Release of a membrane-bound death domain by \( \gamma \)-secretase processing of the p75\( ^{NTR} \) homolog NRADD

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Summary

Neurotrophin receptor alike death domain protein (NRADD) is a death-receptor-like protein with a unique ectodomain and an intracellular domain homologous to p75\( ^{NTR} \). Expression of NRADD results in apoptosis, but only in certain cell types. This paper characterizes the expression and proteolytic processing of the mature 55 kDa glycoprotein. N-terminally truncated NRADD is processed by a \( \gamma \)-secretase activity that requires presenilins and has the same susceptibility to \( \gamma \)-secretase inhibitors as the secretion of amyloid \( \beta \) (A\( \beta \)). The ectodomain of endogenous NRADD is shed by activation of metalloproteinases. Inhibitor studies provide evidence that NRADD is cleaved in two steps typical of regulated intramembrane proteolysis (RIP). Inhibition of \( \gamma \)-secretase abrogates both the production of the soluble intracellular domain of NRADD and the appearance of NRADD in subnuclear structures. Thus, solubilized death domains with close homology to p75\( ^{NTR} \) might have a nuclear function. Furthermore, presenilin deficiency leads to abnormally glycosylated NRADD and overexpression of presenilin 2 inhibits NRADD maturation, which is dependent on the putative active site residue D366 but not on \( \gamma \)-secretase activity. Our results demonstrate that NRADD is an additional \( \gamma \)-secretase substrate and suggest that drugs against Alzheimer’s disease will need to target \( \gamma \)-secretase in a substrate-specific manner.

Key words: \( \gamma \)-Secretase, Death domain, Metalloproteinases, NRADD, Presenilin

Introduction

We recently described neurotrophin receptor alike death domain protein (NRADD; PLAIDD/NRH2) as a death-receptor-like protein that causes apoptosis upon expression in neuronal cells (Wang et al., 2003). This protein is highly conserved across mammalian species and has extensive homology to the cytoplasmic regions of p75\( ^{NTR} \) but retains a unique N-terminal ectodomain. This ectodomain is N-glycosylated and its deletion leads to a dominant negative form that protects cells from death induced by a range of endoplasmic reticulum (ER) stressors. Our studies showed that the NRADD ectodomain is required for subcellular localization and apoptotic function. Whether the short ectodomain creates enough of a surface to bind a specific ligand is not currently known. Alternate mechanisms of NRADD activation (such as proteolytic processing or association with other ligand-binding receptors) could also be operating. Further, the lack of homotypic interaction partners for p75\( ^{NTR} \)-like death domains also suggest an intracellular signaling cascade differing from that of other death receptors (reviewed by Hempstead, 2002).

Regulated intramembrane proteolysis (RIP) is one way by which transmembrane proteins are cleaved to release cytosolic domains from the membrane that are biologically active (Brown et al., 2000). Two sequential cleavages occur during RIP. The first regulated hydrolysis sheds the ectodomain and results in a substrate that is then processed by the second cleavage within the transmembrane domain. An intracellular domain is released that, in many instances, transduces nuclear signals (Cao and Sudhof, 2001) (reviewed by Ebinu and Yankner, 2002). Intramembrane cleaving proteases (I-CLiPs) have catalytic site motifs embedded in predicted transmembrane regions. Presenilins (PSs), a component of the \( \gamma \)-secretase, are I-CLiPs that were initially shown to cleave APP and Notch (De Strooper et al., 1999; De Strooper et al., 1998). Subsequently, other type-I transmembrane proteins have been found to be RIP substrates, including Notch 1-4, the Notch ligands Jagged and Delta, APP, APLP1, APLP2, ErbB-4, E-Cadherin, N-Cadherin, CD44, \( \alpha \)-Nectin, LRP (Martoglio and Golde, 2003) and, more recently, DCC (Taniguchi et al., 2003), ApoER2 (May et al., 2003), p75\( ^{NTR} \) (Kanning et al., 2003) and Syndecan 3 (Schulz et al., 2003). The cytoplasmic domains released from these substrates do not contain a common signaling motif and only p75\( ^{NTR} \) has a death domain. Among these substrates, only RIP of ErB\( ^{NTR} \) has been shown to contribute directly to cell death (Ni et al., 2003). Additional substrates are likely to exist because the \( \gamma \)-secretase cleavage requires only an ectodomain stump of 50 amino acids or less but no consensus sequences (Struhl and Adachi, 2000).

Mutations in the human genes encoding presenilins 1 and 2 (PS1 and PS2) are the main cause of familial early-onset Alzheimer’s disease (FAD). PSs are components of \( \gamma \)-secretase and have been shown to be tightly associated with Nicastrin (Yu et al., 2000), APH-1 (Goutte et al., 2002) and Pen-2 (Francis et al., 2002). Together, these form the currently known minimal essential components of the active \( \gamma \)-secretase complex (Edbauer et al., 2003; Hu and Fortini, 2003; Kimberly
et al., 2003; Takasugi et al., 2003). Mature presenilins are stabilized by Nicastrin, and Pen-2 and APH-1 are associated with presenilins in the secretory pathway. Knockdown of either of them affects PS maturation, Nicastrin levels and overall γ-secretase activity (De Strooper, 2003). Presenilins contain multiple transmembrane domains and mutation of the active site aspartates abolishes γ-secretase activity (Wolfe et al., 1999). Furthermore, aspartate transition state analogs inhibit protease activity and binding to PSs (Esler et al., 2000). PS knockout cells are defective in Aβ production (De Strooper et al., 1998). Despite such evidence, whether PSs provide the proteolytic moiety for γ-secretase is a topic of ongoing debate. In addition to their role as components of the γ-secretase complex, PS have been shown to function as ER resident chaperones affecting the maturation of Nicastrin (Edbauer et al., 2002; Herreman et al., 2003; Leem et al., 2002b), APP (Kaether et al., 2002; Leem et al., 2002a), TrkB (Naruse et al., 1998) and ICAM-5 (Annaert et al., 2001).

Cell surface molecules, including death receptors, are often modified by complex glycosylation. Very little is known about these structures beyond their role in defining the antigenic properties of the proteins. Ligand binding is often affected by glycosylation. For example, N-glycosylation of LOX-1 reduces ligand binding (Kataoka et al., 2000). Ligand specificity can also be modulated by the glycosylation pattern as for Notch (Haltiwanger, 2002). Most recently, it was shown that differential glycosylation regulates RIP of lipoprotein receptors (May et al., 2003).

Here, we show that the fully mature 55 kDa NRADD is a highly glycosylated protein that is widely expressed. NRADD is processed by two protease activities. Inhibitor studies indicate that the first cleavage is by matrix metalloproteases (MMPs) or ADAM family members. Genetic and biochemical assays indicate that γ-secretase is involved in the second cleavage. Furthermore, PS affect the maturation of NRADD and overexpression of PS2 inhibits NRADD maturation by abrogating its N-glycosylation.

Materials and Methods

Cell culture and transfections

NIH3T3, HEK293 and CHO cells, and mouse embryonic fibroblasts (MEFs) were cultured in Dulbecco’s modified Eagle’s medium (DMEM), 10% fetal bovine serum (FBS) (Bio-Whittaker, Walkersville, MD), L-glutamine (2 mM) and penicillin-streptomycin (100 U/ml). N2a, a mouse neuroblastoma line expressing hAPPsw (gift from D. J. Selkoe, Harvard University, Boston, MA) by using the quick change kit. Wt PS1 was provided by S. C. Turner (University of Michigan, Ann Arbor, MI), D285A PS1 by D. J. Selkoe (Harvard University), C99Gal4-VP16 by J. Lundkvist (Karolinska Institute, Huddinge, Sweden) and the pFRLuc-luciferase reporter and thymidine kinase promoter-renilla luciferase reporter plasmid pRL-TK were from Promega (Madison, WI).

Pulse chase

HEK 293 cells were transfected with full length N-terminally FLAG tagged-NRADD in pcDNA3 as described previously (Wang et al., 2003). Cells were labeled with medium containing 35S-methionine and 35S-cysteine for 15 minutes and chased with nonradioactive medium for different times. Immunoprecipitations were performed with anti-NRADD anti-serum (1:100) followed by sodium-dodecyl-sulfate polyacrylamide-gel electrophoresis (SDS-PAGE) and fluorography. Tunicamycin was added (5 μg ml–1) during the labeling and chase where indicated.

In vitro deglycosylation

Cell lysates were treated with glycopeptidase F (PNG-F), endoglycosidase H (Endo H) or N-glycanase, by incubating for 18 hours at 37°C as suggested by the manufacturer (Prozyme, San Leandro, CA). Tissue fractions were diluted to final 0.1% SDS and boiled for 5 minutes. 1 mM DTT and 0.75% Triton X-100 were added, and the reactions were incubated with the enzyme or mock incubated overnight.

Tissue fractionation

Indicated mouse tissues were dispersed by a tissue grinder and lysed by nitrogen cavitation. Nuclei and debris were removed by centrifugation at 900 g. The membrane fraction was collected at 10,000 g (10 minutes) and the soluble fraction S100 was obtained by centrifugation at 100,000 g (1 hour).

Immunocytochemistry

Cells were plated on gelatin-coated coverslips, stained with Hoechst number 37742 (10 μg ml–1 for 10 minutes), fixed in 4% paraformaldehyde and stained with primary antibody against NRADD (1:50) and secondary anti-rabbit-FITC (1:100) in PBS containing 3%...
bovine serum albumin (BSA) and 5% goat serum. For the blocking experiments, antibody was blocked with threefold excess antigen (w/w) for 1 hour before staining. Cells were visualized by confocal microscopy on a Zeiss LSM150.

Luciferase assays
Cells were transfected in 24-well plates, with 100 ng vector or GV constructs along with the 100 ng pFR-Luc the luciferase reporter and 25 ng of pRL-Tk. Cell lysates were assayed using the dual luciferase assay kit from Promega.

Inducers and inhibitors
γ-Secretase inhibitor treatment was for 24 hours. L-658,458 was a gift from S. Sisodia (University of Chicago, Chicago, IL) and was used at 10 μM concentration. N-[N-(3,5-difluorophenacetyl)-L-alanyl]-S-phenylglycine t-butyl ester (DAPT) and compound E were gifts from M. S. Wolfe (Harvard University, Boston, MA). DAPT was used at 500 nM unless indicated otherwise. 4-Aminophenylmercuric acetate (APMA) (Sigma) was used at 336 μg ml–1, phorbol 12-myristate 13-acetate (PMA; Sigma) at 100 ng ml–1, lactacystin (Sigma) at 10 μM.

Cell wounding and detection of γ-secretase product
Cells were scraped from the culture dish with a cell scraper and either immediately lysed or incubated for 3 hours at 37°C before lysis in 1% NP-40 lysis buffer (20 mM Tris pH 8.0, 10% glycerol, 137 mM NaCl, 5 mM EDTA, 1% NP-40, protease inhibitor cocktail (Roche), 5 mM Na3VO4.

In vitro γ-secretase assay
Two confluent 10 cm dishes of N2a cells were harvested by scraping into cold buffer H [20 mM Hapes, pH 7.4, 150 mM NaCl, 10% glycerol, 5 mM EDTA, 1 mM DTT, protease inhibitors (Roche), and 5 mM NaOv(V)]. Nuclei were removed at 800 g (10 minutes) and a membrane pellet was obtained at 100,000 g. Membranes were washed once with buffer H, resuspended in 2 ml buffer H and incubated at 37°C.

Subcellular fractionation
N2a cells were fractionated as described previously (Marambaud et al., 2002).

Results
Mature NRADD is a 55 kDa glycoprotein
Mouse NRADD is predicted to have a molecular weight of 25 kDa. However, exogenous NRADD migrates as a 40 kDa protein with less-intense bands at 55 kDa and 35 kDa. To study the post-translational modifications that lead to this discrepancy, pulse-chase experiments were performed with transfected NRADD in HEK293 cells. A 15 minute pulse gave rise to a strong 40 kDa band and a weak 35 kDa band (Fig. 1A). During the chase period, a 55 kDa band emerged and the fastest migrating band disappeared. NRADD produced in the presence of tunicamycin, a potent inhibitor of N-linked glycosylation, yielded a band that migrated with the 35 kDa band. Therefore the 40 kDa band is immature NRADD (iNR) and the 55 kDa band is the mature glycosylated form (mNR).

In vitro deglycosylations revealed that both iNR and mNR bands were converted to the fully deglycosylated 35 kDa form (dglNR) by PNG-F, a glycosidase that releases N-linked oligosaccharides from the protein backbone (Fig. 1B). Only immature NRADD is converted to dglNR by Endo H, an endoglycosidase that cuts linear oligomannose and hybrid glycans, but not complex glycans. These results suggests that the 40 kDa band carries simple N-linked glycosyl residues typical of those that are added in the ER or pre-Golgi compartments. mNR, however, is modified with complex glycan structures, probably because of modifications in the Golgi network.

NRADD isoforms are expressed in a tissue-specific manner
A polyclonal antiserum was raised using the intracellular domain of murine NRADD as antigen. The antibody was affinity purified and characterized using cell lysates from neuroblastoma (N2a) and kidney epithelial cells (293), which by reverse-transcription polymerase chain reaction (RT-PCR) analysis were found to be positive and negative, respectively, for NRADD (Wang et al., 2003). In the N2a lane, two bands migrating with mNR and iNR were visible, whereas the 293 lane was blank even after overexposure. These bands were specific for NRADD because they are not visible on a duplicate blot probed with antigen-blocked antibody (Fig. 2A, lower panel). To further document the specificity of the antibody, we analysed lysates from 293 cells transfected with NRADD and p75NTR. Even though the cytoplasmic domain of p75NTR is highly homologous to that of NRADD, no signal could be detected on the anti-NRADD blot. FLAG probing revealed that the constructs were expressed at similarly high levels and that NRADD accumulated mostly as iNR and to a lesser extent as dglNR. The antibody used in this study is therefore highly specific for the cytoplasmic domain of NRADD and, in combination with in vitro deglycosylation, it provided the means to evaluate the maturation and proteolytic processing of NRADD.

Mouse tissues were analysed to document the distribution
and maturation status of endogenous NRADD. Tissue homogenates were separated into membrane and soluble fractions, and an aliquot was deglycosylated in vitro and analysed by western blot by probing with purified antibody (Fig. 2B). A fuzzy band migrating with mNR was seen in the membrane fractions of all tissues. This band was converted to a sharp 35 kDa band upon deglycosylation (dglNR). The mNR that accumulated in the S100 fraction, most evident after deglycosylation, is probably caused by the nitrogen cavitation used during tissue extract preparation because mNR was absent from the S100 fraction from cells grown in culture which were disrupted more gently (Fig. 8). In the soluble fraction of kidney and brain a specific ~32 kDa small NRADD band (sNR) was observed. sNR was not detectable in testis or in the sciatic nerve of rats (not shown). The 40 kDa region of the brain membrane lane was partially obscured by a non-specific band (N.S.) that persisted in western blots probed with antigen-blocked antibody (not shown). Endoglycosidase digestions were performed on tissue fractions to confirm that the post-translational modifications determined in transiently expressed NRADD are also present in the endogenous proteins. PNG-F digestion converted the smeary 55 kDa mNR to a sharp band migrating with dglNR in the tissues tested. However, small NRADD seen in the soluble fraction of brain and kidney was resistant to endoglycosidase treatment indicating that it is not N-glycosylated. Endoglycosidase digestions did not affect the mobility of mNR or sNR, consistent with mNR carrying complex glycan structures (only kidney shown but the same results were observed with brain and testis).

This analysis confirms that mature NRADD is expressed widely, as indicated by the northern blots (Wang et al., 2003), whereas sNR is expressed in a tissue-specific manner, leading to its accumulation for example in the central but not in the peripheral nervous system. The solubility and the lack of glycosylation of sNR suggest that it is an NRADD isoform that is lacking at least part of the transmembrane domain and the ectodomain. The existence of such isoforms is indicated by expressed sequence tag and RT-PCR analysis (Frankowski et al., 2002). The molecular weight and lack of glycosylation would suggest that sNR is equivalent to PLAIDD_S as described by Frankowski et al., but sNR was not found in the membrane fraction, whereas exogenously expressed PLAIDD_S localized with membranes. Nevertheless, we conclude that, in addition to full-length NRADD, at least one of the putative NRADD isoforms is expressed at the protein level in adult mice.

NRADD is distributed throughout the cell, including the nucleus

To analyse the subcellular distribution of NRADD, immunocytochemistry was performed on primary and transformed cells (Fig. 3). The NRADD signal was distributed heterogeneously in MEFs. A low-level uniform signal is obtained throughout the cell, including the nucleus. Often, the perinuclear space was strongly stained (Fig. 3, open arrowhead). The edge of the cell reacted more prominently with the antibody, particularly structures resembling lamellipodia (Fig. 3, closed arrowhead). NIH3T3 cells stained similar to MEF cells except that nuclear staining was higher and not uniform, resembling nucleoli. The mouse neuroblastoma line N2a had less cytoplasm, making it more difficult to distinguish between staining of the perinuclear space and lamellipodia. The frequency of nuclear organelle staining was dependent on the density at which the cells were immunostained. Confluent N2a cells lacked nuclear staining altogether (Fig. 9). However, as in NIH3T3 cells, strong nuclear structures were observed when the cells were stained at lower density. Controls with blocked antibody showed speckles distributed randomly throughout the slide except for the nucleus. Thus, both cytoplasmic and nuclear staining were specific for NRADD, confirming the high specificity of the antibody observed on western blots (Fig. 2A). The heterogeneous staining of NRADD was similar to what we previously observed with the exogenous molecule, which was associated with membranes throughout the cell (Wang et

![Fig. 2. NRADD expression in mouse tissues.](image-url)
NRADD is a γ-secretase substrate

NRADD is N-terminally processed by MMP/ADAM proteinases

When probed with NRADD antibody, the exogenously expressed NRADD in various cell lines revealed bands corresponding to a truncated form. An example of this truncated form is shown in Fig. 4A. Transfection of a C-terminally tagged Myc-His6 NRADD yielded, in addition to mNR and iNR, a 32 kDa band that was called N-terminally processed NRADD (NpNR). This band disappeared upon incubation with GM6001, a specific inhibitor of the MMP and ADAM (a disintegrin and metalloprotease) family of proteinases (Levy et al., 1998). Incubation with APMA, an activator of metalloproteinases, greatly increased the ratio of processed to full-length NRADD species. The overall signals are lower owing to the toxicity of APMA. The APMA effect was reversed by co-incubation with GM6001, further confirming that NRADD is processed by one of the members of the MMP/ADAM family of proteinases. N-Terminal processing was also suggested by our earlier observation that processing products are not detected when analysing NRADD that has been N-terminally tagged with FLAG (Wang et al., 2003). To determine how much of the terminus of NRADD was cleaved by MMP/ADAM, we took advantage of the two N-terminal glycosylation sites located on the ectodomain (Fig. 4B). The N-glycosylation sites are at Asn4 and Asn37; doubly, singly and unglycosylated forms have different gel mobilities (Wang et al., 2003). Therefore, a good estimate of the extent of N-terminal processing is obtained by comparing the Mr of the cleavage products with and without deglycosylation. Digestion with PNG-F converted the mature and immature forms of NRADD to the 35 kDa deglycosylated form. The APMA-inducible band was not affected by PNG-F digestion, indicating that it was truncated by at least 37 amino acids. MMP/ADAM proteases therefore removed most of the short NRADD ectodomain.

Fig. 3. Subcellular localization of NRADD. Cells were grown on gelatin-coated coverslips, stained with Hoechst dye 33342 (blue) and processed for immunocytochemistry with anti-NRADD antibody (green) and visualized by confocal microscopy. MEFs are mouse embryonic fibroblasts from day 16.5 embryos. Notice the common perinuclear staining (open arrowhead) in MEFs. The edge of the cells are also stained more prominently, particularly structures resembling lamellipodia (closed arrowhead). In the fibroblast line NIH3T3 and the neuroblastoma line N2a, additional staining of nuclear substructure is observed (arrows). All samples were stained in parallel with antigen-blocked antibody. Notice the complete lack of background in the nuclei.

Fig. 4. NRADD is proteolytically processed. NRADD-MycHis was transfected into CHO lines. (A) Cells were incubated for 24 hours with the compounds as indicated. Cell lysates were analysed by western blot with anti-NRADD antibody. (B) The same as (A) except that the indicated lysates were also digested with PNG-F. NpNR, N-terminally processed NRADD.

N-Terminally truncated NRADD is a γ-secretase substrate

NRADD was N-terminally processed and partially localized in the nucleus, so we explored the possibility that NRADD was released from the membrane by a second cleavage mediated by γ-secretase. A luciferase assay was adopted by inserting the Gal4 DNA-binding domain and the transactivation domain of VP16 close to the cytoplasmic face of the transmembrane region of NRADD (Fig. 5A). Analogous constructs have recently been used to characterize other γ-secretase substrates, including APP (Cao and Sudhof, 2001). Expression of the
constructs was verified in 293 cells (Fig. 5B). As predicted from our analysis of glycosylation sites, only FL-NRGV accumulates as fully and partially glycosylated proteins (mNRGV and iNRGV). The transmembrane-domain-containing construct, NR45GV, yielded a protein with an Mr consistent with a deglycosylated GV fusion protein. NR75GV, lacking the transmembrane domain, was similarly not glycosylated. These constructs were transfected into N2a cells with a GAL4-promoter-driven luciferase reporter (Fig. 5C). Transfection of FL-NRGV did not result in any luciferase activity above background. NR45GV produced a robust ~40-fold induction of luciferase above vector control. The activation by NR75GV was even higher (~100-fold). The specific secretase inhibitor L-685,458 was added to ascertain that the luciferase activity was γ-secretase dependent (Shearman et al., 2000). Luciferase activation by NR45GV was completely abrogated by the inhibitor but it had no significant effect on the activity of the NR75GV control. The expression levels of the NRGV constructs were measured by western blot (Fig. 5D). Both FL-NRGV and NR45GV were expressed at similar levels. L-685,458 did not reduce the expression of NR45GV indicating that the inhibition was not due to non-specific effects on the expression of the γ-secretase substrate. Interestingly, NR75GV was barely detectable on western blot despite its strong induction of the luciferase reporter. NR75GV was degraded during the N-glycanase digestion, suggesting that the γ-secretase cleavage product of NRADD was unstable.

NRADD processing by γ-secretase is presenilin dependent

To further ascertain that NRADD was processed by γ-secretase, genetic deletion of γ-secretase activity was used. FL-NRGV, NR45GV and NR75GV constructs were transfected into wild-type and PS−/− double-knockout blastocyst-derived cells cultured in the presence of mLIF (Sato et al., 2000). Transfection of FL-NRGV did not result in any luciferase activity above background in either wild-type or knockout cells (Fig. 6A). NR45GV produced a ~100-fold induction of luciferase above vector control in the wild-type cells that was completely abrogated in the knockout cells. The activation by NR75GV was even higher (~700-fold) in both wild-type and knockout cells, revealing that the knockout cells are competent at inducing the Gal4 promoter when activated by this control that does not require γ-secretase cleavage. Expression of FL-NRGV and NR45GV was similar in both wild-type and knockout cells. As in N2a cells, NR75GV was rapidly degraded in both blastocyst-derived lines (Fig. 6B).

Distinct γ-secretase activities produce secreted and intracellular Aβ. They are differentiated by their susceptibility to γ-secretase inhibitors and their requirement for presenilins (Grimm et al., 2003; Wilson et al., 2002). Hence, we performed an inhibitor titration to biochemically characterize the NR45GV-processing activity. N2a cells were transfected with NR45GV and treated with DAPT or compound E for 24 hours and the luciferase activity measured and compared to values without inhibitor (Fig. 6B). The inhibition curve revealed an 50% inhibitory concentration (IC50) value of ~30 nM for DAPT and in the low nM range for compound E. Both IC50 values are similar to that reported for the generation of extracellular Aβ from APP (Kornilova et al., 2003). The level of protein expressed was constant and not affected by the inhibitors. Thus, the same activity that leads to the formation of extracellular Aβ also processed NR45GV, even though NRADD is widely distributed in the cell.
NRADD is a γ-secretase substrate

Functional interactions between NRADD and presenilins
Expression and maturation of components of the γ-secretase are interdependent and tightly controlled. For example, deletion of PS leads to a defect in Nicastrin maturation and stable expression of human PS leads to a replacement of the endogenous protein, not overexpression, suggesting competition for limiting endogenous factors. To explore whether NRADD is subject to similar controls, we analysed the status of NRADD in wild-type PS and double-knockout cells (Fig. 7A). Endogenous NRADD migrated slightly more slowly in PS−/− (m+NR) cells than in the wild-type cells (mNR). In vitro deglycosylation converted both forms to deglycosylated NRADD, demonstrating that the difference was due to the extent of N-glycosylation. Incubation with the γ-secretase inhibitor L685,458 had no effect, suggesting that the proteolytic activity is not required for the control of NRADD maturation. To reduce the possibility that aberrant maturation of NRADD was caused by clonal variation, the experiments were repeated using a second pair of double knock out and wt control cell lines (Fig. 7A, right panel). The presenilin-deficient line BD8 also contained a substantial proportion of NRADD with more extensive N-glycosylation.

The effect of PS overexpression on NRADD was investigated by transient expression and pulse-chase experiments in 293 cells. The PS replacement phenomenon is not observed in this experimental system and high levels of PS expression can be obtained. Compared with expressing NRADD alone (Fig. 1A), a different maturation pattern was observed (Fig. 7B). Co-transfection of PS2 led to the retention of the 35 kDa deglycosylated form. A band migrating with singly glycosylated NRADD was visible (dgNR), suggesting a delay or inhibition of NRADD glycosylation by PS2. To confirm that the PS2-induced rapidly migrating band is indeed deglycosylated NRADD and not a proteolytic fragment, in vitro deglycosylations were performed (Fig. 7C). Lysates from cells transfected with FLAG-NRADD alone (Fig. 7C, lanes 1,2) or co-transfected with PS2 (Fig. 7C, lanes 3,4) were immunoprecipitated with anti-FLAG-antibody-bound beads and mock treated (Fig. 7C, lanes 1,3) or digested with PNG-F (Fig. 7C, lanes 2,4). NRADD expressed on its own yielded mNR and iNR that were converted to rapidly migrating dglnR by PNG-F. However, NRADD produced in the presence of PS2 did not change mobility upon PNG-F digestion, indicating that it was not N-glycosylated. NRADD lysates from cells incubated with or without tunicamycin served as mobility standards (Fig. 7C, lanes 5,6).
To find out whether the inhibition of NRADD glycosylation correlated with any other known function of PS, co-transfections were performed with two types of PS mutant (Fig. 7D): (1) an aspartic acid mutant in which the putative active site residue has been altered to produce a protein deficient in supporting γ-secretase activity; and (2) a FAD mutant, which is known to lead to early-onset Alzheimer’s disease. NRADD accumulates in the deglycosylated form in the presence of wild-type PS2, but not wild-type PS1 or D385A PS1. Transfection with the FAD mutant N141I PS2 has the same effect as wild-type PS2, indicating that the inhibition of NRADD maturation is not affected by FAD PS2 mutations. Significantly, D366A PS2, is not as effective as wild-type or FAD PS2, and a significant amount of glycosylated NRADD was observed even when D366A was grossly overexpressed. Lowering the levels of D366A expression abrogates its effect on NRADD maturation completely (not shown). Lack of γ-secretase involvement in the PS2-mediated inhibition of N-glycosylation was also indicated by the inability of the γ-secretase inhibitors DAPT and compound E to promote NRADD maturation expressed with PS2 (not shown). Inhibition of glycosylation was only observed under conditions of overexpression and was not detectable if wild-type or FAD PS2 was stably expressed at replacement levels. Another notable finding is that D366A was processed to yield a C-terminal fragment in our experiments, which is not seen when exogenous aspartic acid mutants replace endogenous PS in stably transfected cells. Significant processing is observed, however, when aspartic acid mutants are expressed in a PS-null background (Nyabi et al., 2003). This is the first time that it has been shown, albeit in a non-physiological system, that the D366A PS2 mutation can affect functions other than its role in proteolysis.

Fig. 7. Presenilins affect NRADD maturation. (A) Lysates from wild-type (+/+ or PS1,2 double knockout (−/−) cells were analysed by western blot with anti-NRADD antibody. Protein loading was determined by probing with anti-GAPDH. mNR, mature NRADD of higher molecular weight. One of four experiments shown. The independently blastocyst derived clonal lines are designated as in Sato et al. (Sato et al., 2000). NRADD and PS2 were co-transfected into 293 cells and pulse-chase experiment was performed as in Fig. 1A (one of three experiments shown). mglNR indicates an NRADD species migrating with N4Q single glycosylation mutant (Wang et al., 2003). (C) In vitro deglycosylation. FLAG-NRADD was transfected alone or together with PS2 in 293 cells. Cell lysates (lanes 1-4) were immunoprecipitated with FLAG beads and either mock treated (lanes 1,3) or treated with PNG-F (lanes 2,4) before western-blot analysis. Lanes 5, 6 were loaded with cell lysates produced in the absence or presence of tunicamycin, respectively (n=4). (D) NRADD was co-transfected with the indicated presenilin constructs and cell lysates prepared 48 hours later. Lysates were analysed on anti-NRADD, anti-PS2 and anti-PS1 western blots. +, * and – indicate aggregated, full-length and processed forms of PS, respectively (n=5).
NRADD is a γ-secretase substrate

To demonstrate the physiological relevance of the results we obtained using truncated NRADD, we sought a stimulus that would induce N-terminal processing of the endogenous protein. Scraping of cells from their substrate has been shown to mimic cell wounding and to induce the shedding of various ectodomains from transmembrane proteins (McNeil et al., 1989; Okamoto et al., 2001). By scraping NIH3T3 cells, we induced NRADD processing (Fig. 8A). Incubating the scraped cells at 37°C for three hours increased the production of the ~28 kDa NpNR band that was inhibited by GM6001. Endogenous and exogenous NpNR differ in size owing to the His6 and Myc tags fused to the C-terminus of the latter (Fig. 4). When lactacystin was included during the incubation, an additional ~17 kDa band was visible that was inhibited by DAPT, a γ-secretase inhibitor. The ~28 kDa NpNR band is therefore a product of the activity of the MMP/ADAM proteinases and is the substrate for γ-secretase, which processes it to yield the γ-secretase product (γ-PNR). The size of this product, ~17 kDa, corresponds to the entire ICD of NRADD. Inhibition of both products by GM6001 establishes a product to substrate relationship between these NRADD forms.

In vitro processing assay was developed to obtain further evidence that γ-secretase activity produced γ-PNR. Because the γ-secretase activity is associated with membranes, isolated membrane fractions should still be able to produce the product. Indeed, when membranes from N2a cells were purified and incubated at 37°C, the γ-PNR band was generated (Fig. 8B). As in the in vivo assay, inclusion of DAPT prevented this product from being generated. Addition of lactacystin had no effect, consistent with the proteosomal component having been removed during the membrane purification.

Subcellular fractionation was performed to corroborate the implication that NpNR and γ-PNR bands are membrane bound and soluble, respectively (Fig. 8C). N2a cells were fractionated into nuclear (N), 100,000 g supernatant (SN), 1% Triton-X-100-soluble membranes (X100SN), and Triton-X-100-insoluble (X100P) fractions. mNR and iNR are exclusively found in the membrane fraction. APMA treatment resulted in an increase of the NpNR form that was also membrane bound. About half of the NpNR present in the untreated cells separated into the cytoskeletal fraction (X100P). The intensity of this band was not affected by APMA treatment, suggesting a separate pool of this NRADD form. APMA treatment, however, did result in the appearance of some NpNR in the nuclear pellet, probably indicating increased binding to

**Fig. 8.** Endogenous NRADD is a RIP substrate. (A) NRADD processing in vivo. NIH3T3 cells were scraped from the tissue culture dish and incubated at 37°C for 3 hours in the presence of the indicated inhibitors. The first lane (4°C) shows cells kept at 4°C after scraping and lysed immediately. Cell lysates were analysed by anti-NRADD western blot. γ-Secretase substrate (NpNR) and product (γ-PNR) are indicated. (B) NRADD processing in vitro. N2a cell membranes were isolated and incubated at 37°C for 1 hour with the indicated inhibitors. (C) Subcellular fractionation. N2a cells were treated for 40 minutes with APMA or left untreated, then washed and incubated for 3 hours in the presence of lactacystin (10 μM). Cells were harvested and fractionated as described in Materials and Methods. N, Nuclear fraction; S100SN, Cytosol; X100P, Triton-X-100-insoluble fraction; X100SN, 1% Triton-X-100-soluble membranes (n=3). (D) Detection of ECD in conditioned medium. 293 cells were transfected with NRADD tagged at the N-terminus with AU1 (AU1 NR) or vector (Vect.). 18 hours after transfection PMA (100 ng ml–1) was added as indicated and, after 70 hours, medium was harvested and cell lysates prepared. Lysates and conditioned medium were PNG-F treated. Conditioned media were immunoprecipitated with anti-AU1 antibody and analysed by anti-AU1 western blot of a 16% Tricine gel. *NRADD truncated at the C-terminus.
membranes bound to the nuclei (Fig. 9). γ-PNR was only detectable upon APMA addition and appeared almost exclusively in the soluble fraction (S100SN). Treatment with the protein kinase C activator PMA or cell scraping resulted in the same effects on NRADD processing and fractionation (not shown).

To investigate the fate of the ectodomain upon proteolytic processing, NRADD tagged at the N-terminus with AU1 was expressed and the appearance of tagged fragments was monitored both in the conditioned media and cell lysate (Fig. 8D). Because glycosylation interferes with immunoprecipitation, all samples were treated in vitro with PNG-F. A band of ~15 kDa was detectable upon AU1 transfection. Treatment with PMA greatly increased the intensity of these cleavage products. Several products of similar size are generated suggesting multiple closely spaced cleavage sites close to the extracellular face of the ectodomain. A small amount of these products was also apparent in the cell lysate, suggesting that intracellular cleavage can also give rise to similar products.

Inducers of NRADD processing enhance its nuclear immunoreactivity

Immunocytochemistry was performed to observe changes in subcellular localization by stimuli that induced NRADD processing (Fig. 9). When N2a cells were fixed at a high density, little NRADD signal is observed in the nucleus. Incubation with lactacystin resulted in the appearance of faint, diffuse nuclear staining, suggesting that there was constitutive turnover of NRADD. The addition of APMA and PMA caused the appearance of subnuclear structures. Almost every nucleus contained about three NRADD-containing regions with bright staining. High-power micrographs revealed that these nuclear bodies did not localize with subnuclear structures that stained brightly with Hoechst (not shown). However, these brightly staining regions were clearly nuclear, as best documented in the z-axis stack, in which the same focal planes that stain brightly with Hoechst also stain brightly with anti-NRADD antibodies. DAPT negated the effect of PMA, eliminating almost all the nuclear staining. The lack of signal when antigen-blocked antibody was used indicates that the signal is specific. These observations are consistent with the hypothesis that γ-secretase cleavage of NRADD results in the nuclear translocation of a NRADD fragment containing the intracellular domain (NRICD). The subcellular fractionation experiments indicate that this fragment was loosely bound and solubilized during the cell disruption and fractionation procedure.

Discussion

NRADD maturation

Mature NRADD is widely expressed as a 55 kDa protein carrying Endo-H-resistant modifications typical for transmembrane proteins. Immature NRADD and its processing products were only detectable in cells grown in culture, suggesting cell-growth-driven biosynthesis and rapid degradation, respectively. Single point mutations of the N-glycosylation sites N4 and N37 are more stable than the wild type but membrane localization is not affected (Wang et al., 2003) (not shown). We have not yet found a function for the N-glycan modification but, given the small size of the ectodomain, the bulky glycans will probably determine the binding of putative NRADD ligands.
As with another γ-secretase substrate, APP, presenilin deficiency leads to hyperglycosylated NRADD (Leem et al., 2002a). However, in contrast to APP, no increase in cell surface localization was apparent (not shown). Components of the γ-secretase complex are co-regulated, which leads to replacement of the endogenous molecules when these proteins are expressed exogenously (Thinakaran, 2001). This phenomenon is thought to be caused by competition for limiting maturation factors. NRADD is not co-regulated in a similar manner, because exogenous expression of NRADD yields mature NRADD without replacing the endogenous protein (not shown). Intriguingly, however, gross overexpression of PS2 inhibits NRADD glycosylation, indicating a role for PS2 in NRADD maturation in the ER (Fig. 7).

Proteolytic processing of NRADD

Here, we show that the first proteolytic product of the RIP process is membrane bound. Because it was not N-glycosylated, cleavage occurred between amino acid 37 and the transmembrane domain (amino acid 50); the bulk of the ectodomain is shed into the medium. The second cleavage by the γ-secretase produced soluble NRICD with a Mr corresponding to the entire cytoplasmic domain of NRADD.

Immunocytochemistry detected NRADD in multiple cellular compartments, including the perinuclear space, nuclei and lamellipodial structures. The inducers of the first cleavage, cell wounding, PMA and APMA, do not target specific compartments and we cannot deduce where in the cell NRADD is processed. The significant amounts of intracellularly localized NRADD do suggest that NRADD might not only be at the cell membrane. The small proportion of cleaved ectodomain found in the cell lysates is also consistent with this possibility.

The inhibitor experiments clearly establish a substrate-product relationship between mNRADD, NpNR and γ-PNR. The luciferase experiments also suggest that the γ-secretase activity responsible for the hydrolysis at the second peptide bond is dependent on PS and has the same inhibitor profile as the γ-secretase activity that produces extracellular Aβ. As for APP, there is no close spatial correlation between the substrate, NRADD, and the putative protease, presenilin (Annaert and De Strooper, 1999; Cupers et al., 2001). This might indicate that NRADD processing occurs only in select subcellular locales.

The RIP cleavage of NRADD was observed in all cells that we assayed (fibroblasts, neuroblastoma, blastocyst-derived cells). Cell winding and APMA and PMA treatment induce the processing of many other RIP substrates and an NRADD-specific inducer remains to be identified. Even though NRADD has only a 50-residue-long ectodomain, further truncation is necessary for γ-secretase cleavage to occur, highlighting the strict requirement by γ-secretase for a short extracellular stump. Glycosylation is likely to contribute to the inability of γ-secretase to cleave full-length NRADD as unmodified ectodomains of this length can be a γ-secretase substrate (Struhl and Adachi, 2000). Discovering which MMP or ADAM perform the first cleavage in response to a specific stimulus will require further study of these large families of proteinases (Nagase and Woessner, 1999; Primakoff and Myles, 2000).

NRADD also stands out among the other γ-secretase substrates as the only transmembrane protein that does not contain a signal peptide. NRADD is therefore transported to the cell membrane and to the cell compartments where γ-secretase activity is present using other trafficking signals. We have previously shown the cell membrane targeting requires signals both in the ectodomains and in the cytoplasmic tail.

NRADD isoforms

The tissue western blots revealed the presence of an additional NRADD-specific band, sNR (Fig. 2). This band was expressed in a tissue-specific manner and migrated differently from the NRADD processing products described above. It was also not detected in any of the cell lines before or after induction of NRADD processing. Previous analysis of the expressed sequence tags and RT-PCR assays revealed several potential isoforms (Frankowski et al., 2002). Our interpretation is that the tissue western blots reveal the expression of at least two NRADD isoforms at the protein level. The identity of sNR will have to await purification and amino acid sequencing. sNR is not likely to be cleaved by γ-secretase because it is not membrane associated.

NRADD signaling

NRADD joins a growing list of γ-secretase substrates, many of which are processed according to the paradigm established for Notch. Their ICDs often translocate to the nucleus to regulate transcription. Immunocytochemical staining indicates that NRICD is also translocated to the nucleus. The NRICD contains a death domain, which commonly functions in cytoplasmic signaling. However, a recent report indicating that p75NTR is also a RIP substrate suggests that some death domain proteins might have nuclear functions (Kanning et al., 2003). NRICD probably does not function as a co-activator because deletion of the VP16 domain from NR45GV abrogated its transcriptional activation function (not shown). To date, no functions have been identified for death domains in the nucleus. However, the death domain of the NRADD homolog p75NTR interacts with SC-1 and NRIF. Both of these proteins have predominant nuclear localization but the functional consequences of their interaction with p75NTR remain to be elucidated (Casademunt et al., 1999; Chittka and Chao, 1999).

The function of ectodomain shedding is not well understood, although it is frequently observed in a range of cell surface proteins including death receptors (Guo et al., 2002; Reddy et al., 2000). The site of peptide bond hydrolysis by ADAMs has not been precisely determined but, given that ADAMs are membrane bound, it is thought to be close to the extracellular face of the cell membrane. It is therefore likely that, in addition to shedding, the C-terminal fragment of all death receptors is further cleaved and released to the cytoplasm by the constitutive activity of γ-secretase. ICD release to the cytoplasm could easily have escaped detection given the dearth of antibodies recognizing ICDs and the rapid degradation of γ-secretase products.

Implications for PS function

Our studies show that PSs are involved in the maturation of
yet another transmembrane protein. First, PS deficiency led to increased N-glycosylation of NRADD but, unlike APP, no changes in NRADD localization were visible in PS-deficient cells (Cai et al., 2003). Second, PS2 overexpression led to complete inhibition of N-glycosylation. Significantly, the PS2 D366A mutant was a less potent inhibitor of NRADD N-glycosylation. Thus, in this experimental setting, the aspartic acid residue is required for another function of PS in addition to proteolysis. Even though this effect is only observed upon overexpression of PS2, it might indicate that the aspartic acid moiety is affecting the PS more broadly than originally proposed for PS1 (Wolfe et al., 1999). Inhibition of glycosylation by PS2 was not affected by DAPT, compound E or L-685,458, further confirming that it is not dependent on proteolysis (not shown). By contrast, Leem et al. showed that increased cell surface exposure of APP, was dependent on the proteolytic activity of γ-secretase and D385 of PS1 (Leem et al., 2002a). A recent report shows that PS1 aspartic acid mutants expressed in a PS null background are incorporated into a 400 kDa complex and promote Nicastrin maturation and Pen-2 stabilization without reconstituting γ-secretase activity (Nyabi et al., 2003). Our results suggest that, when overexpressed, additional defects in PS2 D366A function can be identified that pertains to the non-proteolytic function of PS. It is also curious that expression only of PS2 and not of PS1 affected NRADD maturation. Different effects of PSs are indicated by the only partially overlapping phenotypes of the PS1 and PS2 knockout mice, and by the greater role PS1 plays in the maturation of Nicastrin (Chen et al., 2003; De Strooper, 2003). PS1 and PS2 also seem to participate in diverse γ-secretase complexes distinguishable by their differing susceptibility to inhibitors (Lai et al., 2003). However, further studies are needed to characterize more clearly the composition of the various γ-secretase complexes.

Drug screening efforts aimed at identifying small molecule inhibitors of γ-secretase have been fruitful and have produced a range of compounds. Although single dose administration of these inhibitors results in no apparent toxicity and some pharmacological benefits, long term use might reveal serious side effects (Dovey et al., 2001; Lanz et al., 2003). In vertebrates, application of γ-secretase inhibitors is toxic to the development of embryos and, in view of the large number of γ-secretase substrates recently described, even adults might be affected (Geling et al., 2002). Given the diversity of γ-secretase complexes, more specific inhibitors might be useful. This task will be facilitated by the discovery of more components of the γ-secretase complex and the description of non-APP substrates such as NRADD.

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