A Sensitive and Quantitative Assay for Measuring Cleavage of Presenilin Substrates*

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The presenilin (PS) proteins are components of the γ-secretase activity, which is central in the pathogenesis of Alzheimer’s disease. Here we present a novel cell-based reporter gene assay for the quantification of PS-controlled γ-secretase cleavage of the Alzheimer amyloid precursor protein (APP). We show that this assay offers several advantages, including increased sensitivity and specificity, improved quantification of cleavage, and simultaneous detection of all γ-secretase cleavages in APP. Furthermore, the APP assay can be used in parallel with a similar assay that records γ-secretase cleavage of a Notch receptor. The use of these assays to analyze the effects of two known γ-secretase inhibitors and postulated PS active site mutants on APP and Notch processing demonstrated that inhibitors and mutants that differently affect Notch and APP cleavage can be identified rapidly. The possibility in using these assays for high throughput screening of candidate γ-secretase inhibitors for APP and Notch in parallel opens up new vistas to systematically search for novel inhibitors that selectively block APP cleavage while not affecting Notch signaling.

PS1 proteins and APP are frequently mutated in familial Alzheimer’s disease (AD), and PS controls the γ-secretase-mediated processing of APP. The level of γ-secretase activity controls the amount of amyloid β-peptide (Aβ) formation from the APP intermediate fragment C99 (see Fig. 1A). Familial AD-associated mutations in PS lead to a specific increase in production of the more fibril-prone Aβ42 variant (1), a peptide species linked to the early pathological changes seen in AD (2,3). It is therefore of interest to develop pharmaceutical approaches that reduce the level of γ-secretase activity acting on APP. However, a complicating factor for this approach is that PS also controls cleavage of Notch receptors (4), which are critical for many functions during development and in the adult organism. γ-Secretase inhibitors aimed at reducing Aβ formation might thus also affect Notch cleavage and have unwanted side effects. Therefore, high throughput screening assays that record γ-secretase cleavage of both APP and Notch would be useful for the identification of novel APP-specific γ-secretase inhibitors. In this study we present a novel cell-based reporter gene assay for monitoring γ-secretase cleavage on APP.

EXPERIMENTAL PROCEDURES

DNA Constructs—Silent mutations were first incorporated in a pcDNA3-C99 construct to create an Ascl site immediately 3’ of the nucleotides encoding the transmembrane-spanning region of C99. A cDNA encoding the DNA binding/transactivation domains Gal4/VP16 (GVP) was subsequently amplified by PCR and cloned into the Ascl site of C99 generating the C99-GVP construct. Both PS1 and PS2 cDNAs were obtained in the pcDNA3 backbone. All PS1 mutants were generated by PCR using Ffu Turbo polymerase according to the QuikChange mutagenesis protocol (Stratagene, La Jolla, CA). The PS1 D257A/D385A double mutant construct was generated out of the PS1 D257A and PS1 D385A-encoding plasmids by a subcloning approach. All constructs were verified by sequencing using the ABI377 automated sequencer (PerkinElmer Life Sciences). The construct encoding Notch ΔE-GV and the UAS-responsive reporter gene construct MH100 and the CMV-βgal plasmid have been described previously.2

Cell Transfection Experiments and Reporter Gene Assay—HEK293 cells and blastocyst-derived embryonic stem cells lacking PS1 and PS2 expression, BD8 cells, were cultured as described previously.2 All transfections with BD8 cells were carried out in 24-well tissue culture plates (Costar), and transfection of 293 cells was carried out in 10-cm cell culture dishes (Costar). For each well of the 24-well tissue culture dish, 200 ng of BD8 cells were transfected and analyzed for reporter gene activity 48 h post-transfections. For the 10-cm dishes, 2 μg of MH100, 400 ng of CMV-βgal, and either 2 μg of C99-GVP or Notch ΔE-GVP were mixed and used to transfect the 293 cells according to the LipofectAMINE PLUS protocol. The transfected 293 cells were split and seeded into 24-well plates, and γ-secretase inhibitors, were added. Twelve hours posttransfection, the cells were harvested and analyzed as described previously.2

RESULTS AND DISCUSSION

In this report we describe a new sensitive and quantitative assay for γ-secretase-mediated cleavage of APP. To generate a reporter APP protein, from which γ-secretase cleavage can be recorded, we incorporated a Gal4 DNA-binding/VP16 transactivation (GVP) domain into the C99 fragment of APP (Fig. 1A), which is an immediate substrate for γ-secretase in vivo (5). Cleavage of the resulting hybrid protein, hereafter referred to as C99-GVP, liberates the C-terminal region, including the GVP moiety, which is translocated to the nucleus due to the nuclear localization signals in GVP. In the nucleus, GVP specifically signals through a UAS-luciferase reporter gene by virtue of the strong transactivation domain and specific binding to a UAS promoter (Fig. 1A). The assay has several attractive features. First, it should be specific for γ-secretase cleavage.
since the GVP moiety is inserted only 13–15 amino acid residues C-terminal of the γ-secretase cleavage sites, thus minimizing unspecific cleavage events. Second, it should be very sensitive since VP16 is a strong transactivation domain on the UAS promoter. Third, it will record the total γ-secretase cleavage activity, i.e., γ-cleavage generating both Aβ40, the more abundant Aβ variant (6), and the pathogenic Aβ42 species as well as minor cleavage products generated by processing at
cleavage at the site 2 and site 3 in a ligand-independent constitutive manner. Following Notchceptible to presented as -fold activation of mock-transfected cells and show mean for Notcherase expression from the UAS promoter.

We first examined the specificity of the C99-GVP assay by recording γ-cleavage in cells expressing different combinations of PS genes. After transfection of C99-GVP into HEK293 cells, which express endogenous levels of wild-type PS1 and PS2, a strong activation of the UAS-luciferase gene was observed (Fig. 1B). In contrast, transfection of C99-GVP into BD8 cells, which lack PS1 and PS2 expression, did not activate the reporter gene (Fig. 1B), indicating that cleavage is completely dependent on the presence of PS proteins. To test the assay for PS1 and PS2 individually, we transfected expression constructs of PS1 and PS2 into the BD8 cells and measured the cleavage of C99-GVP. This resulted in strong reporter gene activation in both cases, demonstrating that both PS1 and PS2 are capable of mediating γ-cleavage of C99-GVP (Fig. 1B). These results are in agreement with recent reports from Drosophila in which a similar construct was successfully used for genetic analysis of PS-controlled γ-cleavage (10).

To address whether the assay was quantitative, we transfected different amounts of PS1 into BD8 cells. The level of reporter gene activation increased with the amount of PS expressed, primarily in the range from 10 pg to 10 ng of added PS1 plasmid DNA (Fig. 1C). Interestingly, above 10 ng, only a minor increase in cleavage activity was observed. This may suggest that the production of the activated form of PS required for cleavage is limiting (i.e. by adding too much PS, the proteolytic machinery generating the N- and C-terminal PS fragments is saturated) or, alternatively, that a component other than PS in the active γ-secretase complex is the rate-limiting determinant of cleavage (11). We also tested whether the assay responded quantitatively to the addition of two known γ-secretase inhibitors, MW167 (12) and L-685,458 (13). Addition of both MW167 and L-685,458 resulted in a dose-dependent decrease in γ-secretase activity (Fig. 1D).

As discussed above, γ-secretase inhibitors that do not discriminate between APP and Notch cleavage may have dangerous side effects if used in a therapeutic setting. Both APP and Notch are cleaved at defined sites in the intramembranous region, and thus it should be possible to use a very similar GVP-based assay to monitor γ-secretase-mediated cleavage (site 3 cleavage) of Notch (Fig. 2A). We have recently shown that a truncated Notch receptor that acts in a ligand-independent constitutive manner (14) and harbors the GVP moiety inserted immediately C-terminal of the site 3 cleavage site (Notch ΔE-GVP) is cleaved specifically in the presence of PS in adjacent residues will be measured simultaneously. This is a marked improvement over current γ-secretase assays that rely primarily on analyzing secreted Aβ species with specific antibodies while excluding the large, and structurally distinct (7), intracellular pool of Aβ. Moreover, the C99-GVP construct harbors the native APP cytoplasmic tail, ensuring correct intracellular trafficking by the sorting signals contained within the tail (8, 9). Finally, the assay records cleavage only from the C99-GVP protein and is thus insensitive to the levels of endogenous γ-secretase substrates in the cell.

### Figure 2

**Processing of Notch ΔE-GVP is PS-dependent and susceptible to γ-secretase inhibitors.**

*Panel A* shows an overview of the ligand-induced processing events of the Notch receptor and the γ-secretase assay. Notch ΔE is a truncated form of a Notch receptor, which is cleaved at site 2 and site 3 in a ligand-independent constitutive manner. Following cleavage at the γ-secretase site (site 3) the intracellular domain containing the GVP domain translocates to the nucleus and initiates luciferase expression from the UAS promoter. *B. PS proteins are required for Notch ΔE-GVP processing.* Cleavage of Notch ΔE-GVP in BD8 cells was analyzed in the presence of different amounts of transfected PS1 encoding cDNA. As for C99-GVP (Fig. 1C), only a very minor increase in γ-secretase-mediated cleavage of the Notch ΔE-GVP substrate was observed above 10 ng of transfected PS1-encoding cDNA. Data are presented as -fold activation of mock-transfected cells and show mean ± S.E. of two independent experiments with triplicates of each treatment.

***, p < 0.001; **, p < 0.01; *, p < 0.05 versus 0 ng PS1 cDNA. C. γ-secretase inhibitors inhibit processing of Notch ΔE-GVP. As for C99-GVP, both MW167 and L-685,458 caused a dose-dependent decrease in γ-secretase activity. The experiment was repeated at least three times with duplicates of each treatment. Data are presented as percent activity where 100% activity corresponds to the reporter gene activity obtained in the absence of γ-secretase inhibitors. The graph shows mean ± S.E. of three independent experiments with duplicates of each treatment.***, p < 0.001; **, p < 0.01; *, p < 0.05 versus Notch ΔE-GVP with Me2SO. Protein levels of Notch ΔE-GVP in the presence of different concentrations of MW167 were assessed by Western blotting using the monoclonal c-Myc antibody 9E10 (1:200). luc, luciferase.
cultured cells. We show here that Notch ΔE-GVP, like C99-GVP, can respond in a graded fashion to varying amounts of PS (Fig. 2B) and γ-secretase inhibitors (Fig. 2C). As in the case for C99-GVP, addition of PS above 10 ng does not significantly increase Notch cleavage efficiency. In addition, preliminary experiments suggest that expression of increasing amounts of Notch ΔE results in a dose-dependent attenuation of the processing of C99-GVP (data not shown). These data further demonstrate the similarities in processing between APP and Notch.

Both γ-secretase inhibitors displayed similar potency in inhibiting Notch ΔE-GVP and C99-GVP processing. The IC50 value for MW167 was calculated to be ~50 μM, which is slightly higher than the previously reported value of 20 μM (20). This discrepancy in IC50 values probably reflects the different methodologies used in the two investigations. L-865,458 inhibited C99-GVP and Notch ΔE-GVP processing with an IC50 value of 200 nM. The IC50 for C99-GVP was comparable to the IC50 reported for L-865,458 in intact cells (48–775 μM depending on the cell line used and whether secreted Aβ40 or Aβ42 was measured) (13). Thus, the similarity of the data obtained in this study and other reports confirm the validity of the C99-GVP reporter gene assay to address γ-secretase activity on APP.

To learn whether the assay was capable of recording differences between Notch and APP processing, we used artificial nonsense mutations in PS that affect two aspartate residues at positions 257 and 385 thought to be involved in the PS active site that have been shown to differentially alter APP and Notch processing (17). We generated single or double aspartate to alanine mutants at positions 257 and 385 in PS1 and tested the effects in the C99 and Notch GVP assays in the PS-deficient BD8 cells (Fig. 3). Transient expression of the D257A, D385A, and D257A/D385A mutants into BD8 cells resulted in no detectable signaling from the C99-GVP reporter construct. In contrast, the D385A mutant rescued 15% of the signaling for Notch ΔE-GVP, while the other two mutants did not activate the reporter gene. This finding is interesting in light of previous data. Our data on APP processing support the original finding by Wolfe and co-workers (18) that the aspartate residues are critical in PS-mediated cleavage of APP but disagree with data from Haass et al. (17). The D385A mutant has in all previous reports been shown to inhibit processing in a potent way (17, 18). The residual cleavage activity of the D385A PS1 mutant in our assay was therefore unexpected but may reflect the sensitivity of the GVP-based assay and that it was conducted in a PS-null background, avoiding possible interference of wild-type, endogenously expressed PS. Moreover, recent data have emerged that challenge the view that PS proteins are direct executors of the γ-secretase cleavage (19).

In conclusion, we provide a novel assay to monitor γ-secretase processing of APP and show that it can be used together with a similar assay for Notch processing. The APP assay is highly sensitive and quantitative and can simultaneously record all γ-secretase-mediated cleavages. The fact that the GVP-based assays for APP and Notch are based on transfected cells also render these assays suitable for large scale testing of multiple compounds for γ-secretase inhibition in an unbiased high throughput fashion. In principle, it should be possible to screen several thousands of compounds for their effect on γ-secretase activity in a few days. The observation that these assays can score differences in processing between APP and Notch makes the assays very attractive as tools to identify novel γ-secretase modulators that differentially affect APP and Notch processing.

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REFERENCES

1. Hardy, J. (1997) Trends Neurosci. 20, 154–159
2. Iwatsubo, T., Odaka, A., Suzuki, N., Minasawa, H., Nakina, N., and Iyama, Y. (1994) Neuron 13, 45–53
3. Nashlund, J., Haroutunian, V., Mohs, R. C., Davis, K. L., Davies, P., Greengard, P., and Buxbaum, J. D. (2000) J. Am. Med. Assoc. 283, 1571–1577
4. De Strooper, B., Annaert, W., Cupers, P., Saftig, P., Cruts, K., Mummen, J. S., Schroeter, E. H., Schrijvers, V., Wolfe, M. S., Ray, W. J., Goate, A., and Kopan, R. (1999) Nature 398, 518–522
5. Herreman, A., Sercels, L., Aerts, W., Colleen, D., Schonnors, L., and De Strooper, B. (2000) Nat. Cell Biol. 2, 461–462
6. Schueber, D., Eckman, C., Jensen, M., Song, X., Citron, M., Suzuki, N., Bird, T. M., Hardy, L., Hutton, M., Kakuk, W., Larson, E., Levy-Lahad, E., Viitanen, M., Peiskind, E., Poorkaj, P., Schellenberg, G., Tanzi, R., Wase, W., Lannfelt, L., Selkoe, D., and Younkin, S. (1996) Nat. Med. 2, 864–870
7. Skowronska, D. M., Dome, R. W., and Lee, V. M.-Y. (1998) J. Cell Biol. 141, 1031–1039
8. Lai, A., Sasidhar, S. S., and Trowbridge, I. S. (1995) J. Biol. Chem. 270, 3565–3573
9. Haas, C., Koo, E. H., Capell, A., Teplow, D. B., and Selkoe, D. J. (1995) J. Cell Biol. 129, 537–547
10. Struhl, G., and Greenwald, I. (1999) Nature 399, 522–525
11. Yu, G., Chen, F., Nishimura, M., Steiner, H., Tandon, A., Kawasaki, T., Arawaka, S., Supapa, A., Song, Y.-Q., Rogaeva, E., Holmes, E., Zhang, D. M., Milman, P., Prater, F. E., He, S., and St George-Hyslop, P. (2000) J. Biol. Chem. 275, 27253–27258
12. Wolfe, M. S., Xia, W., Moore, C. L., Leatherwood, D. D., Ostaszewski, B. L., Diehl, T. S., Kimberly, W. T., and Ray, W. J. (1999) J. Biol. Chem. 274, 4730–4737
13. Shearman, M. S., Beher, D., Clarke, E. E., Lewis, H. D., Harrison, T., Hunt, P., Nadin, A., Smith, A. L., Stevenson, G., and Castro, J. L. (2000) Biochemistry 39, 8648–8654
14. Mumm, J. S., and Kopan, R. (2000) Dev. Biol. 228, 151–165
15. Deleted in proof
16. Deleted in proof
17. Capell, A., Steiner, H., Ronig, H., Keck, S., Baeader, M., Grimm, M., Baumeister, R., and Haas, C. (2000) Nat. Cell Biol. 2, 205–211
18. Wolfe, M. S., Xia, W., Ostaszewski, B. L., Diehl, T. S., Kimberly, W. T., and Selkoe, D. J. (1999) Nature 399, 513–517
19. Armogida, M., Petit, A., Vincent, B., Scardello, A., Alves da Costa, C., and Checler, F. (2001) Nat. Cell Biol. 3, 1030–1033
20. Berezovska, O., Jack, C., McLean, P., Auster, J. C., Hicks, C., Xia, W., Wolfe, M. S., Kimberly, W. T., Weissthal, G., Selkoe, D. J., and Hyman, B. T. (2000) J. Neurochem. 75, 583–593

Fig. 3. PS1 active site mutants differentially affect γ-secretase-mediated cleavage of C99-GVP and Notch ΔE-GVP. cDNAs encoding PS1 D257A, D385A, or D257A/D385A were transfected into BD8 cells together with either C99-GVP or Notch ΔE GVP. γ-Secretase-mediated processing was recorded as luciferase activity where the activity obtained in the presence of wild-type PS1 was set to 100%. Note that the expression of none of the mutants resulted in any detectable processing from either the C99-GVP or the Notch ΔE-GVP construct, except for the D385A mutant, which rescued cleavage from the Notch ΔE GVP construct by 15%. The graph shows mean ± S.E. of three independent experiments with triplicates of each transfectant. ***, p < 0.001 versus C99-GVP without PS1 cDNA. +++, p < 0.001 versus Notch ΔE-GVP without PS1 cDNA. ***, p < 0.001 versus C99-GVP + PS1 wild-type cDNA. ***, p < 0.001 versus Notch ΔE-GVP + PS1 wild-type cDNA. WT, wild type.
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