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Sequential Proteolytic Processing of an Interferon-Alpha Receptor Subunit by TNF-Alpha Converting Enzyme and Presenilins

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It is well established that interferons trigger tyrosine-kinase-dependent signaling via JAK kinases and STAT transcription factors. However, we have observed both IFNaR2 receptor cleavage and functional activity of the liberated intracellular domain (ICD), suggesting that interferon-alpha (IFN-alpha) can also signal via regulated intramembrane proteolysis (RIP), an evolutionarily conserved mechanism of receptor-mediated signaling. Sequential cleavage of the receptor ectodomain and transmembrane domain is a hallmark of the most common class of RIP. To investigate the mechanisms of IFNaR2 RIP signaling, we examined IFNaR2 cleavage by TNF-alpha converting enzyme (TACE) and presenilin proteases. We tracked the fate of epitope-tagged and fusion variants of IFNaR2 in cells expressing wild-type, mutant, or null versions of TACE and presenilins 1 and 2. Cleavage and subcellular location were determined by immunoblot, fluoresence microscopy, and reporter assays. We found that both TACE and presenilin 1/2 cleave IFNaR2, in a sequential manner that allows the ICD to move to the nucleus. TACE cleavage was induced by IFN-alpha but was not consistently required for the antiproliferative effects of IFN-alpha. In conclusion, IFNaR2 is cleaved by TACE and Presenilin 1/2, suggesting that interferons signal by both kinase and RIP-mediated pathways.

Introduction

Regulated intramembrane proteolysis (RIP) (Brown and others 2000) is a simple, highly conserved signaling mechanism, in which a transmembrane protein is cleaved in response to an extracellular cue to release the intracellular domain (ICD) of the receptor. Once liberated from the plasma membrane, the ICD typically functions as a signaling mediator, often by translocating to the nucleus and regulating gene transcription. The proteases that catalyze RIP signaling, known as intramembrane cleaving proteases (I-Clips), are polytopic integral membrane proteins that possess the remarkable ability to hydrolyze peptide bonds of membrane-embedded protein helices. I-Clips can be divided into 3 classes based on enzymatic mechanism (Lal and Caplan 2011): aspartyl proteases (eg, presenilins), zinc metalloproteases (eg, site-2 protease), and serine proteases (eg, rhomboids).

Notch is one of the most thoroughly studied substrates for RIP (Schroeter and others 1998). As is the case for most RIP substrates—rhomboid substrates being a significant exception—intramembrane proteolysis of Notch is preceded by cleavage of the extracellular domain (ECD), in this case by TNF-alpha converting enzyme (TACE), a member of the ADAM (a disintegrin and metalloprotease domain) family of metalloproteases (Brou and others 2000). TACE cleavage occurs in a juxtamembrane region of Notch, shedding the bulk of the ECD and leaving a very short (13 amino acids) residual extracellular stub. The stub is the proximal substrate for intramembrane proteolytic cleavage by the gamma-secretase complex, containing presenilins 1 and 2 (Kimberly and others 2003). TACE cleaves many substrates (Edwards and others 2008) and can be activated by phorbol esters, suggesting that protein kinase C (PKC) is one mediator of TACE activation (ERK and p38 have also been implicated) (Montero and others 2002). While the physiological mechanism of TACE activation is still being unraveled (Xu and Derynck 2010), in most situations TACE cleavage is the regulated step of RIP signaling (Weskamp and others 2004; Glenn and van der Geer 2007). In contrast, presenilin cleavage appears to be constitutive once the target protein has been trimmed to a stub. Presenilins also cleave a large number of substrates, but proteomic profiling of presenilin substrates demonstrates that presenilin cleavage does involve a degree of specificity (Hemming and others 2008). This indicates that the gamma-secretase/presenilin complex does not simply function as a membrane proteosome but instead triggers specific signaling events. Thus, sequential cleavage of a transmembrane protein by

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TACE and presenilins is strongly suggestive of one form of RIP signaling.

The best characterized signaling pathway triggered in response to interferon-alpha (IFN-alpha) binding to its receptor utilizes the JAK family tyrosine kinases and the STAT transcription factors (Darnell and others 1994). The Tyk2 (Firmbach-Kraft and others 1990) and Jak1 (Wilks and others 1991) kinases stably associate with the receptor subunits IFNaR1 and IFNaR2, respectively (Colamonici and others 1994a, 1994b; Yan and others 1996b; Domanski and others 1997). After binding of IFN-alpha, these JAK kinases phosphorylate IFNaR1 (Krishnan and others 1997), creating a docking site for the SH2 domain of Stat2 (Yan and others 1996a). Stat2 is then phosphorylated, and in turn recruits Stat1 via its SH2 domain. The phosphorylated STATs heterodimerize via SH2 domains, translocate to the nucleus, and direct gene transcription (Darnell 1997). Thus, this signaling circuitry is driven by STAT SH2–phosphotyrosine interactions (Gupta and others 1996).

Previously, we found that in contrast to phosphotyrosine-dependent inducible recruitment to IFNaR1, Stat2 binds IFNaR2 constitutively (Nguyen and others 2002). Such binding was not required for canonical Stat2-Stat1 heterodimer-driven signaling in response to IFN-alpha (Nguyen and others 2002). Moreover, Stat2 binds with significantly higher affinity to the constitutive site on IFNaR2 than to the phosphotyrosine site on IFNaR1, arguing that IFNaR2 does not represent a predocking site for the JAK-STAT signaling mechanism (Saleh and others 2002). This suggested that the IFNaR2-Stat2 complex might signal via an alternative mechanism. Since some transmembrane proteins [eg, HER4 (Ni and others 2001) and CSF-1 (Wilhelmsen and van der Geer 2004)] can signal via canonical tyrosine kinase mechanisms and RIP, we searched for evidence that IFNaR2 can be proteolytically cleaved. We showed that IFNaR2 was indeed cleaved, after IFN-alpha treatment or PKC activation, to generate a small ICD-sized fragment (Saleh and others 2004) and that recombinant versions of the ICD could translocate to the nucleus (El Fiky and others 2008) and modulate gene expression (El Fiky and others 2005). Here, we provide additional support that IFNaR2 can signal via RIP, by demonstrating that TACE and presenilin 1/2 sequentially cleave IFNaR2.

Materials and Methods

Reagents

IFN-alpha2 was provided by Roche. Protein N-glycosidase F (PNGase F) was purchased from New England Biolabs, and used as described in the legend of Fig. 3. Zeocin (100 μg/mL final concentration), G418 (500 μg/mL final concentration), and puromycin (500 ng/mL final concentration) were purchased from Invitrogen. Doxycycline (50 ng/mL final concentration) was purchased from Sigma.

Recombinant gene constructs

The double-tagged version of the IFNaR2 cDNA (Fig. 1A) was generated via multiple polymerase chain reaction (PCR) cloning steps and inserted into pcDNA3.1 (Invitrogen). The construct has a 12-amino-acid insertion encoding the octapeptide FLAG tag (DYKDDDDK) and residues corresponding to flanking *Eco*RV and *Mlu*I restriction sites between amino acid residues 33 and 34 (the predicted signal sequence extends to residue 28). An hemagglutinin (HA) tag (YPYDVPDYA) was added at the extreme C-terminus. The cDNA encoding a GFP-IFNaR2-HA fusion protein (Fig. 3A) was constructed by inserting a PCR fragment corresponding to the *Aequorea victoria* (jellyfish) green fluorescent protein (GFP) gene into the *Nde*I restriction site between IFNaR2 residues 28 and 29 of a carboxyl-terminal-tagged IFNaR2 construct (IFNaR2-HA) described previously (Nguyen and others 2002). The IFNaR2-HA construct was also inserted into pcDNA3.1-Zeo (+) (Invitrogen) for use in establishing stable transfectants.

The IdelE construct (Fig. 10A) was made via a series of PCR and restriction fragment cloning steps. It encodes the N-terminal 32 amino acids of IFNaR2, 2 amino acids corresponding to an *Eco*RV cloning site (ie, not encoded by the *IFNaR2* gene), and 11 amino acids of the ECD, followed by the IFNaR2 transmembrane domain (TMD) and ICD, and a C-terminal HA-tag. Thus, after signal sequence removal, the encoded protein is predicted to have an 18-amino-acid ECD (the intact, mature ECD is 215 residues). The IdelE coding region was also joined to a tetO enhancer and a CMV minimal promoter (from pUHD 10-3) and inserted into pcDNA3.1 (+)-Zeo (Invitrogen), to create a tetO-regulated gene encoding IdelE-HA. All PCR-derived fragments of recombinant cDNA inserts were sequenced.

Construction of the IFNaR2 full-length cleavage reporter (Fig. 5A) was previously described (Saleh and others 2004). An IdelE version of the cleavage reporter (Fig. 12C) was constructed as a fusion between the full-length cleavage reporter and the deleted ECD of the IdelE construct, by joining the appropriate DNA fragments at the endogenous IFNaR2 *Nco*I restriction site (at residue 299).

Plasmid DNA encoding murine TACE (Black and others 1997), a mutant version of IFNaR2 (m1; in which residues DDED at positions 435–438 were changed to AAAA) that binds Stat2 weakly (Nguyen and others 2002), a mutant version of presenilin 1 (PS1 D257A) (Wolfe and others 1999), and the tet-regulator system plasmids [pUHD172-1neo and pUHD 10-3 (Gossen and Bujard 1992)] were all previously described.

Cell culture and transfection

Individuals providing cell lines are listed in the Acknowledgements. All cell lines were grown in Dulbecco's modified Eagle's medium plus 10% fetal calf serum (FCS), except Daudi cells (grown in RPMI plus 10% FCS). HEK293T cells, TACE and presenilin-deficient mouse embryo fibroblast (MEF) cell lines, CHO cells expressing presenilin constructs, and U5A cells were plated at subconfluence and then transfected 18 h later using calcium-phosphate DNA precipitates, as described (Jordan and Wurm 2004). In some experiments (noted in the figure legends), TACE-deficient MEFs were transfected using the Nucleofection protocol (Amaxa). In brief, 5×10^6 cells were resuspended in 100 μL of V-buffer and shocked at the A23 setting, and then plated in the medium containing serum.

To create a stable clone expressing the IdelE-HA construct shown in Fig. 10A, U5A cells were transfected with a plasmid encoding the reverse-tet transcriptional activator (rtTA; pUHD172-1neo) and stable clones were selected using G418 and then assayed for rtTA function [after transient transfection with a tetO-linked luciferase construct,

as described (Gossen and Bujard 1992), data not shown (DNS)]. A stable clone expressing rtTA was then transfected with a plasmid encoding a tetO-regulated IdelE-HA construct and a zeocin resistance gene. After drug resistance selection, a U5A cell line expressing a doxycycline-regulated IdelE-HA protein was identified (employed in Figs. 10 and 11).

Cell lysis and immunoprecipitation

Combined cytoplasmic and plasma membrane protein extracts were prepared by lysing cells in 1% NP40, 150 mM NaCl, 40 mM Tris-HCl, pH 8, 1 mM sodium vanadate, 100 mM NaF, 2.5 μM ZnCl2, and 400 μM phenylmethylsulphonylfluoride, and separating nuclei and cellular debris by centrifugation, as described previously (Nguyen and others 2002). The supernatant was used for most of the immunoblotting experiments. Two subcellular fractionation protocols were employed. In some studies (Fig. 10C) plasma membrane and cytosolic proteins were separated by first lysing cells by repeatedly passing a cell suspension through a syringe fitted with a 23-gauge needle (50 strokes). The lysate was centrifuged at 1,500×*g* and the supernatant was recovered and further centrifuged at 14,000×*g*. The supernatant is used as the cytosol and the pellet (after resuspension in 2× sodium dodecyl sulfate (SDS) sample buffer) was used as the plasma membrane fraction. In other experiments (Fig. 11B), nuclear and cytoplasmic/plasma membrane fractions were prepared. Cells were swollen in a hypotonic buffer (10 mM HEPES, pH 7.9; 10 mM KCl; 0.1 mM EDTA; 1 mM DTT), lysed by the addition of NP40 to a final concentration of 0.3%, and centrifuged at 14,000×*g*. The supernatant was used as the nonnuclear fraction and pelleted nuclei were extracted with a high salt buffer (20 mM HEPES, pH 7.9; 400 mM NaCl; 1 mM EDTA; 1 mM DTT) to obtain the nuclear fraction. For immunoprecipitation, combined cytoplasmic and plasma membrane extracts were diluted in lysis buffer and immunoprecipitated overnight at 4°C with 1 μL of anti-GFP (Abcam, diluted 1:10) or isotype control. Immune complexes were incubated at 4°C for 1 h with Protein A sepharose beads (Sigma), washed 3 times with lysis buffer, and resuspended in 2× SDS sample buffer.

Immunoblotting

SDS-PAGE and immunoblotting to nitrocellulose membranes was performed as described previously (El Fiky and others 2008). Immunoblotting antibodies (source, identification number, dilution) used were as follows: anti-TACE (Chemicon, AB19027, 1:10,000); anti-HA (Santa-Cruz, Y-11, 1:5,000); anti-FLAG (Sigma, F7425, 1:5,000); anti-VP16 (Clontech, 3844-1, 1:1,000); anti-elongation factor 2 (EF2) [K. Nastiuk (Marin and others 1997), G271, 1:15,000]; anti-integrin beta 1 [E. Marcantonio (Marcantonio and Hynes 1988), 363.1; 1:10,000]; anti-rabbit IgG (Pierce, PI31460, 1:20,000); and anti-GFP (Abcam, AB290, 1:50,000). Secondary goat anti-rabbit or goat anti-mouse horseradish peroxidase-conjugated antibodies (Pierce) were used, and chemiluminescence (SuperSignal West Pico, Pierce) detection was captured on film or in some cases using a cooled charge-coupled digital (CCCD) camera system (Kodak 4000R) and Carestream Molecular Imaging software, which allows blot image quantification.

Reporter gene assays

Cells were lysed in Passive Lysis buffer (Promega) and sequentially assayed in a luminometer (Turner) for experimentally regulated firefly luciferase activity and constitutive Renilla luciferase activity using Dual Luciferase Reporter reagents (Promega), which discriminate between the 2 types of luciferase activities. Normalized firefly luciferase activity, in relative light units, was calculated by dividing each determination of firefly luciferase activity by the corresponding transfection control (Renilla luciferase) activity value. In some cases, fold-induction was calculated by dividing all values by an appropriate control.

Fluorescence microscopy

Cells were plated on glass coverslips and 24 h later the coverslip was incubated for 3 min in 150 mM NaCl, 10 mM Tris-HCl pH 7.4, 1 mM sodium vanadate, 100 mM NaF, 2.5 μM ZnCl2, 400 μM phenylmethylsulphonylfluoride, and 0.1% NP40 to lyse the cells *in situ*. Nuclei were fixed to the cover slips with 4% (w/v) paraformaldehyde in phosphate-buffered saline. Coverslips were incubated for 1 h at room temperature with phosphate-buffered saline containing 10% goat serum, and then incubated for 2 h at room temperature with the primary antibody (anti-HA antibody diluted 1:500) by inverting the coverslip on parafilm. The coverslip was washed with phosphate-buffered saline, and then incubated with a fluorescent secondary antibody (Alexifluor-594-labeled goat anti-rabbit immunoglobulin, diluted 1:1,000; Molecular Probes), washed, counterstained with Hoechst 33258 (2 μg/mL; Invitrogen), and visualized under a Nikon Eclipse E600 fluorescent microscope. Images were captured with a Spot RT digital camera and accompanying software. All cells were photographed under identical exposure time and gain settings.

Gene silencing by RNA interference

Daudi cells (2,000) were infected at a multiplicity of 10 with a recombinant lentivirus containing either shRNA targeting human TACE mRNA (4 different target sequences, corresponding to clones 1–4; Sigma Mission shRNA, catalog number SHVRS-NM003183) or a nontargeting shRNA (Sigma, catalog number SHC002V). Cells were centrifuged in the presence of virus at 800×*g* for 60 min at 32°C, and then resuspended in Daudi-cell-conditioned RPMI and serially diluted in 96-well plates to obtain single-cell populations (limiting dilution). Cultures were expanded under puromycin selection and cell lysates were immunoblotted with anti-TACE. The extent of silencing was calculated by quantitating TACE protein expression using the software and CCCD camera described above, following the manufacturer's directions.

Proliferation assays

In some cases (Fig. 13) cell proliferation rates were determined by monitoring mitochondrial dehydrogenase activity, measured as the hydrolysis of a tetrazolium salt (WST-1; Roche) to formazan. After transfection by the Nucleofection protocol, MEFs were seeded into 96-well plates, in triplicate at 10,000 cells/well. The next day, WST-1 (10 μL) was added to each well, plates were incubated at 37°C, and formazan

concentration was measured at 5-min intervals at 450 nm (minus background absorbance at 600 nm) in a microplate reader. Conversion rates were derived from the slope of the curve in the linear range for each sample. In other cases (Fig. 14) proliferation was determined by seeding 2×10^5 Daudi cells in 2 mL of the medium in 12-well plates, treating cultures with 30 U/mL IFN-alpha or vehicle control and counting viable cells (those excluding Trypan blue dye) at 24 h intervals.

Results

The ECD of IFNaR2 is shed from the cell surface

In previous studies documenting proteolysis of IFNaR2, we observed that either phorbol ester treatment or PKC overexpression enhanced the cleavage of an IFNaR2 cleavage reporter construct (Saleh and others 2004), suggesting that TACE is involved in IFNaR2 RIP. To investigate this hypothesis, TACE and a double-tagged version of IFNaR2 (containing a C-terminal Flag epitope and an N-terminal HA epitope; Fig. 1A) were expressed in HEK293T cells. Increasing levels of TACE protein (Fig. 1B, middle panel) reduced the level of anti-Flag-detected IFNaR2, consistent with the release of the ECD by TACE (Fig. 1B, upper panel). Blots in this figure and throughout the article were re-probed with anti-EF2 to control for sample loading (Fig. 1B, lower-most panel).

To demonstrate that the ECD is shed in an intact form, and not simply degraded, we performed an *in vitro* cleavage experiment. Protein lysates from HEK293T cells transfected separately with DNA encoding either TACE or IFNaR2 were mixed and incubated *in vitro*. After 2 h, the amount of full-length IFNaR2 decreased relative to the starting material (Fig. 2, uppermost panel, lane 3 versus 7) accompanied by an increase in an anti-Flag reactive 40-kDa fragment that corresponds to the cleaved ECD (Fig. 2, second panel from the top).

To determine if the IFNaR2 ECD is shed from live cells, we constructed a variant of IFNaR2 with a GFP domain inserted downstream of the signal sequence (Fig. 3A), transiently expressed this construct into HEK293T cells, recovered the medium from these cultures, and immunoprecipitated and probed for presence of GFP (Fig. 3A). We detected a diffuse band of the predicted size ($\sim$70 kDa), consistent with the expected glycosylated ECD fragment. To confirm that the recovered fragment is glycosylated, additional media were immunoprecipitated and digested with PNGase F. The immunoprecipitating antibody (rabbit IgG) was digested by this treatment, confirming that the PNGase F was active under these conditions (Fig. 3C, lower panel). This treatment reduced the apparent molecular weight of the recovered fragment (Fig. 3C, upper panel), indicating that the fragment was glycosylated. A similar reduction in apparent size was also observed when the intact full-length receptor was digested with PNGase F (Fig. 3D).

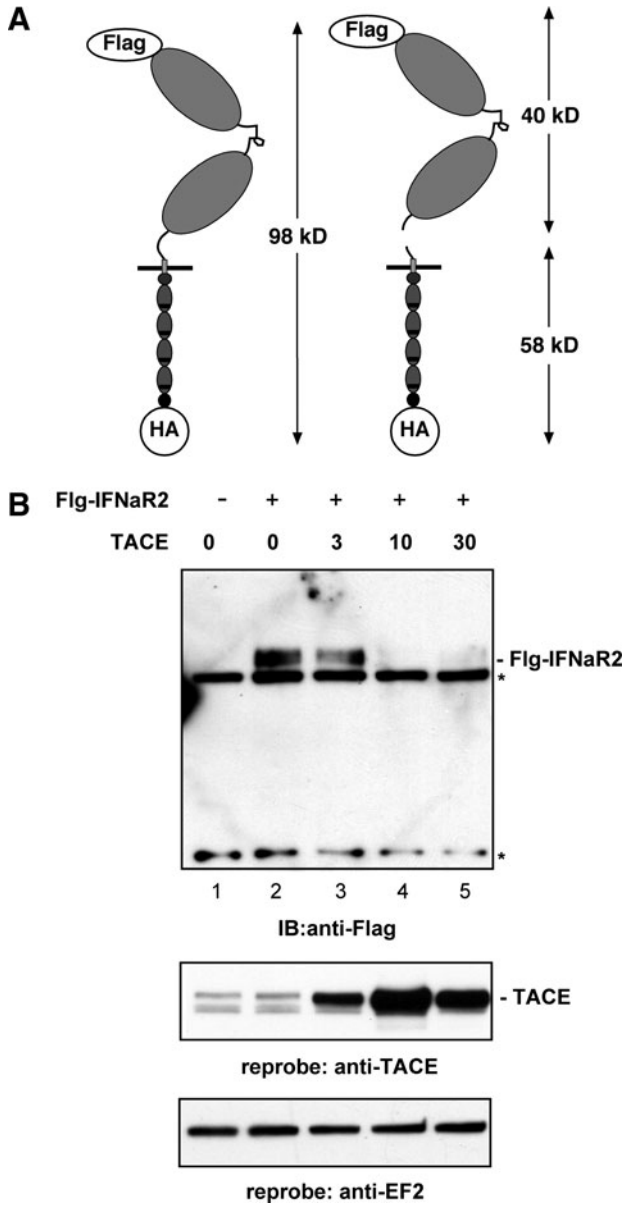


FIG. 1. Cleavage of the IFNaR2 ECD. **(A)** Schematic of IFNaR2 cleavage products. The IFNaR2 subunit of the type I IFN receptor consists of a 211-amino-acid ECD composed of cytokine homology regions (gray ovals), a short juxtamembrane domain, a TMD (small rectangle), and a 215-amino-acid ICD that contains repeats (ovals) with acidic regions (black bars) (Mogensen and others 1999). Some constructs contain a HA epitope tag at the extreme C-terminus; others (depicted) contain both the HA tag and a FLAG tag 5 amino acids from the predicted mature N-terminus. The known size of full-length IFNaR2 (98 kDa), the predicted size of the ECD shed after TACE cleavage (40 kDa), and the known size of the residual membrane-bound stub (58 kDa) are indicated. **(B)** TACE induces IFNaR2 cleavage. HEK293T cells were transfected with variable amounts (μg) of plasmid DNA encoding TACE and/or N-terminal FLAG-tagged IFNaR2 (Flg-IFNaR2-HA), as indicated. NP-40 lysates were prepared 18 h later and immunoblotted with anti-FLAG (*upper panel*). FLAG-IFNaR2-HA runs as a diffuse band at about 98 kDa, as indicated. Asterisks indicate artifact bands. The filter was sequentially stripped and re-probed with anti-TACE and anti-EF2. The immunoblots in **(B)** are representative of 3 replicates. IFN, interferon; ICD, intracellular domain; TACE, TNF-alpha converting enzyme; ECD, extracellular domain; EF2, elongation factor 2; TMD, transmembrane domain.

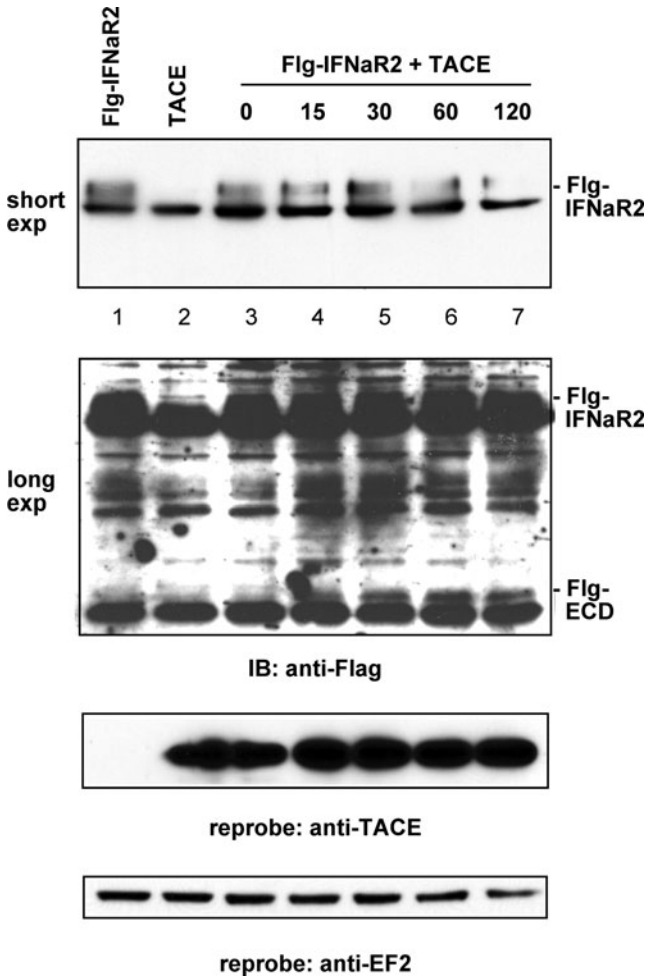


FIG. 2. TACE mediates *in vitro* cleavage of the ECD of IFNaR2. HEK293T cells were separately transfected with plasmid DNA encoding either Flg-IFNaR2 (25 μg) or TACE (25 μg) and 18 h later lysed in buffer containing 1% NP-40. The 2 corresponding lysates were incubated at 37°C, either separately for 120 min (lanes 1–2) or in combination (lanes 3–7) for the indicated time (min), and then immunoblotted with anti-FLAG. Filters were stripped and sequentially reprobed with anti-TACE and anti-EF2 (*lower panels*). The *top panel* is a short exposure of the upper portion of the anti-FLAG immunoblot; the second panel from the top is a long exposure of the entire immunoblot (IB). The ECD cleavage product (Flg-ECD) migrates at 40 kDa (DNS). Unlabeled bands in the *upper 2 panels* are due to antibody cross reaction. These immunoblots are representative of 2 replicates.

TACE is required for production of the 58-kDa processed form of IFNaR2

To confirm the role of TACE in the processing of IFNaR2, we employed MEFs derived from a TACE knockout (KO) mouse. Anti-HA immunoblotting of MEFs transfected with C-terminally HA-tagged IFNaR2 revealed a 58-kDa stub in the wild-type cells, but not in the TACE KO counterparts, indicating that TACE is required to generate the stub (Fig. 4, upper panel). The zinc binding domain has been homozygously deleted in the *TACE* gene in the germline of the KO mouse, producing a smaller, nonfunctional TACE protein (Black and others 1997) (Fig. 4, middle panel). A similar experiment was performed using the IFNaR2 cleavage reporter

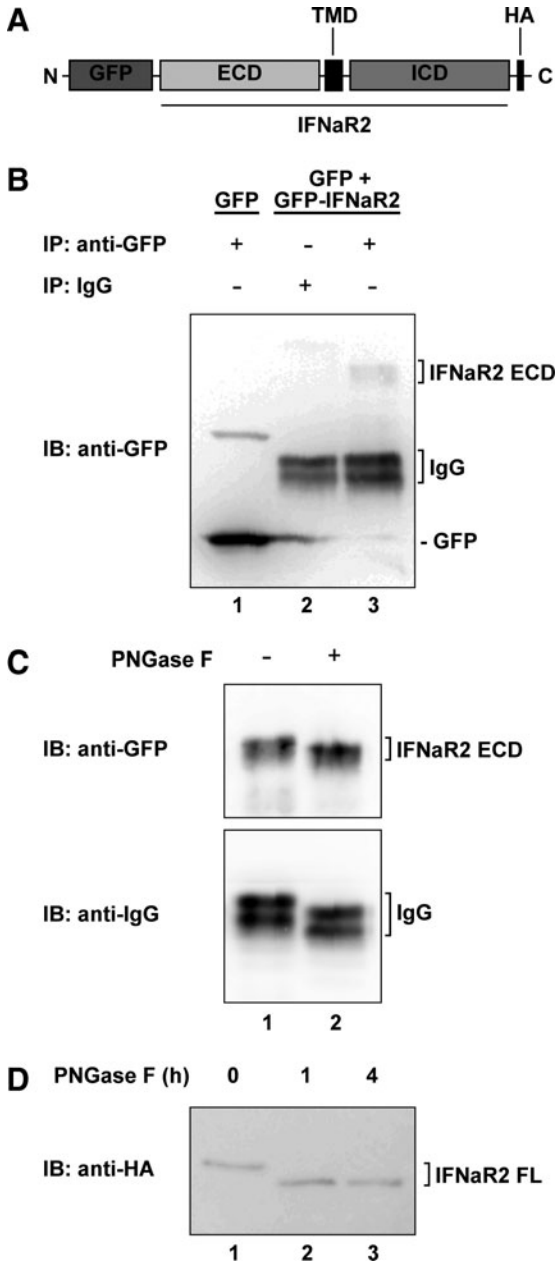


FIG. 3. The IFNaR2 ECD is shed from live cells. **(A)** Schematic of GFP-IFNaR2 construct. The ECD, TMD, ICD, and HA epitope tag are indicated. **(B)** Immunoprecipitation of the shed ECD from the culture medium. HEK293T cells were transfected with plasmids encoding GFP or GFP plus the construct shown in **(A)**. Lane 1 is a control immunoprecipitation of lysate from GFP-transfected cells. In lanes 2 and 3, the cell culture medium was immunoprecipitated with the indicated antibodies and immunoblotted with anti-GFP. The GFP signal in lane 2 represents spill-over from lane 1. **(C)** The recovered IFNaR2 ECD is N-glycosylated. Immunoprecipitates prepared as in **(B)** were incubated with 1 U PNGase F at 37°C for 1 h and then immunoblotted with anti-GFP (upper portion of filter) or anti-rabbit IgG (lower portion of filter). **(D)** Full-length IFNaR2 is similarly N-glycosylated. Lysates from HEK293T cells transfected with the construct shown in Fig. 1A were incubated with PNGase F for 0, 1, or 4 h and immunoblotted with anti-HA. These immunoblots are representative of 2 replicates. PNGase F, protein N-glycosidase F; GFP; IP; HA.

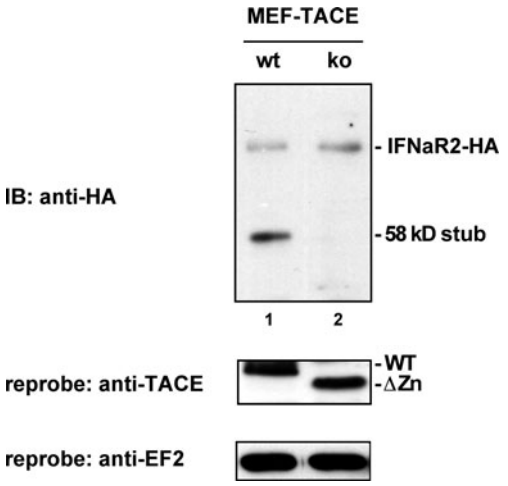


FIG. 4. IFNaR2 is cleaved in wild-type, but not in TACE-deficient MEFs. Plasmid DNA encoding IFNaR2-HA was introduced, via Nucleofection, into either wild-type (wt) or TACE-deficient (ko) MEFs. Eighteen hours later lysates were prepared and immunoblotted with anti-HA (*upper panel*). The filter was sequentially stripped and re-probed with anti-TACE and anti-EF2. The positions of full-length (98 kDa) IFNaR2-HA, the corresponding 58-kDa cleavage product (stub), wild-type TACE (WT), and the inactive, deleted version of TACE (ΔZn) are indicated. These immunoblots are representative of 2 replicates. MEFs, mouse embryo fibroblasts.

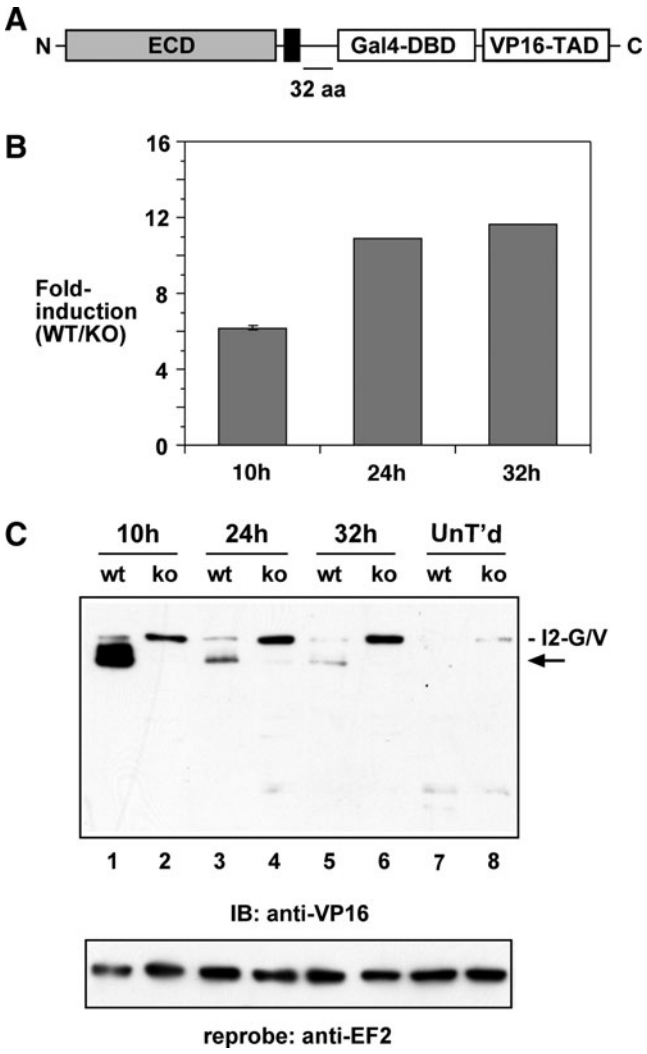


FIG. 5. TACE is required for processing of a chimeric IFNaR2 cleavage reporter. **(A)** Cleavage reporter construct. The construct contains the ECD, TMD (black square), and 32 amino acids of the ICD of IFNaR2 fused to the yeast Gal4-DBD and the Herpes virus VP16-TAD. **(B)** Reporter assay. Plasmid DNA encoding the construct depicted in **(A)**, a Gal4-UAS-driven firefly luciferase gene and a constitutively expressed Renilla luciferase gene were transfected via Nucleofection into either wild-type (wt) or TACE-deficient (ko) MEFs. Luciferase activity was measured at the indicated times post-transfection, normalized, and plotted ($n=3$; error bars correspond to the standard error of the mean). **(C)** Immunoblot of cleavage reporter. Lysates from cultures assayed in **(B)** were prepared and immunoblotted with anti-VP16. Filters were re-probed with anti-EF2. The position of the cleavage-reporter construct shown in **(A)** (I2-G/V) is indicated. The *arrow* corresponds to the position of the putative cleavage product. These experiments are representative of 3 replicates. Gal4-DBD, Gal4 DNA binding domain; VP16-TAD, VP16 transactivation domain; UAS, upstream activating sequence.

we employed previously to demonstrate IFNaR2 RIP (Saleh and others 2004). In brief, this protein consists of the IFNaR2 ECD and TMDs fused to a hybrid Gal4-VP16 transcription factor domain, which substitutes for the IFNaR2 ICD (Fig. 5A). Cleavage within the IFNaR2 sequences liberates a Gal4-VP16 transcription factor fragment, which stimulates expression of luciferase under the control of a Gal4 upstream activating sequence (UAS). Plasmids encoding the cleavage reporter and UAS-luciferase were co-transfected into both the wild-type and TACE KO MEFs and then assayed for luciferase activity and immunoblotted with anti-VP16 antibodies. The ratio of luciferase activity in wild-type compared to KO MEFs was increased 6- to 12-fold, demonstrating that TACE greatly enhanced cleavage (Fig. 5B). Similarly, there is a significant reduction in the amount of the full-length cleavage-reporter protein in the wild-type relative to the TACE KO MEFs (Fig. 5C), consistent with nearly complete cleavage in cells expressing TACE.

In addition to the transient transfection experiments in Fig. 4, we prepared a set of stable clones expressing IFNaR2-HA in both wild-type and TACE KO MEFs. Although the transfection efficiency was similar in both wild-type and TACE KO cells (Fig. 6C), the level of IFNaR2 expression is significantly higher in the TACE KO cells relative to the wild-type TACE-proficient cells (compare Fig. 6A and 6B). This suggests that cells expressing TACE cleave the majority of the exogenous IFNaR2 protein, which may be further processed into fragments that are not detected in these blots. To further characterize these stable transfectants, we complemented 2 of the KO clonal lines (ko-5 and ko-6) with a plasmid encoding TACE or vector DNA only (Fig. 7). TACE expression in these clones leads to IFNaR2 cleavage, either eliminating detectable IFNaR2 protein (ko-6) or producing the expected 58-kDa stub (ko-5).

IFN-alpha induces TACE-dependent cleavage of IFNaR2

We previously observed IFN-alpha-induced cleavage of IFNaR2, employing the cleavage reporter shown in Fig. 5 (Saleh and others 2004). To determine if IFN-alpha-dependent

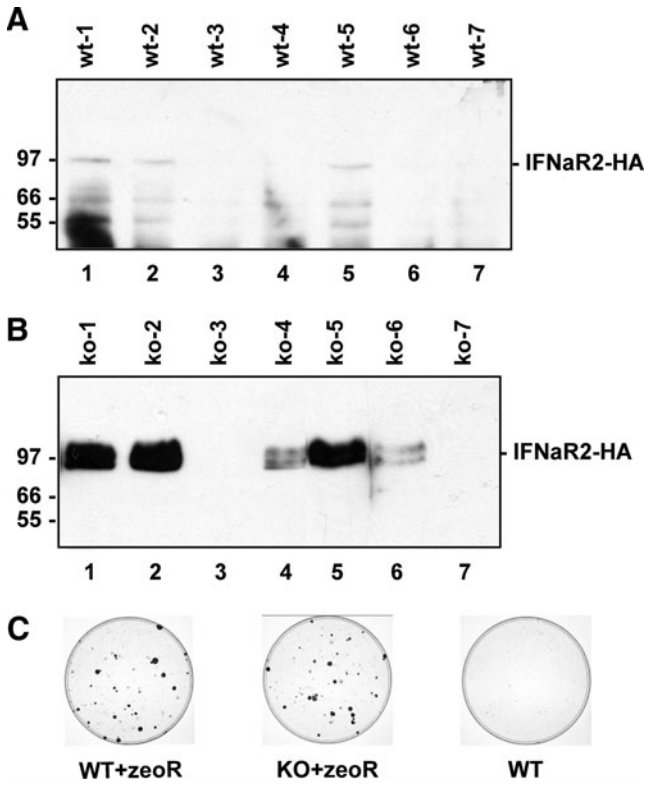


FIG. 6. Stable IFNaR2 transgene expression in wild-type and TACE-deficient MEFs. A plasmid encoding IFNaR2-HA and zeocin resistance was transfected into either wild-type (wt) or TACE-deficient (ko) MEFs. Clones were selected for zeocin resistance and corresponding lysates were prepared and immunoblotted with anti-HA. **(A)** Representative IFNaR2 transfectants in wild-type MEFs. **(B)** Representative IFNaR2 transfectants in TACE KO MEFs. The position of IFNaR2 is indicated. The amount of protein loaded was similar in each lane of **(A)** and **(B)**, as judged by Ponceau Red staining of the blot filter (DNS). **(C)** MEF transfection efficiency. A plasmid encoding Zeocin resistance (zeoR) was introduced into the same cell lines as **(A)** (WT + ZeoR) and **(B)** (KO + ZeoR). After 2 weeks of zeocin selection, dishes were stained with crystal violet and photographed. The dish on the far right (containing WT MEFs) did not receive plasmid DNA before zeocin selection. The clones shown in **(A)** and **(B)** were probed 2 times (representative immunoblots shown). KO, knockout; DNS, data not shown.

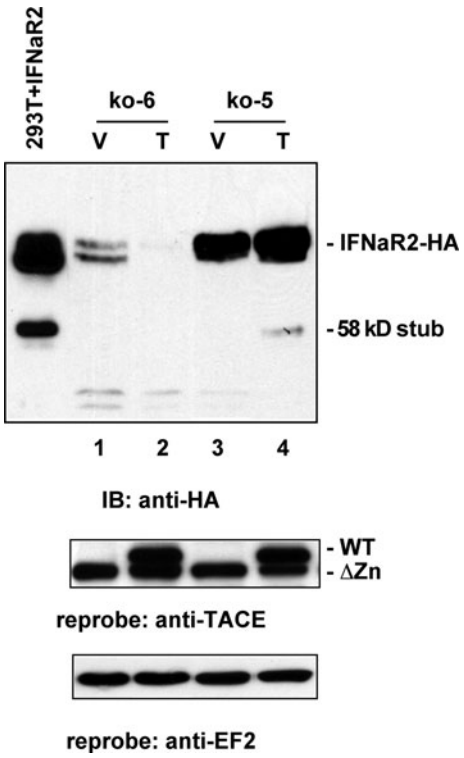


FIG. 7. Complementation of KO clones with TACE induces IFNaR2 cleavage. Two clones from Fig. 6B (ko-6 and ko-5) were transiently transfected with plasmid DNA encoding TACE (T) or vector (V). Lysates were prepared and immunoblotted with anti-HA. Filters were stripped and reprobed with anti-TACE and anti-EF2. The left-most lane contains lysate from HEK293T cells overexpressing full length IFNaR2 to provide a size marker for the 58-kDa stub. Lysates from clone ko-5 were probed 2 times, and clone ko-6 lysates were probed 3 times. Representative blots are shown.

cleavage is mediated by TACE, cells were co-transfected with plasmids encoding IFNaR2-HA and TACE (or the corresponding vector), and then treated with IFN-alpha. Immunoblotting demonstrated an IFN-alpha-dependent increase in the level of the 58-kDa IFNaR2 stub (Fig. 8), suggesting that IFNaR2 RIP is physiologically relevant.

Presenilin processes the IFNaR2 stub generated by TACE cleavage

Since TACE cleaves the ECD of IFNaR2, we next sought to determine if the gamma-secretase complex, containing presenilin 1 and 2, proteolytically cleaves the 58-kDa IFNaR2 stub. First, HEK293T cells were co-transfected with plasmids encoding TACE and IFNaR2. We observed that cultures receiving the highest levels of TACE-encoding plasmids contained the lowest levels of full-length IFNaR2 and the 58-kDa stub (Fig. 9, lanes 1–3), suggesting that increased levels of TACE resulted in the processing of IFNaR2, consistent with the results in Figs. 4–7. Note that in the absence of IFN-alpha, the 58-kDa stub is only detected in extended exposures of the immunoblots (compare Fig. 8, lane 4 with Fig. 9, lane 3). In a second, companion experiment we found that the transfection of an additional plasmid, encoding a protease-deficient variant of presenilin 1 (PS1 D257A), increased the levels of both IFNaR2-HA and the 58-kDa stub (compare Fig. 9, lane 3 versus 6). This suggests that full-length IFNaR2 may be a precursor of the 58-kDa stub.

To address this directly, a variant of IFNaR2 (IdelE) containing only 18 amino acids of the mature (after signal sequence processing) ECD and a C-terminal HA tag was constructed (Fig. 10A). First, a cDNA encoding this recombinant version of the 58-kDa stub was placed under a tetracycline-responsive promoter in a cell line (U5A) that lacks IFNaR2 expression (Lutfalla and others 1995) (see Materials and Methods). Doxycycline treatment induced expression of the expected 58-kDa band (Fig. 10B), which was localized to the plasma membrane by cell fractionation studies (Fig. 10C). Anti-HA immunofluorescence microscopy of the same cells demonstrated the presence of C-terminally tagged IFNaR2 co-localized with nuclear (Hoechst) staining, only in the induced cells (Fig. 11A). HEK293T cells were then transfected with either the IdelE or the full-length IFNaR2

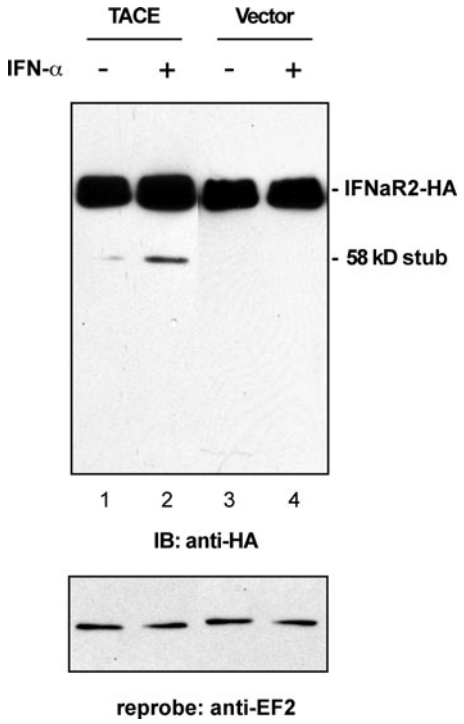


FIG. 8. IFN-alpha induces IFNaR2 cleavage in a TACE-dependent manner. HEK293T cells were transiently transfected with plasmids encoding IFNaR2 plus either TACE (Lanes 1–2) or vector DNA (Lanes 3–4). Each culture was split after 5 h and one half received IFN-alpha (1,000 U/mL) for 18 h, as indicated. Lysates were prepared and immunoblotted with anti-HA. Filters were stripped and re-probed with anti-EF2. These immunoblots are representative of 2 replicates.

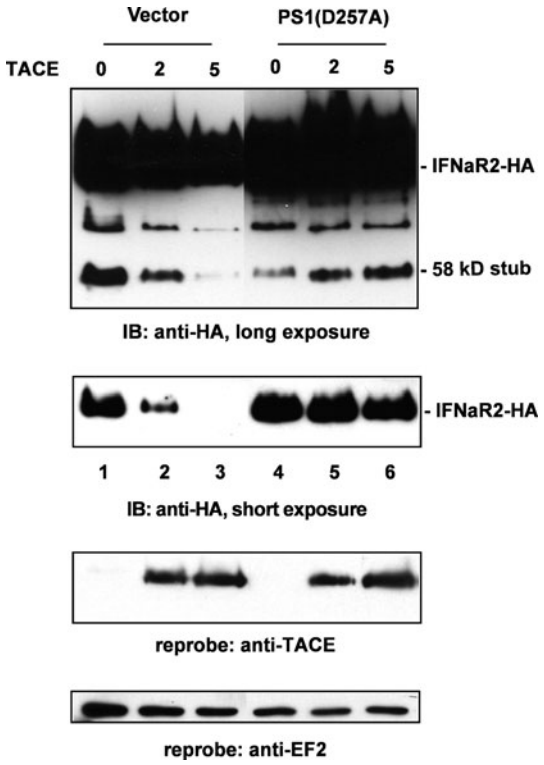


FIG. 9. Accumulation of the 58-kDa IFNaR2 stub in cells overexpressing protease-deficient presenilin-1. HEK293T cells were transiently transfected with variable amounts (μg) of plasmid DNA encoding TACE and either vector DNA or a plasmid encoding protease-deficient Presenilin-1 D257, as indicated. In addition, all cultures received plasmid DNA encoding IFNaR2-HA. Lysates were prepared and immunoblotted with anti-HA. Filters were stripped and sequentially re-probed with anti-TACE and anti-EF2. These immunoblots are representative of 4 replicates.

construct. Immunoblotting of cells separated into nuclear and non-nuclear fractions revealed a 27-kDa C-terminal fragment of IFNaR2 in the nuclear fraction of the IdelE-transfected cells, consistent with the ICD (Fig. 11B). Only a trace amount of this fragment was detected in cells transfected with the full-length IFNaR2 construct, suggesting that TACE cleavage is the rate determining step in the RIP of IFNaR2.

Next, we transfected plasmids encoding the IdelE-HA construct into 2 sets of isogenic cell lines with variable levels of presenilin activity, to determine if IdelE processing correlated with presenilin activity. The first set comprised CHO cells stably transfected with either wild-type presenilin 1 (WT^+) or protease-deficient forms of both presenilin 1 and 2 (PS1/2 DN), which act together in a dominant inhibitory manner to reduce presenilin activity (Kimberly and others 2000). Figure 12A shows that cells with increased presenilin activity (WT^+) have reduced levels of IdelE-HA, while those with reduced activity (PS1/2 DN) have increased levels of IdelE-HA, consistent with presenilin processing of IdelE. The IdelE construct was co-transfected with a GFP-encoding plasmid to provide a transfection efficiency control, based on the assumption that expression of the 2 genes was proportional. Thus, IdelE quantitation was normalized to GFP protein levels (Fig. 12A, lower panel)

The second set of cell lines are MEFs derived from wild-type mice or KO mice deficient in presenilin 1, presenilin 2, or both genes (De Strooper and others 1999). Co-transfection of constructs encoding IdelE-HA and GFP and subsequent analyses were performed as in Fig. 12A. The GFP-normalized level of IdelE protein was inversely related to the number of functional presenilin genes (Fig. 12B). To confirm this result, a cleavage-reporter version of the IdelE construct (compare Figs. 12C and 5A) was prepared and transfected into wild-type and presenilin 1/presenilin 2 double-KO (DKO) MEFs. Wild-type MEFs displayed an ~10-fold increase in luciferase activity relative to the presenilin-deficient cell line (Fig. 12D), confirming that the presenilins can cleave the 58-kDa stub form produced by TACE cleavage.

Role of TACE in IFNaR2-mediated growth inhibition

In the final set of experiments, we sought to determine if TACE-catalyzed RIP signaling was required for the growth inhibitory effects of IFN-alpha. Overexpressed IFNaR2 was cleaved in the presence of endogenous levels of TACE expression, thereby initiating RIP of IFNaR2 (Figs. 3–7). Therefore, we first sought to determine if IFNaR2 overexpression affects cell growth in a TACE-dependent manner. Figure 13A demonstrates that in the absence of TACE, overexpression of IFNaR2 does not alter the proliferation rate of MEFs (right hand columns). In contrast, in wild-type cells expressing TACE, overexpression of IFNaR2 significantly reduced the proliferation rate (Fig. 13A, left-hand columns). Figure 13B replicates the findings of Fig. 13A in TACE wild-type cells,

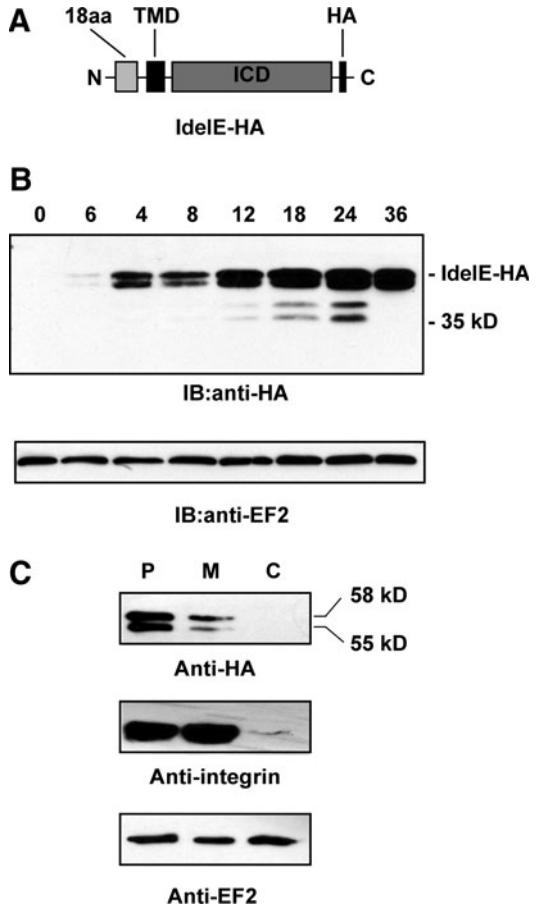


FIG. 10. Inducible IFNaR2 stub (IdelE-HA) localizes to the plasma membrane. **(A)** The IdelE-HA construct consists of 18 amino acids of the IFNaR2 ECD, plus the TMD, ICD, and a C-terminal HA-tag. **(B)** IdelE-HA is doxycycline induced. U5A cells expressing a reverse-tet transcriptional activator and a tetO-regulated IdelE-HA construct were treated with 50 ng/mL doxycycline for the indicated time. Lysates were prepared and immunoblotted with anti-HA. Filters were stripped and re-probed with anti-EF2. These immunoblots are representative of 4 replicates. **(C)** Membrane localization of IdelE-HA encoded protein products. U5A cells expressing tetO-IdelE were induced with doxycycline for 24 h and fractionated as described in Materials and Methods. Equivalent volumes of the plasma membrane fraction (M) and the cytosolic fraction (C) and an unfractionated lysate containing both cytoplasmic and plasma membrane protein extracts (postnuclear lysate; P) were immunoblotted with the indicated antibodies. These immunoblots are representative of 3 replicates.

employing 2 versions of IFNaR2, differing only in the addition of epitope tags. We also tested the effect of a mutant version of IFNaR2 (IFNaR2m1), in which the constitutive Stat2 binding site has been altered to substantially reduce Stat2 binding (Nguyen and others 2002). Figure 13B shows that IFNaR2m1 is not effective in inhibiting the proliferation of the MEFs, despite the presence of TACE. We previously demonstrated that Stat2 binding to IFNaR2 mediates at least some of the transcriptional effects of the IFNaR2 ICD, after translocation of the ICD to the nucleus (El Fiky and others 2005); therefore, this result suggests a similar requirement for Stat2 in mediating the anti-proliferative effects.

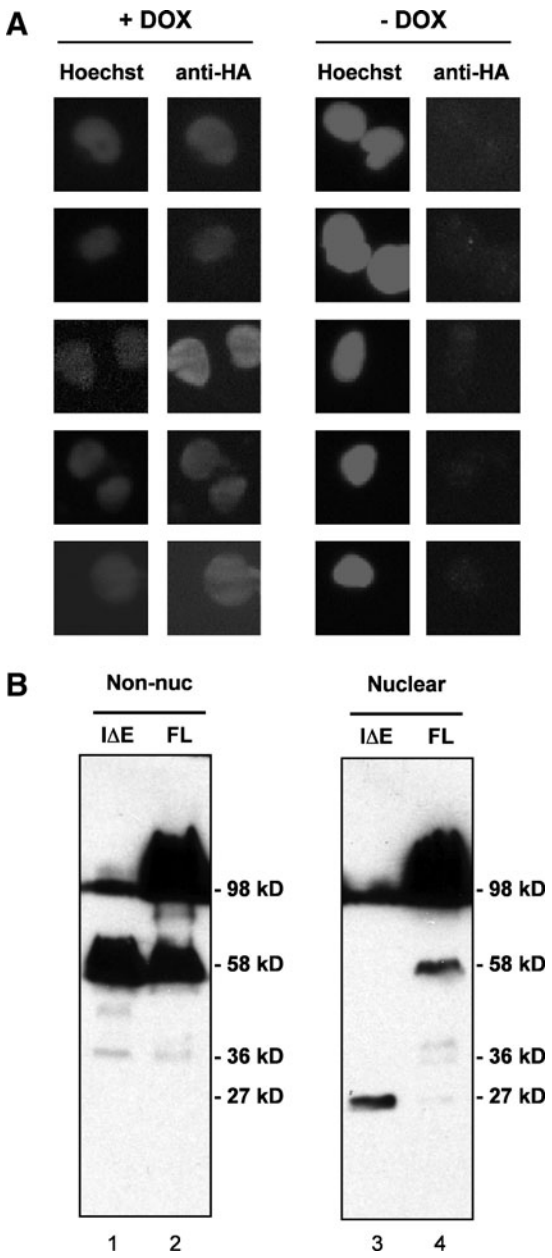
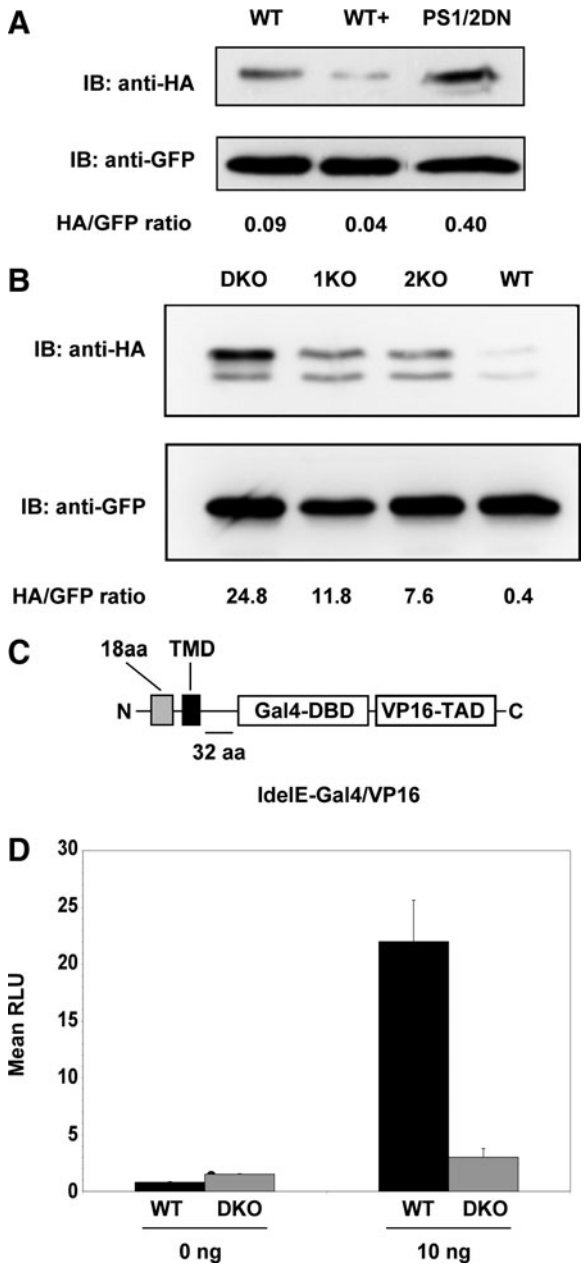


FIG. 11. Nuclear translocation of the IFNaR2 ICD. **(A)** Nuclear translocation detected by indirect immunofluorescence. U5A cells expressing a reverse-tet transcriptional activator and a tetO-regulated IdelE-HA construct were plated on glass coverslips, and either induced (+DOX) (50 ng/mL) for 24 h or left uninduced (−DOX), lysed *in situ* (see Materials and Methods), fixed, and incubated with anti-HA antibody followed by an Alexifluor-594-labeled secondary antibody. Nuclear counterstaining was performed with Hoechst 33258. Representative cells visualized under a fluorescent microscope are shown. These images are representative of 3 replicate experiments. **(B)** Nuclear translocation detected by immunoblotting. Plasmids encoding constitutively expressed full-length IFNaR2-HA (FL) or IdelE-HA were transiently transfected into HEK293T cells, harvested 24 h later, swollen in hypotonic buffer, lysed in 0.3% NP40, and centrifuged. The supernatant was used for non-nuclear fractions and the pelleted nuclei were extracted with high salt to obtain nuclear proteins. The protein fractions were immunoblotted with anti-HA. Each lane contains lysate from the same number of cells. These immunoblots are representative of 2 replicates. Non-nuc, non-nuclear.

To further assess the TACE dependence of the anti-proliferative effects of IFN-alpha, we employed Daudi cells, a human lymphoma cell line that is very sensitive to the anti-proliferative effects of IFN-alpha (Davis and others 1996). TACE-specific shRNA was introduced into Daudi cells, stable clones were isolated, and the levels of TACE protein determined (Supplementary Fig. S1; Supplementary Data are available online at www.liebertonline.com/jir). Three clones with reduced levels of TACE expression, along with the corresponding control clones, were treated with IFN-alpha and proliferation rates measured (Fig. 14). Although 2 clones with partial (50%) TACE protein reduction showed a reduced anti-proliferative response, a third clone, with ~90% reduction in TACE expression, was as responsive as the uninfected control clone. Thus, TACE silencing does not consistently block the anti-proliferative effects of IFN-alpha in Daudi cells.



Discussion

Transmembrane receptor proteins can be cleaved, in a 2-step process, to initiate nuclear signaling. The initial juxtamembrane cleavage, releasing the ectodomain from the cell surface, is thought to be the regulated step. The second cleavage, acting on the TMD of the residual stub, is typically constitutive. The ICD that is liberated and mediates signaling is often labile, and difficult to monitor. Therefore, identifying the proteases involved can provide critical evidence that an RIP pathway is physiologically relevant.

We viewed TACE as a candidate juxtamembrane protease for IFNaR2 because it has been frequently implicated in the shedding of cell surface receptor ectodomains (Arribas and Borroto 2002). Furthermore, as noted above, IFNaR2 RIP is stimulated by PKC (Saleh and others 2004), which activates TACE and type I IFNs activate both PKC-delta (Uddin and others 2002) and IFNaR2 RIP (Saleh and others 2004). We present two lines of evidence supporting a role for TACE in the cleavage of the 98-kDa intact IFNaR2 receptor to generate a membrane-bound 58-kDa stub. One set employed TACE overexpression in HEK293T cells. Figure 1 demonstrates that an N-terminal Flag-tag is lost from IFNaR2 as a consequence of TACE overexpression, while Figs. 2–3 demonstrate that the ECD is released intact, and not simply degraded. In particular, we recovered a glycosylated ECD from the medium of cells overexpressing IFNaR2, which has the molecular weight (40 kDa) predicted for the intact, shed IFNaR2 ECD (Fig. 3).

In a second set of experiments, we examined IFNaR2 processing in TACE-deficient MEFs. When the IFNaR2 cDNA was expressed in these cell lines, the 58-kDa stub was

FIG. 12. Presenilin activity is required for processing the IFNaR2 stub. **(A)** Plasmids encoding GFP and IdelE-HA were transiently transfected into CHO cells expressing the following: only endogenous presenilin genes (WT), endogenous genes plus a wild-type presenilin 1 transgene (WT^+), or endogenous genes plus transgenes encoding catalytically inactive presenilins 1 and 2 (PS1/2 DN). Twenty-four hours later, lysates were prepared and immunoblotted with anti-HA. Filters were stripped and re-probed with anti-GFP. Blot images were quantified as described in Materials and Methods. The anti-HA signal was divided by the anti-GFP signal to obtain the HA/GFP ratio shown. These immunoblots are representative of 3 replicates. **(B)** IdelE-HA encoding plasmid was transiently transfected into wild-type (WT), presenilin 1 knockout (1KO), presenilin 2 knockout (2KO), or presenilin 1 and 2 double-knockout (DKO) MEFs. Lysates were prepared and immunoblotted with anti-HA. Filters were stripped and re-probed with anti-GFP and the anti-HA/GFP ration determined as per **(A)**. These immunoblots are representative of 3 replicates. **(C)** IFNaR2 stub cleavage-reporter construct. Similar to Fig. 5A, except the deleted version of the ECD of the IdelE construct (Fig. 10A) is substituted for the full-length ECD. **(D)** Reporter assay. Plasmid DNA encoding the construct depicted in **(C)**, a Gal4-UAS-driven firefly luciferase gene, and a constitutively expressed Renilla luciferase gene were introduced via Nucleofection into either wild-type (WT) or presenilin 1/2 DKO MEFs. Luciferase activities were assayed, normalized, and plotted as the RLU ($n=3$; error bars correspond to the standard error of the mean). RLU, relative luciferase units.

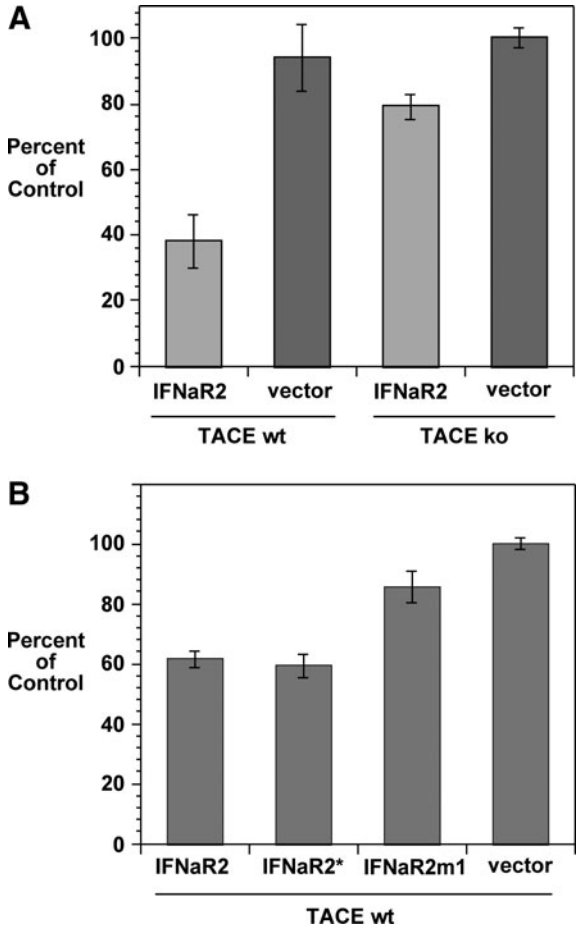


FIG. 13. IFNaR2 inhibits cell growth in a TACE-dependent manner. **(A, B)** The indicated constructs were transfected via Nucleofection into either wild-type (TACE wt) or TACE-deficient (TACE ko) MEFs as indicated, plated into 96-well plates, and the rate of WST1 conversion to formazan was assayed 24 h later. The conversion rates were normalized to vector-transfected control (the right-most column in each panel) and plotted as percentage of this control ($n = 3$; error bars correspond to the standard error of the mean). The IFNaR2 construct was HA-tagged, while the IFNaR2* and IFNaR2m1 constructs were tagged with both Flag (N-terminus) and HA (C-terminus), as per Fig. 1A.

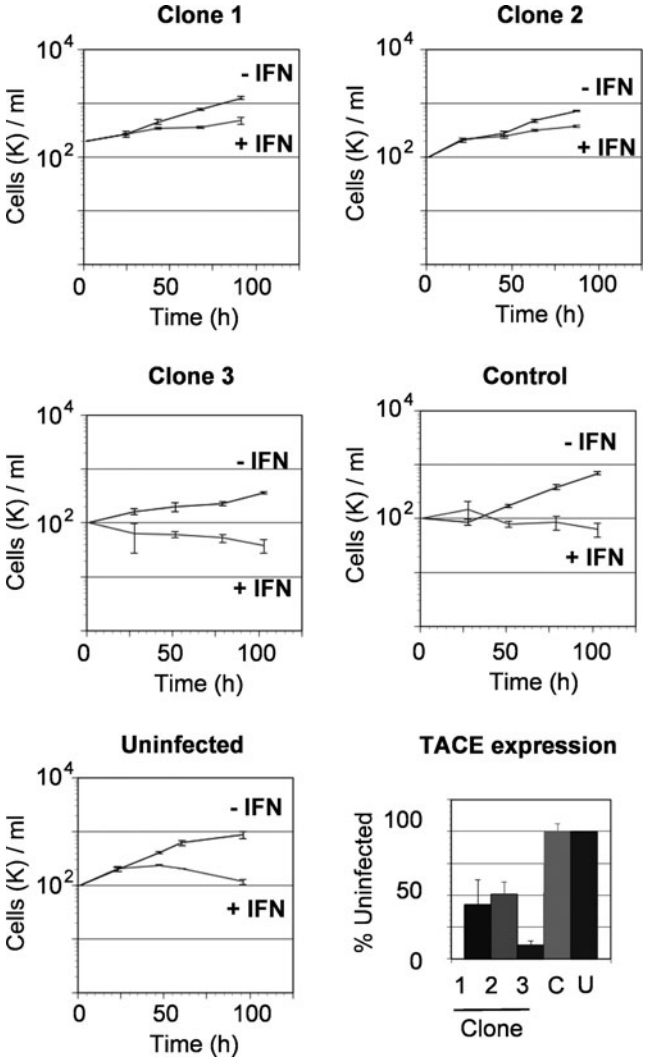


FIG. 14. Silencing of TACE does not abrogate the antiproliferative effects of IFN-alpha on Daudi cells. Stable clones of Daudi cells expressing either TACE-specific shRNA or a control shRNA or uninfected cells were seeded in triplicate, treated with or without 30 U/mL of IFN-alpha, and monitored for 4 days. Cell growth (viable cell counts) was plotted versus time (in hours; $n = 3$; error bars correspond to the standard error of the mean). Quantification of TACE expression for individual clones is shown in the *lower right panel* (immunoblotting data from Supplementary Fig. S1).

observed in wild-type but not the deficient cells (Fig. 4), demonstrating TACE-dependent processing. Stable clones established in the same MEFs showed higher levels of IFNaR2 expression in the TACE-deficient background (Fig. 6), consistent with IFNaR2 processing in TACE-proficient MEFs. Two clones in the TACE-deficient background, expressing different levels of the 98-kDa form of IFNaR2, were complemented with a TACE cDNA (Fig. 7). In clone ko-6, which expressed a lower level of the 98-kDa IFNaR2 receptor, there was a significant reduction in the 98-kDa band but no discernable 58-kDa product. In this case, the relatively high level of TACE may have resulted in nearly complete processing of full-length IFNaR2. Any 58-kDa intermediate that had been generated was probably rapidly processed by presenilin. In the clone (ko-5) originally expressing higher levels of the 98-kDa receptor, we observed the steady-state appearance of the 58-kDa stub. This might indicate that TACE complementation of clone ko-5 produces enough 58-kDa stub to saturate the intramembrane protease. While these experiments are not designed to assess the relative rates of TACE and presenilin proteolysis, the results demonstrate that cells expressing physiologic levels of TACE (as demonstrated in the anti-TACE re-probed blot in Fig. 7) process IFNaR2 via juxtamembrane cleavage, generating the expected cleavage product of 58 kDa.

We also examined IFNaR2 processing using a chimeric construct in which a Gal4DBD-VP16TAD fusion replaced the native ICD (Fig. 5). Once released by cleavage, the fusion protein induces the production of firefly luciferase. Thus, assuming that presenilin cleavage is constitutive and uniform within the experimental design, we use this assay to measure a stable end product and therefore to quantitate cleavage accurately. In contrast, the 58-kDa form of IFNaR2

is subject to further processing and, therefore, detection of this cleavage product by immunoblotting represents an integration of the rates of TACE and intramembrane proteolysis. Specifically, high rates of intramembrane cleavage can mask lower rates of TACE cleavage if the endpoint is the 58-kDa stub. For these reasons, the data in Fig. 5 provide strong confirmation for the role of TACE in the processing of IFNaR2. It should be noted that the luciferase measurements reported in Fig. 5B underestimate the relative level of reporter activity in TACE-proficient cells, since there is some level of background transcription of the reporter, independent of specific cleavage, when the reporter plasmids are introduced into TACE-deficient MEFs (DNS).

In Figs. 1–7 we examined the processing of ectopically expressed IFNaR2, rather than endogenous IFNaR2. This was done because we lack a suitable antibody against the ICD and because overexpression facilitates detection of the cleavage products. We do not believe that cleavage is an artifact of the higher levels of expression afforded by the transgenes. For instance, processing occurs in both clones examined in Fig. 7 despite very different levels of IFNaR2 expression. Furthermore, TACE and presenilin processing of the growth hormone receptor was shown to be very similar for both the endogenous protein as well as an overexpressed, exogenous protein encoded by a recombinant adenovirus (Cowan and others 2005).

We mainly observed TACE-dependent cleavage of IFNaR2 in untreated cells overexpressing the receptor. We have previously demonstrated that cleavage is enhanced after PKC activation via overexpression or PMA treatment (Saleh and others 2004). HER4 (Vecchi and others 1996; Vecchi and Carpenter 1997) and growth hormone receptor (Zhang and others 2000) are also cleaved spontaneously as well as in response to PMA. It is unclear what activates spontaneous juxtamembrane cleavage in untreated cells. It may be a result of basal PKC activation due to serum or autocrine growth factors. In the case of HER4, ligand (neuregulin-1)-induced cleavage has also been established (Zhou and Carpenter 2000), but growth hormone binding inhibits proteolysis of the growth hormone receptor (Zhang and others 2001). We previously demonstrated that IFN alpha can induce IFNaR2 cleavage, although we observed very little of the 58-kDa stub relative to the ICD in the prior experiments (Saleh and others 2004), suggesting that as the stub was generated it was efficiently processed by presenilin or another intramembrane protease. To more clearly demonstrate that IFN-alpha induces juxtamembrane cleavage of IFNaR2, cells overexpressing TACE and IFNaR2 were treated with IFN-alpha. Under these conditions, the production of the 58-kDa stub was induced in a TACE- and IFN-dependent manner (Fig. 8).

Previously, we found that processing of a full-length IFNaR2 cleavable-reporter (Fig. 5) was presenilin dependent (Saleh and others 2004). Figure 9 confirms this. Specifically, if TACE proteolysis of IFNaR2 is generating a substrate for presenilin cleavage, then the 58-kDa form should accumulate in the presence of presenilin 1 D257A, which is catalytically inactive and dominantly inhibits presenilin activity. Indeed, co-expression of IFNaR2 and presenilin 1 D257A and increasing levels of TACE produce a progressive increase in the 58-kDa form of IFNaR2.

TACE cleavage sites are not characterized by recognizable consensus sequences but, instead, reside in juxtamembrane regions that have structural features of so-called stalks: short regions of apparently little secondary structure spanning the transition between globular ligand binding domains and the helical TMD (Baumann and Frank 2002; Conte and others 2002). We have previously demonstrated that a recombinant version of IFNaR2, in which the bulk of the ECD (spanning amino acids 33–232) was deleted (IdelE), co-migrates with the 58-kDa stub protein we have observed in this report (Saleh and others 2004). Assuming accurate removal of the predicted leader sequence (amino acids 1–27), this recombinant construct should have an ECD stub of 18 amino acids. Thus, we predict that the TACE cleavage site is in the vicinity of IFNaR2 residue 262. NMR structural studies of IFNaR2 indicate that the predicted juxtamembrane cleavage site lies immediately distal to a well-defined fibronectin III-like domain in the IFNaR2 ectodomain, within a short, stalk-like region (Chill and others 2003). Sequencing of the N-terminus of the 58-kDa stub will be required to identify the *bone fide* cleavage site.

To further examine the role of presenilin in IFNaR2 RIP, independent of TACE, we employed the IdelE version of IFNaR2 (Figs. 10–12). In a prior report we observed that a GFP-ICD fusion protein accumulates in the nucleus (El Fiky and others 2008). Here, we show that when expression of the IdelE protein is induced acutely, the ICD migrates to the nucleus, as shown by fluorescence microscopy as well as cell fractionation (Figs. 10 and 11). In Fig. 12 we employed the IdelE construct in a cellular background of presenilin overexpression or in cells deleted at the *presenilin 1* and/or *presenilin 2* loci. IdelE levels were elevated, consistent with stabilization of the protein, in cells co-expressing catalytically inactive presenilin 1 and 2 or when either presenilin gene was deleted. The stabilization effect was additive in cell lines deficient in both presenilin 1 and 2. These experiments strongly imply that TACE and presenilin act sequentially to process IFNaR2 and release the ICD from the cell surface, and strengthen our assertion that the TACE cleavage site is near residue 262.

IFN-alpha inhibits the proliferation of a variety of cells, including lymphoid lineages (Erickson and others 1999; Romerio and Zella 2002) and some epithelial cells (Ceballos and others 2011). Since we were unable to establish stable clones expressing significant amounts of the 98-kDa form of the IFNaR2 receptor in wild-type MEFs (Fig. 6), we hypothesized that cleavage of IFNaR2 might inhibit proliferation of these cells. In fact, we observed that transient transfection of IFNaR2 significantly inhibited the proliferation of wild-type MEFs (Fig. 13). Importantly, the anti-proliferative effect was dependent on the Stat2 binding activity of IFNaR2, consistent with the idea that the ICD mediates this effect, perhaps via transcriptional mechanisms. However, murine IFNβ did not differentially inhibit the proliferation of the wild-type and TACE-deficient MEFs (DNS). This may indicate that type I interferons can trigger anti-proliferative effects via more than one signaling pathway, or that interferon fails to stimulate sufficient IFNaR2 cleavage to produce an anti-proliferative effect. Moreover, when we silenced TACE in Daudi cells and measured proliferation in the presence or absence of IFN-alpha (Fig. 14), we did not observe a consistent TACE-dependent effect on the IFN-alpha sensitivity of these cells. Thus, we conclude that it remains uncertain if signaling via RIP contributes to the anti-proliferative effects of IFN-alpha.

In summary, we have demonstrated that TACE can cleave IFNaR2 and that it is the predominant, if not sole, protease responsible for the juxtamembrane cleavage of IFNaR2.

Furthermore, presenilin also cleaves IFNaR2, and the TACE cleavage product is the substrate for presenilin cleavage, strongly suggesting that these 2 proteases act sequentially. Although our studies have focused on the possibility that sequential TACE and presenilin cleavage of IFNaR2 release the ICD of IFNaR2 to trigger signaling, it remains possible that ectodomain shedding or receptor degradation are the sole outcome of this regulated proteolytic processing. Thus, to determine if TACE plays a role in IFN signaling, it will be necessary to show that mutations which prevent juxtamembrane and/or intramembrane cleavage of IFNaR2 also block one or more physiologic effects of IFN-alpha.

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Author Disclosure Statement

No competing financial interests exist.

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