffer (50 mM HEPES, 150 mM NaCl, 2 mM EDTA, 0.1% Nonidet P-40, 10 mM sodium orthovanadate, and protease inhibitor mixture (Complete™, Roche Applied Science). Cells were lysed on ice for 15 min and then centrifuged at 14,000 rpm for 20 min, and the supernatants were collected. Lysates were then normalized using the bicinchoninic acid method (Pierce) such that equivalent amounts of protein were present in each sample. Lysates were pre-cleared for 2 h with 30 µl of protein G-agarose beads (Roche Applied Science). The lysates were immunoprecipitated with the indicated antibodies, and then the immunoprecipitates were washed five times in lysis buffer. Samples were resolved by SDS-PAGE, transferred to polyvinylidene difluoride or nitrocellulose membrane (Millipore), and subjected to Western blot analysis with the indicated antibody. For detection of exogenously expressed or endogenous proteins, cells were lysed as described above and normalized such that an equivalent amount of protein was fractionated on SDS-polyacrylamide gels. Blots were subjected to Western blot analysis with the indicated antibodies, and proteins were detected with the ECL reagent (Amersham Biosciences).

MAPK and IκB Signaling Assays—MEFs were serum-starved overnight in Dulbecco’s modified Eagle’s medium and either supplemented with DMSO or the indicated γ-secretase inhibitors 2 h before stimulation with ligand (IL-1β 10 ng/ml) for the indicated times. Subsequently, cells were lysed and analyzed for activation of MAPK (p38 MAPK or JNK) or NF-κB by Western blotting.

ELISA—After incubation in low serum Dulbecco’s modified Eagle’s medium (2.5% fetal bovine serum), MEFs were incubated for 16 h with IL-1β (10 ng/ml) supplemented with 100 nM XIX, and the concentration of IL-6 in culture supernatants was determined with a murine-specific ELISA kit (R & D Systems and eBioscience) and normalized for total cellular protein. Each experiment was done in triplicate, and data are expressed as picograms/mg IL-6 (mean ± S.D.) for a representation of at least three independent experiments.

Immunocytochemistry—Cells were grown on polylysine-coated glass coverslips, and subsequent to experimental treatment, cells were fixed for 20 min at room temperature with 3.6% paraformaldehyde in PBS, washed twice for 5 min with PBS, and permeabilized in freezing methanol at −20 °C for 20 min. Coverslips were then washed twice for 5 min with PBS and blocked for 1 h at room temperature with 5% bovine serum albumin (Sigma) in PBS. Cells were subsequently incubated for 1 h at room temperature with primary antibody, anti-IL-1R1 (Santa Cruz Biotechnology; 1:200) in 5% bovine serum albumin (Sigma) in PBS. Cells were subsequently incubated for 1 h at room temperature with primary antibody, anti-IL-1R1 (Santa Cruz Biotechnology; 1:200) in 5% bovine serum albumin in PBS, washed three times for 5 min with PBS, and incubated with rhodamine-labeled anti-mouse secondary antibody (Molecular Probes, 1:200) and Hoescht (Molecular Probes; 1:1000) for 1 h at room temperature. Coverslips were then washed twice for 5 min with PBS, rinsed in water, and mounted in Mowiol mounting media (Calbiochem) and stored overnight at room temperature in the dark prior to analysis. Fluorescence images were captured using an Olympus IX71 inverted fluorescence microscope equipped with Spot-RT™ cooled digital camera (Diagnostic Instruments).

Densitometric Analysis—Autoradiograms (x-ray films) of the Western blots were analyzed by densitometry using a SynGene bio-imaging system and the corresponding software GeneTools (version 3.06). Experiments were done in triplicate. First, subtracting background levels normalized signal intensities on the films that contained anti-phospho-JNK and the corresponding anti-β-actin immunoreactive bands. Subsequently, anti-phospho-JNK immunoreactivity was normalized for anti-β-actin immunoreactivity and averaged. Value for control situation at t = 0 min was set at 1, and relative fold inductions compared with control values were calculated.

Statistics—All experiments were done multiple times independently and yielded similar results. All values represent mean ± S.D. for the number of wells or experiments indicated. Statistical analysis was assessed by Student’s t test. Level of significance was set at p < 0.05.

RESULTS

PS1 Contains a TRAF6-binding Site and Interacts with TRAF6 and IRAK2—A characteristic of several proteins involved in the regulation of inflammatory responses is the existence of discrete protein-protein interaction domains (19, 72) that allow for the recruitment and assembly of intracellular signaling pathways (73). TRAF6 interacts with p75NTR (74), IL-1R1 (75), B cell antigen CD40 (76), IRAKs (67, 75), p62 (77, 78), and RIP2 (79) through a homologous domain at the C-ter-
minal region of TRAF6, the TRAF domain. Previous studies have indicated that the TRAF6-binding consensus (PXEXXXAc/Ac, where Ar is any aromatic residue, and Ac is an acidic residue) is a protein-protein interaction motif (80, 81), mediating binding of several proteins to TRAF6 (80, 82, 83). Alignment of the TRAF6-binding sequences of CD40, RIP2, IRAK-M, IRAK1, IRAK2, Mal, PS1, and PS2 revealed a conserved consensus TRAF6-binding site (PEERGV) in PS1 (Fig. 1A). Alignment of PS1 and PS2 demonstrated that the consensus TRAF6-binding site was not as highly conserved in PS2 (EEERGV). Because this homologous region is conserved in PS1 and only partially conserved in PS2, it seems likely that the consensus TRAF6-binding site may mediate a specific PS1 function.

To examine this hypothesis, first we examined if PS1 or PS2 could interact with TRAF6. It is well documented that the presenilins are synthesized as holoproteins that are subsequently endoproteolyzed into NTF/CTF heterodimers (84–87). We therefore performed immunoprecipitation and Western blotting with PS1 and PS2 antibodies directed to each fragment. Subconfluent HEK293T cell cultures were transfected with expression constructs that directed the synthesis of PS1 or PS2 and cotransfected with FLAG-TRAF6 (Fig. 1B). Analyses of PS1 and PS2 immunoprecipitates revealed that TRAF6 clearly coprecipitated with PS1 and only weakly coprecipitated with PS2. In reciprocal analyses, immunoblotting with an antibody raised to the PS1-NTF (Chemicon 1563) revealed a prominent ∼45-kDa band corresponding to PS1 holoprotein but no band resembling PS1-NTF (∼30kDa). In contrast, immunoblotting with an antibody raised against the PS1-CTF (Scios 3.6.1) (70, 88) detected a prominent band resembling PS1 CTF (∼20 kDa) (Fig. 1C, lower panel), demonstrating that under these coprecipitation conditions PS1-CTF preferentially interacts with TRAF6. Analysis of TRAF6 immunoprecipitates with an antibody raised to PS2-CTF (Cell Signaling catalog number 2192) revealed an ∼50-kDa band corresponding to full-length PS2 and another band resembling PS2-CTF (∼20 kDa). From the data presented (Fig. 1B), it is clear that TRAF6 interacts with both PS1 and PS2 holoproteins and C-terminal fragments.

It has been shown previously that mutating consensus TRAF6-binding site(s) can disrupt the association between TRAF6 and some of its interacting partners (81). To determine whether the newly identified homologous TRAF6-binding domain shared between PS1 and other TRAF6-binding proteins (Fig. 1A) facilitates the interaction between PS1 and TRAF6, a mutant version of PS1 (PS1P374G/E376A) was generated in which amino acids, proline 374 and glutamate 376, were mutated by site-directed mutagenesis. Coimmunoprecipitation analysis demonstrated that the ability of PS1 to coprecipitate with TRAF6 was dramatically reduced when the TRAF6-binding consensus (PXEXXXAr/Ac) was mutated (Fig. 1C). In reciprocal immunoprecipitation experiments, PS1 wild type but not PS1P374G/E376A was found in immunoprecipitates prepared with an anti-FLAG antibody (Fig. 1C). Therefore, these data show a genuine TRAF6-binding site in PS1 that contributes to the association between TRAF6 and PS1.

IL1-R1 signaling involves the recruitment of TRAF6 which, in turn, initiates signaling cascades that result in activation of the stress-activated mitogen-activated protein kinases (MAPK), c-Jun N-terminal kinase (JNK) and p38, as well as transcription factor NF-κB (5, 89). We next examined whether endogenous PS1 could interact with TRAF6 in response to IL-1β ligand. Serum-starved subconfluent HEK293T cell cultures were either untreated or stimulated with recombinant IL-1β, after which cell extracts were immunoprecipitated with an anti-TRAF6 antibody, and the immunoprecipitates were analyzed by immunoblotting with an anti-PS1 NTF antibody. Interestingly, although little association was seen in unstimulated cells, the addition of IL-1β (Fig. 1D) reproducibly enhanced the association between endogenous TRAF6 and PS1. The rapid and transient response of HEK293T cells to IL-1β is not unique because the association of TRAF6 with p75NTR and IL1-R1 occurs within minutes after ligand treatment and reaches a maximum after 10 min (74). Collectively, these data suggest that PS1 contains a legitimate TRAF6-binding domain.

As TRAF6 can function as part of a larger multiprotein complex (5, 75, 76), we next examined whether other IL-1R/TLR adapter proteins, including IRAK2 or MyD88, could interact with PS1. HEK293T cell cultures were transfected with PS1 and cotransfected with IRAK2-Myc, AU1-MyD88, or FLAG-TRAF6 expression constructs. As anticipated, immunoprecipitation of PS1 quantitatively coprecipitated TRAF6 (Fig. 2A), but surprisingly PS1 also coprecipitated IRAK2 (Fig. 2A). The interaction between PS1 and IL-1R/TLR adapter proteins was selective as PS1 failed to coprecipitate MyD88 (Fig. 2A), Mal/TIRAP, or p62 (data not shown). Further analyses revealed that IRAK2 also interacted with full-length PS1 (Fig. 2B), PS1-CTF (Fig. 2C), PS2 holoprotein, and the FAD PS1 variant E280G (PS1 E280G) (Fig. 2B).

In contrast to TRAF6, where a highly conserved binding domain has been identified and extensively characterized (5, 80), to date no consensus IRAK-binding domain has been described. Therefore, to examine and further characterize the PS1 and IRAK2 interaction, we performed reciprocal coimmunoprecipitation experiments with either IRAK2-Myc or kIRAK2-Myc, a truncated version of IRAK2 that lacks the N-terminal intermediate and death domains and cotransfected with a PS1-expressing construct (67) (Fig. 2D). Immunoprecipitation of PS1 with an antibody raised against the PS1-NTF quantitatively coprecipitated IRAK2 but failed to coprecipitate significant amounts of kIRAK2 (Fig. 2D). In reciprocal immunoprecipitation experiments, PS1 holoprotein was predominantly found in immunoprecipitates of cells expressing IRAK2 but barely detected in cells expressing kIRAK2 (Fig. 2D, lower panel), indicating that the interaction between PS1-FL and IRAK2 most likely occurs outside the IRAK2 kinase domain and requires the IRAK2 intermediate or death domain. Because TRAF6 and IRAK2 interact with each other (67), and we have demonstrated that each interact with PS1 and PS2, we next determined if TRAF6 and IRAK2 competitively interacted with PS1. HEK293T cell cultures were transfected with IRAK2 and cotransfected with either PS1 wild type or PS1P374G/E376A as indicated (Fig. 2E). Immunoprecipitation of IRAK2 and subsequent analysis of coprecipitated PS1 indicated that disruption of the TRAF6-binding site and TRAF6 binding to PS1 had no apparent effect of the ability of PS1-FL to interact with IRAK2.
FIGURE 1. PS1 contains a TRAF6-binding domain and interacts with TRAF6. A, sequence alignment of amino acid sequences recognized as TRAF6-binding domains of human CD40, receptor interacting protein 2 (RIP2), interleukin-1 receptor-associated kinases (IRAK) 1 or 2, Mal, PS1, and PS2. Shading indicates amino acids that are identical in all proteins. Alignments were done with AlignX (Vector NTI) software. B, HEK293T cells were transfected with expression constructs encoding PS1 or PS2 and FLAG-TRAF6. C, HEK293T cells were transfected with expression constructs encoding wild type PS1 or PS1P374G/E376A and FLAG-TRAF6. Cell extracts were prepared, normalized to ensure equal protein levels in all samples, and immunoprecipitated (IP) with protein G-beads (designated C) or an anti-FLAG monoclonal antibody. In reciprocal coimmunoprecipitation experiments, lysates were immunoprecipitated (IP) with protein G-beads (designated C) or monoclonal antibody to PS1 or PS2. Coprecipitating TRAF6, PS1, or PS2 was detected by immunoblotting (IB) with anti-FLAG, anti-PS1 and anti-PS2 monoclonal antibodies, respectively. D, HEK293T cell cultures were unstimulated or treated with IL-1β (10 ng/ml) for increasing times (0, 5, 10, and 15 min), after which cell lysates were immunoprecipitated with an anti-TRAF6 antibody. Coprecipitated endogenous PS1 and immunoprecipitated TRAF6 were detected by immunoblot analysis with an anti-PS1 NTF or anti-TRAF6 monoclonal antibody. WB, Western blot.
FIGURE 2. Biochemical characterization of the interaction between presenilins and IRAK2. A, HEK293T cell cultures were transiently transfected with PS1 and cotransfected with Myc-IRAK2, AU1-MyD88, or FLAG-TRAF6 expression vectors, as indicated. B, HEK293T cell cultures were transfected with wild type PS1 (PS1-WT), FAD PS1 variant E280G (PS1-E280G), or PS2 and cotransfected with Myc-IRAK2 expression vectors as indicated. C, HEK293T cell cultures were transfected with PS1 alone or cotransfected with Myc-IRAK2 as indicated. D, HEK293T cell cultures were transfected with PS1 and cotransfected with IRAK2-Myc or kIRAK2-Myc expression vectors. E, HEK293T cell cultures were transiently transfected with expression constructs encoding Myc-IRAK2 and cotransfected with PS1 or PS1P374G/E376A and expression vectors as indicated. Cell extracts were prepared 24–36 h post-transfection extracts, normalized to ensure equal protein levels in all samples, and immunoprecipitated (IP) with a control (designated C) or monoclonal antibody to PS1 (PS1) or Myc (IRAK2) as indicated. Coprecipitating IRAK2, TRAF6 or MyD88 were analyzed by immunoblotting (IB) with anti-Myc, anti-FLAG, or anti-AU1 antibodies. Coprecipitating PS2, PS1, or PS1P374G/E376A was detected by immunoblotting with anti-PS2 or anti-PS1(CTF or NTF) monoclonal antibodies. Immunoblot analysis of transfected cell lysates with the indicated antibodies confirmed expression of all constructs and equivalency of protein expression. WB, Western blot.
Regulated Intramembrane Proteolysis of IL-1R1

A HEK293T cells were transiently transfected with IL-1R1 and cotransfected with IRAK2-Myc, AU1-MyD88, or FLAG-TRAF6 expression vectors, as indicated. Parallel cultures were stimulated for 2 h with the phorbol ester PMA (200 ng/ml). Thirty-six hours post-transfection, cell lysates were prepared, normalized to ensure equal protein loading. Immunoblot analysis of transfected cell lysates with the indicated antibodies confirmed expression of all constructs, and equivalent β-actin expression levels are also indicated (lower panel) to demonstrate equivalency of protein loading. Immunoblot of cell lysates from HEK293T cells expressing IL-1R1 and FLAG-TRAF6 as indicated. Eight hours prior to lysis, cell cultures were serum-starved and treated with DMSO as a control or the indicated inhibitor for 4 h, as indicated. The anti-IL-1R1, C-terminal specific antibody was used to detect full-length (FL) proteins and their C-terminal fragments in the immunoblot. C Immunoblot analysis of soluble IL-1R1 from trichloroacetic acid-precipitated culture media from HEK293T cells transfected, and treated as in B. The anti-IL-1R1, N-terminal specific antibody was used to detect soluble IL-1R1 in the immunoblot. WB, Western blot.

B Allogeneic, these data suggest that PS1 and PS2 are novel binding partners for IRAK2 and that PS1 can independently interact with TRAF6 and IRAK2. The adapter proteins MyD88, TRAF6, and IRAK2 were first characterized as important mediators of IL-1R1 signaling events (67), and an interaction between these adapter proteins and PS1 is further suggestive of a role for presenilins in the regulation of immune responses. Taken with the reported γ-secretase cleavage of IL-1R2 (32), another way in which PS1 may be involved in IL-1R1 signaling is through proteolysis of IL-1R1. RIP of many cell surface receptors is promoted by treatment with phorbol esters, such as PMA (57, 90). To test this idea, we first determined whether stimulation with PMA or expression of the adapter proteins TRAF6, IRAK2, or MyD88 had any effect on the proteolytic cleavage pattern of IL-1R1. HEK293T cells were stimulated with PMA, transiently transfected with IL-1R1 alone, or cotransfected FLAG-TRAF6, IRAK2-Myc, or AU1-MyD88 expression vectors as indicated (Fig. 3A, long and short exposure). Similar to many other reported γ-secretase substrates, subsequent to PMA stimulation we observed an increase in detection of anti-IL-1R1 immunoreactive fragments (Fig. 3A, 3rd lane, long exposure). Interestingly, Western blot analysis of cell lysates from cells coexpressing IL-1R1 and IRAK2 or TRAF6 revealed that IRAK2 expression noticeably increased IL-1R1 fragment generation, but TRAF6 expression dramatically increased the generation of anti-IL-1R1 fragments (Fig. 3A, 5th and 6th lanes, long exposure). In contrast, no significant increase in IL-1R1 immunoreactive fragments was observed in cell cultures coexpressing MyD88 with IL-1R1 (Fig. 3A, compare 2nd with 4th lane). TRAF6-induced proteolysis of IL-1R1 was so dramatic and robust compared with PMA-treated cultures that detection of any synergistic proteolysis could not be determined.

These data suggest that the exogenous expression of TRAF6 may function to promote or mediate proteolysis of IL-1R1. To determine whether TRAF6-induced cleavage of IL-1R1 resulted in the production of a soluble IL-1R1 ectodomain and a subsequent substrate for R1NP, HEK293T cells were transiently transfected with IL-1R1 and cotransfected with FLAG-TRAF6 as indicated (Fig. 3B). The cell lysates (Fig. 3B) and conditioned media (Fig. 3C) from the same experiment were analyzed by SDS-PAGE and immunoblotting with anti-IL-1R1 C-terminal domain or anti-IL-1R1 N-terminal domain-specific antibodies. It was again evident that coexpression of IL-1R1 with TRAF6 leads to a dramatically increased in the detection of IL-1R1 immunoreactive cytosolic fragments (Fig. 3B) and soluble IL-1R1 ectodomain (Fig. 3C). Notably, in cell cultures treated with the γ-secretase inhibitor XIX (68), the intracellular lower molecular weight fragment disappeared (Fig. 3B, 4th lane) with minimal effect on TRAF6-induced production of soluble IL-1R1 ectodomain (Fig. 3C). These data demonstrate that exogenous expression of TRAF6 induces ectodomain shedding of IL-1R1, most likely resulting from oligomerization of TRAF6 and IL-1R1, and generation of an IL-1R1 C-terminal domain (CTF) that is subsequently cleaved by γ-secretase to generate an IL-1R1 ICD. This responsiveness to PMA (Fig. 3A) and a γ-secretase inhibitor (Fig. 3B) strengthens our hypothesis that IL1-R1 may be a novel γ-secretase substrate.

Metalloprotease-mediated Ectodomain Shedding of IL-1R1— IL-1R1 is predominantly a membrane-bound protein, but it can
also exist as a soluble receptor and modulate the biological signaling potential of IL-1 (91–93). To more closely examine and characterize the potential RIP of IL-1R1, we first examined the constitutive and PMA-stimulated release of the soluble IL-1R1 ectodomain into cell-tissue culture supernatant. Constitutively released IL-1R1 ectodomain was detected in the control medium of HEK293T cells transiently transfected with a plasmid encoding human IL-1R1 and was greatly increased by PMA treatment resulting in the detection of soluble IL-1R1 fragments (Fig. 4A). The most prominent fragment at about 60 kDa, which is also visible under nonstimulated conditions, most likely corresponds to mature and fully glycosylated soluble IL-1R1, with the approximate 47-kDa fragment corresponding to unglycosylated soluble IL-1R1 (13, 94). Using the same cultures, the cells were harvested, and detergent-soluble proteins were separated by SDS-PAGE and immunoblotted with anti-IL-1R1 antiserum that recognizes the receptor cytoplasmic domain. Consistent with the detection of PMA-stimulated soluble IL-1R1 in culture media, PMA caused the increased appearance of two predominant anti-IL-1R1 reactive C-terminal domains (CTF) of ∼32 and 26 kDa (Fig. 4B). Notably, in the absence of PMA, the detection of the 32- and 26-kDa anti-IL-1R1-reactive fragments was evident, although reduced, suggestive of constitutive receptor ectodomain shedding and intracellular fragment generation. Stimulation of IL-1R1 ectodomain shedding by PMA is consistent with functionality of metalloproteases of the ADAM family (22, 95). Therefore, we examined the effect of pharmacological inhibition of metalloproteases on PMA-stimulated IL-1R1 cleavage. Generation of PMA-stimulated soluble IL-1R1 ectodomain fragments was inhibited by TAPI, a potent but broad spectrum inhibitor of ADAMs (Fig. 4C) (96). Importantly, the increase in intracellular IL-1R1 CTF generated in the presence of PMA could be inhibited by pretreatment with TAPI, indicating that like other characterized γ-secretase substrates ectodomain shedding is important for generation of the intracellular IL-1R1 CTF (Fig. 4D) (32, 52, 97).

**Presenilin-dependent γ-Secretase Cleavage of IL-1R1—**
Ectodomain shedding of IL-1R1 and subsequent formation of intracellular fragments are reminiscent of γ-secretase-mediated RIP (33, 52, 57). In this proteolytic event, cleavage of type I membrane proteins by γ-secretase is preceded by an initial cleavage event outside of the membrane, which results in the shedding of the large ectodomain, production of a membrane-anchored CTF, and subsequent cleavage of the CTF by γ-secretase to produce a soluble ICD (27, 31, 33, 47, 98). We next addressed the possibility that the IL-1R1 CTF, derived from PMA-induced ectodomain shedding, is subject to γ-secretase cleavage. Independent studies have shown that synthetic γ-secretase inhibitors enhance the accumulation of substrate CTFs by inhibiting γ-secretase-mediated turnover and generation of ICDs (29, 52). HEK293T cells were transiently transfected with empty vector (1st lane) or a plasmid encoding human IL-1R1, and cell cultures were subsequently treated with either PMA, the γ-secretase inhibitor, XIX (68), or both (Fig. 5A). The cell lysates were analyzed by SDS-PAGE and immunoblotting with the anti-IL-1R1 antibody directed against the intracellular domain of IL-1R1. PMA-induced accumulation of two prominent anti-IL-1R1-reactive fragments (Fig. 5A, 3rd lane). In cell cultures treated with XIX, the fragment that migrated at an apparent mass of ∼26 kDa disappeared (Fig. 5A, compare 2nd and 4th lanes or 3rd and 5th lanes). This responsiveness to PMA and γ-secretase inhibitor is consistent with the ∼32-kDa band being the predicted IL-1R1 CTF, whereas the ∼26-kDa band is the predicted γ-secretase-mediated intramembrane proteolytic fragment of IL-1R1.
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FIGURE 5. Ectodomain shedding-derived IL-1R1 C-terminal domains (CTF) are substrates for presenilin-dependent γ-secretase cleavage. A, immunoblot of cell lysates from HEK293T cells expressing empty vector or human IL-1R1. Cells were treated with either DMSO as a control or the γ-secretase inhibitor XIX (30 nM), alone or in combination with PMA (200 ng/ml) for 2 h as indicated. The anti-IL-1R1, C-terminal specific antibody was used to detect full-length (FL) proteins and their C-terminal fragments in the immunoblots. Immunoblot analysis of cell lysates for β-actin expression levels is also indicated (lower panel) to demonstrate equivalency of protein loading. B, immunoblot of cell lysates from HEK293T cells expressing p75NTR. Cells were treated with DMSO as a control or the γ-secretase inhibitor XIX, alone or in combination with PMA (200 ng/ml) for 2 h to induce ectodomain shedding, as indicated. The anti-p75NTR, C-terminal specific antibody was used to detect full-length (FL) proteins and their C-terminal fragments in the immunoblots. C, immunoblot of cell lysates from HEK293T cells expressing human IL-1R1 and coexpressing PS1 WT or dominant negative PS1 mutant (PS1 D257A/D385A). D, immunoblot of cell lysates from HEK293T cells expressing human IL-1R1 and coexpressing PS1 WT or dominant negative PS1 mutant (PS1 D257A/D385A) or PS1 mutant (PS1 P374G/E376A) expression vectors, as indicated. Cells were treated with either DMSO as a control or the γ-secretase inhibitor XIX (30 nM), alone or in combination with PMA (200 ng/ml) for 2 h prior to cell lysis as indicated. The anti-IL-1R1, C-terminal specific antibody was used to detect full-length (FL) proteins and their C-terminal fragments in the immunoblots. β-Actin, PS1 WT, and PS1 D257A/D385A expression levels are also indicated (lower panel). E, immunoblot of cell lysates from mouse embryonic fibroblasts from wild type (MEF WT) or presenilin-deficient (PS KO) mice transiently transfected with human IL-1R1. Cells were pretreated with DMSO as a control or the γ-secretase inhibitor XIX (30 nM) for 4 h and subsequently treated with PMA (200 ng/ml) for 2 h to induce ectodomain shedding, as indicated. An IL-1R1 C-terminal specific antibody was used to detect full-length (FL) proteins and their C-terminal fragments in the immunoblots. β-Actin expression levels are also indicated (lower panel) to verify equivalency of protein loading. F, immunoblot of cell lysates from wild type (PS WT) and presenilin-deficient (PS KO) MEF cell lines. The anti-PS1 C-terminal specific antibody was used to detect endogenous PS1-CTF. Immunoblot with anti-β-actin antibody confirmed equivalency of protein loading. WB, Western blot.

dependent ICD. Similar responsiveness to PMA and the γ-secretase inhibitor was seen for p75NTR, a well characterized γ-secretase substrate (Fig. 5B) (18, 57, 90, 99).

Presenilins are necessary and required for γ-secretase activity (37). We next analyzed the cleavage of IL-1R1 in HEK293T cells expressing human IL-1R1 and coexpressing either wild type or a dominant negative PS1 (PS1 D257A/D385A), which abolishes PS1-dependent γ-secretase activity (38). Immunoblot analysis of IL-1R1 cleavage in response to PMA revealed that in cells expressing dominant negative PS1, the constitutive and PMA-stimulated generation of IL-1R1 ICD was reduced as compared with cells expressing comparable levels of wild type PS1 (Fig. 5C). In contrast, by employing the PS1 mutant (PS1 P374G/E376A) that reduces the association between PS1 and TRAF6, we demonstrate that the interaction between PS1 and TRAF6 may not be absolutely necessary for IL-1R1 processing (Fig. 5D, 7th and 8th lanes). Together, these data indicate that catalytically active PS1 is required for generation of IL-1R1 ICD. IL-1R1 and other family members are found at low levels on the cell surface of primary cell types (15), precluding detection and examination of endogenous receptor proteolysis. Therefore, to analyze further the cleavage of human IL-1R1 and requirement for presenilins-dependent γ-secretase activity, we used mouse embryonic fibroblasts (MEFs) lacking both PS1 and PS2 (PS KO) (Fig. 5F) (37). In wild type MEFs overexpressing human IL-1R1, PMA-stimulation increased CTF and ICD generation (Fig. 5E, 2nd lane), whereas the γ-secretase inhibitor, XIX (68), selectively inhibited PMA-induced production of the IL-1R1 ICD (Fig. 5E, 4th lane). In contrast, and consistent with the requirement for presenilins for γ-secretase substrate cleavage, generation of IL-1R1 ICD was not observed in PS-deficient MEFs (PS KO) overexpressing human IL-1R1 (Fig. 5E, 5th to 8th lanes). Furthermore, and consistent with a deficiency in γ-secretase activity, IL-1R1 CTF accumulated to higher levels in PS-deficient cells when compared with wild type MEFs (Fig. 5E, compare 1st with 5th lane). Our results collectively demonstrate that the IL-1R1 is a substrate for γ-secretase-mediated receptor intramembrane proteolysis.

Induction of IL-1R1 Cleavage by Ligand Binding—Thus far we have shown that generation of IL-1R1 CTF and ICD are dependent on metalloprotease and γ-secretase activities. Next we determined the physiological conditions when IL-1R1
Regulated Intramembrane Proteolysis of IL-1R1

Inhibition of γ-secretase activity alters IL-1β-mediated responsiveness and IL-6 secretion. A, immunoblot of cell lysates from HEK293T cells expressing human IL-1R1. Cells were pretreated with the γ-secretase inhibitor XIX (30 nM) for 2 h after which the cells cultures were cultured with IL-1β for the indicated times. The anti-IL-1R1 C-terminal specific antibody was used to detect full-length (FL) proteins and their C-terminal fragments in the immunoblot. Vertical line on the blot indicates that the samples were analyzed on the same gel but not adjacent to each other. Wild type MEFs were pretreated with γ-secretase inhibitors XIX (B) or DAPT (C) for 2 h and subsequently stimulated with IL-1β for the indicated times. JNK protein levels and phosphorylation status were detected with anti-JNK and anti-phospho-JNK antibodies. P- indicates phosphorylated. As a control, treatment did not affect β-actin levels (lower panel). D, densitometry scanning and quantification of phosphorylated JNK normalized to total β-actin. Wild type MEFs were pretreated with γ-secretase inhibitors XIX (E) or DAPT (F) for 2 h and subsequently stimulated with IL-1β for the indicated times. IL-1β-induced IkB phosphorylation and proteolytic degradation were detected with anti-IkB and anti-phospho-IkB antibodies. G, ELISA of IL-1β-stimulated IL-6 production in supernatants of MEFs untreated or pretreated with pharmacological inhibitors of γ-secretase activity, XIX, or DAPT overnight (16 h). Data are means ± S.D. of three independent experiments in duplicate. *, p < 0.05, versus control.

undergoes receptor intramembrane proteolysis, and we examined whether generation of IL-1R1 CTF and ICD is stimulated by the IL-1R1 physiological ligand IL-1β. HEK293T cells were transiently transfected with a plasmid encoding human IL-1R1, and cell cultures were subsequently stimulated with IL-1β for increasing times (Fig. 6A). The cell lysates were analyzed by SDS-PAGE and immunoblotting with the anti-IL-1R1 antibody. IL-1β induced a time-dependent accumulation of IL-1R1 ICD. In contrast, cell cultures cotreated with the γ-secretase inhibitor XIX completely inhibited IL-1β-induced generation of IL-1R1 ICD (Fig. 6A). Although IL-1β-induced IL-1R1 CTF and ICD generation was clearly detected (Fig. 6A), it was much less than that induced by PMA and therefore precluded detection of IL-1β-induced generation of soluble IL-1R1 (data not shown). Collectively, these data are consistent with a role for γ-secretase-mediated proteolytic cleavage of IL-1R1 in IL-1β-mediated signaling events.

RIP has been reported to initiate intracellular signaling events whereby the ICD of specific receptors can either perform non-nuclear signaling functions (34, 35, 57) or translocate to the nucleus and regulate the transcription of target genes (29, 33, 52–56). To examine the ability of IL-1R1-ICD to translocate
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to the nucleus, we analyzed wild type and PS-deficient MEF cells transiently transfected with a cDNA encoding IL-1R1 (supplemental Fig. 1). Cell cultures were subsequently treated with either PMA, the γ-secretase inhibitor XIX, or both as indicated. The expression pattern of IL-1R1 was confirmed by Western blot analysis (Fig. 5D), and cell cultures were analyzed by fluorescence microscopy with the anti-IL-1R1 antibody raised against the intracellular domain of IL-1R1. Fluorescence imaging revealed that IL-1R1 accumulated predominantly in the cytoplasmic region. Likewise, following stimulation with PMA, the γ-secretase inhibitor XIX, or both, IL-1R1 retained a predominantly diffuse cytoplasmic staining profile (supplemental Fig. 1). These data suggest a non-nuclear function for IL-1R1 ICD.

Pharmacological Inhibition of γ-Secretase Negatively Regulates IL-1β Responsiveness—It has been shown that γ-secretase cleavage of Notch, epidermal growth factor receptor, and many other substrates is important for ligand-mediated signaling events (100, 101). It is well established that following IL-1β stimulation, recruitment and activation of IRAKs and TRAF6 result in activation of both the MAPK and NF-κB pathways (75, 102). Having performed an analysis of γ-secretase cleavage of IL-1R1, we next determined whether inhibition of γ-secretase activity antagonized IL-1β-stimulated activation of MAPK and NF-κB. Immortalized MEFs were pretreated with the γ-secretase inhibitors XIX (Fig. 6B) or DAPT (Fig. 6C) for 2 h and subsequently stimulated with IL-1β for increasing times (0–60 min). Activation of JNK was detected by immunoblotting with a phospho-specific JNK antibody. As anticipated, stimulation of MEF cell cultures with IL-1β induced a time-dependent, transitory activation of JNK (Fig. 6, B–D) and p38 activity (data not shown). In contrast, we found that pretreatment of cultures with either γ-secretase inhibitor XIX or DAPT abrogated the responsiveness of MEFs to IL-1β-induced JNK activation (Fig. 6, B–D). Phosphorylation of JNK was on average 4-fold over base line in IL-1β-stimulated cells (Fig. 6D). In contrast, IL-1β-stimulated JNK was only 2-fold over base line in cultures pretreated with a γ-secretase inhibitor (Fig. 6D). In the classical NF-κB activation pathway, NF-κB dimers are held in the inactive state by a family of inhibitors called IkB. IL-1β stimulation leads to phosphorylation and degradation of IkB, which thereby enables NF-κB dimers to translocate to the nucleus, bind DNA, and activate transcription. Next, the effect of inhibition of γ-secretase activity on IL-1β-stimulated NF-κB activation was examined by measuring IkB phosphorylation and protein degradation profiles (Fig. 6, E and F). In contrast to JNK activity, inhibition of γ-secretase activity did not significantly affect IL-1β-stimulated IkB phosphorylation or IkB degradation (Fig. 6, E and F). To assess the effects of inhibitors of γ-secretase on IL-1β-stimulated cytokine production, we also measured the production of IL-6 in MEFs after IL-1β stimulation (Fig. 6G). Pretreatment with either γ-secretase inhibitor, XIX or DAPT, prior to stimulation with IL-1β antagonized IL-6 production. Taken together, these data provide additional supporting evidence that presenilin and γ-secretase activity are directly involved in IL-1β-induced responsiveness and IL-1R1 signaling.

DISCUSSION

A strong link between presenilins and the immune system has been reported from phenotypic characterization of several in vivo models of presenilin biology and identification of γ-secretase substrates. The phenotype of PS1+/−/PS2+/− “partial deficient” mice revealed a novel in vivo function for presenilins in the immune system (63), which has been substantiated in T cell-specific (51) and B cell-specific (50) presenilin-deficient animals. The PS1+/−/PS2+/− animals are relatively normal up to about 6 months of age, after which the majority of the mice develop a systemic lupus erythematosus-like autoimmune disease (103). Ablation of presenilin in T cells results in inefficient generation of CD4+ T cells, a phenotype that correlates with evidence of impaired T cell receptor signaling (51), whereas selective loss of presenilins in B cells compromises responsiveness to lipopolysaccharide and B cell antigen receptor-induced proliferation and signal transduction events (50). Additionally, HLA-A2, a major histocompatibility complex class I protein and IL-1R type 2 are reportedly γ-secretase substrates (32, 104). In this regard, our observations that PS1 contains a highly conserved TRAF6-binding domain, interacts with the adapter proteins TRAF6 and IRAK2, and that IL-1R1 is a γ-secretase substrate further substantiates the importance of presenilins in regulation of the immune system.

First, the data obtained in our study have demonstrated that the presenilins are novel binding partner for the IL-1R1 adapter proteins TRAF6 and IRAK2. TRAF6 possesses an N-terminal RING domain that confers its ubiquitin–protein isopeptide ligase activity, and a C-terminal TRAF-domain that enables oligomerization and recruitment of TRAF6 to the interleukin and toll-like receptors (5). Additionally, TRAF6 has been reported to interact with numerous proteins through a highly conserved TRAF6-binding domain (PXEXXXAc/Ar) (80, 82, 83). IRAK2, on the other hand, is a serine/threonine pseudokinase and an important modulator of TRAF6 activity and function (105). In our studies we found the following: (i) PS1 and PS2 interact with exogenously expressed IRAK2; (ii) PS1 contains a conserved TRAF6-binding domain (PEERGY); (iii) TRAF6 interacts with both functionally inactive PS1 holoprotein and mature PS1 heterodimer; and (iv) endogenous TRAF6 interacts with endogenous PS1 in response to IL-1β ligand stimulation, confirming the possible physiological relevance of the interaction.

The interaction between TRAF6 and PS1 was mapped to a highly conserved TRAF6-binding domain, present in the hydrophilic domain of PS1. Though PS2 contains only a partial consensus TRAF6-binding motif, it still interacts with TRAF6. As it is well documented that TRAF6 and IRAK2 associate and function in the same multiprotein complexes (5, 67), we speculate that the PS2 that did precipitate with exogenously expressed TRAF6 was probably mediated by endogenous IRAK2. In this regard, under various experimental conditions we were unsuccessful in our attempts to coimmunoprecipitate endogenous presenilins with exogenously expressed or endogenous IRAK2 (data not shown). This may be due to the fact that IRAK2 preferentially coprecipitated with PS1 and PS2 holoproteins, which have a short half-life and are difficult to detect in
endogenous conditions (86). Furthermore, by employing the FAD PS1 variant PS1ΔE9, and the catalytically inactive PS1 D257A/D385A mutants, we found that IRAK2 and TRAF6 also associate with the endoproteolytically defective forms of PS1 (data not shown), suggesting that PS1 endoproteolysis is not required for the interaction between PS1 and TRAF6 or IRAK2, thus showing that these proteins possibly interact prior to and independent of assembly of the active γ-secretase complex.

The precise physiological roles of the PS1-TRAF6 and PS1-IRAK2 interactions are at present unknown, although we can speculate to a number of potential functions. First, the association of PS1 with TRAF6, a ubiquitin–protein isopeptide ligase (102, 106–108), and its modulator IRAK2 (105) is consistent with studies reporting the ubiquitination of PS1 and suggests that, like Sel-10 (109), TRAF6 might ubiquitinate and affect presenilin protein stability and degradation. Second, the association may identify PS1 as a chaperone or scaffold important for the regulation and function of TRAF6 and/or IRAK2. Third, as TRAF6 and IRAK2 are common adapter proteins shared by many cytokine and growth factor receptors other than IL-1R1 (5), our reported association between PS1 and TRAF6 in response to ligand stimulation suggests that the interaction might play an important role in regulating other cytokine and growth factor signaling pathways.

Both TRAF6 and IRAK2 are proximal and essential mediators of IL-1β-mediated signaling events that include activation of MAPKs and the NF-κB transcription factor (110). The MAPKs include three subfamilies as follows: extracellular signal-regulated kinase (ERK), SAPK/JNK, and p38 MAPK (111). Each pathway consists of several unique and overlapping upstream kinases that can be activated by a variety of distinct cellular stresses that include proinflammatory cytokines such as TNF-α or IL-1β (111) When activated, SAPK/JNK can phosphorylate and activate c-Jun or other transcription factors, including ATF-2 and Elk-1 (112). In this study we have demonstrated that inhibition of γ-secretase activity antagonizes IL-1β-induced JNK activation. These observations are in keeping with previous independent reports, which have demonstrated that PS1 expression and γ-secretase activity are required for activation of the SAPK/JNK pathway (65) and further endorse a role for presenilins in cytokine and growth factor signaling.

Second, we identify IL-1R1 as a novel substrate for metalloproteolytic- and γ-secretase-mediated RIP. We demonstrate that γ-secretase is capable of catalyzing the proteolytic cleavage of membrane-anchored fragments of ectodomain-shed IL-1R1 to generate the IL-1R1-ICD. The γ-secretase complex is responsible for the proteolytic cleavage of at least 50 substrates (34, 39, 113). Limited homology among the putative cleavage sites of known γ-secretase substrates demonstrates that the intramembrane cleavage event is not dependent on strict amino acid sequence specificity. This broad substrate spectrum strongly suggests that additional members of the IL-1R receptor superfamily and other cytokine receptors are putative candidate substrates for RIP. Consistent with this, the IL-1R2 was reported to undergo RIP by α-, β-, and γ-secretase (32). Additionally it was reported that homologues of signal peptide peptidases (SPPL2a and SPPL2b) catalyze the intramembrane proteolysis of TNFα to generate a TNFα ICD, which in turn induces expression of the pro-inflammatory cytokine IL-12 in human dendritic cells (114).

IL-1R1 and IL-1R2 exist in both membrane-bound and soluble forms (91). Soluble IL-1 receptors (sIL-1R) are naturally occurring regulatory proteins that influence the biological activities of IL-1 (92, 93). Soluble recombinant forms of IL-1R1 (sIL-1R1) have been developed, and administration of sIL-1R1 in vivo improves the survival of heart allografts (115), protects from experimental autoimmune diabetes (116), and improves the severity of active arthritis (117, 118). Although it has been shown that γ-secretase cleavage of the notch or ErbB-4 receptors occurs in the presence of their corresponding ligands (101, 119), ligand-induced cleavage has not been shown for other receptors such as the growth hormone receptor (120). Here we demonstrate that IL-1β stimulation can induce ectodomain shedding (as evident by increased IL-1R1 CTF generation) and subsequent γ-secretase cleavage of IL-1R1, perhaps pointing to a general mechanism of ligand-mediated RIP.

The proinflammatory IL-1β cytokine exerts its biological effects by binding IL-1R1 on the plasma membrane and initiating the assembly of a well-characterized intracellular receptor-associated protein complex accountable for transducing IL-1β cytokine signals (1, 4, 121). Formation of an active IL-1R1 signaling complex involves the stepwise recruitment of several accessory proteins to the receptor, including IL-1RACp, MyD88, Tollip, and IRAKs. IL-1β cytokine-mediated receptor endocytosis has also been regarded as a mechanism for regulation of IL-1R1-mediated signaling at the plasma membrane. However, recent independent studies have provided evidence suggesting that IL-1R1 endocytosis has an important role in the activation and amplification of IL-1β-initiated receptor signaling events (15, 122). These studies have elucidated a series of signaling events, subsequent to ligand binding that regulates IL-1R1 ubiquitination and endocytosis. Following Tollip-dependent receptor ubiquitination and endocytosis, it is proposed that TRAF6 is recruited to the MyD88/IL-1R1 complex in the endosomal compartment and not plasma membrane as proposed previously. Although determination of the precise sequence of events and underlying molecular mechanisms will require further investigation, our results suggest a more complex role for IL-1R1 and its adapter proteins (TRAF6 and IRAK2) in cell signaling (Fig. 7). In our model we demonstrate that following ligand binding or exogenous expression of TRAF6, a metalloprotease-mediated cleavage event within the extracellular domain of IL-1R1 liberates soluble IL-1R1 and generates a membrane-bound IL-1R1 CTF (Fig. 7B). Subsequent to ectodomain shedding, intracellular cleavage of IL-1R1 CTF by presenilin-dependent γ-secretase releases the intracellular soluble fragment, IL-1R1 ICD (Fig. 7C). Considering that it has been recently reported that RIP of the p75 neurotrophin receptor (18), EphB2 receptor (123), and Notch (124) occurs subsequent to endocytosis on the endosome, determining whether γ-secretase-dependent proteolysis of IL-1R1 CTF occurs at the plasma membrane or endosome requires further experimentation. Highlighting
the physiological importance of this model, we show that pharmacological inhibitors of γ-secretase activity inhibit generation of IL-1R1-ICD and antagonize IL-1β-induced JNK activation. It is noteworthy that despite the induction of IL-1R1 ectodomain shedding and subsequent γ-secretase cleavage of IL-1R1 following overexpression of TRAF6, disruption of the interaction between PS1 and TRAF6 has no obvious effect on IL-1R1 proteolysis (Fig. 5D). This suggests that the interaction of PS1 with TRAF6 may be functionally redundant in the RIP of IL-1R1, but not so in the proteolysis of other cytokine or growth factor receptors that require TRAF6 and IRAK2 to transduce a physiological signal. This hypothesis is supported by the fact that disrupting the interaction between PS1 and β-catenin alters RIP of N-cadherin but not other γ-secretase substrates (70, 125). Alternatively, it suggests that the function of PS1-TRAF6 and PS1-IRAK2 interactions are independent of assembly of the γ-secretase complex and IL-1R1 proteolysis, a theory supported by our observation that TRAF6 and IRAK2 interact with endoproteolytically defective PS1ΔE9 and PS1D257A/D385A mutants (data not shown).

Finally, in this study we also show that presenilin-dependent γ-secretase activity can regulate IL-1R1 signaling through the proteolysis of membrane-anchored CTF species and generation of a cytosolic ICD. Inhibition of IL-1R1 proteolysis attenuates activation of JNK, a finding that is consistent with previous reports (65). We also found that inhibition of γ-secretase activity also antagonized IL-6 cytokine secretion, suggesting that presenilins and γ-secretase have a role in the regulation of cytokine responsiveness. The release of the IL-1R1 ectodomain and subsequent generation of CTF and ICD may affect cell responsiveness through multiple mechanisms. First, to reduce IL-1 toxicity, the liberated ectodomain of the receptor may compete with cell surface IL-1R1 for ligand binding and act as a competitive inhibitor of endogenous ligand-receptor interactions. Second, γ-secretase-mediated cleavage of IL-1R1 may also be a mechanism to disrupt or prevent overactivation of IL-1-responsive cells. Third, the cleavage of IL-1R1 and generation of soluble ICD may suggest a novel function for IL-1R1 in cytosolic signaling, as the IL-1R1 ICD has several potential nuclear localization signals (126), and IL-1R1 has been reported previously to localize to the nucleus (126). Furthermore, independent reports have demonstrated that endogenous IRAK1 and TAB2, which are adapter and/or signaling proteins for IL-1R1, can localize to the nucleus (127). Moreover, subsequent to γ-secretase cleavage of ErbB4, ErbB4 ICD associates with TAB2, translocates to the nucleus, and interacts with nuclear proteins (128). Future studies will no doubt determine whether γ-secretase or IL-1R1 ICD generation is important for the reported nuclear translocation of IRAK and TAB2. In our studies, although we can detect a moderate increase in nuclear IL-1R1 immunoreactivity in PMA-stimulated cells, which is antagonized by inhibition of γ-secretase activity or presenilin deficiency (supplemental Fig. 1), when coupled with low detectable levels of endogenous IL-1R1 and difficulty in distinguishing between full-length and γ-secretase-generated ICD, further analysis is warranted before definitive conclusions can be reached. Likewise, because of the complexity of activation and redundant antagonist mechanisms that regulate IL-1 responsiveness, and as both IL-1R1 and IL-1R2 are γ-secretase substrates, more experiments are required to elucidate precisely.
how γ-secretase is involved in IL-1R1 and IL-1R2 cleavage and subsequent IL-1α/β signaling.

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