An in vivo assay for the identification of target proteases which cleave membrane-associated substrates

Harald Steiner[a,b], Brigitte Pesold[a], Christian Haass[a,b,*]

[a] *Central Institute of Mental Health, Department of Molecular Biology, J5, 68159 Mannheim, Germany*
[b] *Adolf-Butenandt-Institute, Department of Biochemistry, Ludwig-Maximilians-University, Schillerstr. 44, 80336 Munich, Germany*

Received 12 November 1999

Edited by Jesus Avila

Abstract Proteases not only play a fundamental role in numerous physiological processes, but are also involved in several human diseases including Alzheimer's disease (AD). A key protease implicated in AD is the so far unidentified γ-secretase, which cleaves the membrane-bound β-amyloid precursor protein (βAPP) at the C-terminus of its amyloid domain within the membrane to release the neurotoxic amyloid β-peptide. In order to allow the isolation of proteases, which specifically cleave membrane-bound substrates within or in the vicinity of a transmembrane domain, we developed a reporter gene assay in *Saccharomyces cerevisiae*. This assay may allow the identification of genes encoding target proteases that specifically cleave membrane bound substrates by transforming expression libraries.

Key words: Alzheimer's disease; Amyloid β-peptide; Notch; Proteolytic processing; γ-Secretase

1. Introduction

Proteolytic processing is of great importance for the regulation of numerous biological processes. Site specific proteolysis is also required for the generation of amyloidogenic polypeptides involved in various neurodegenerative diseases. Proteases of the caspase family [1] are involved in generating cytotoxic proteolytic fragments of gene products associated with triple nucleotide extensions in disorders like Huntington's disease and others [2]. The pathology of Alzheimer's disease (AD), the most abundant neurodegenerative disorder world wide, is closely associated with the generation of the neurotoxic amyloid β-peptide (Aβ) by the site specific cleavage of two proteolytic activities [3,4]. Both activities require a membrane bound substrate, the β-amyloid precursor protein (βAPP). One activity (β-secretase), located within the lumen of membranous compartments generates the N-terminus while the second activity (γ-secretase) cleaves at the C-terminus of the amyloid domain within the transmembrane domain of βAPP. This cut finally results in the secretion of Aβ into biological fluids [3,4]. Therefore the secretases which are directly involved in amyloidogenesis are obvious targets for the development of amyloid lowering drugs.

A cleavage event, which closely resembles γ-secretase cleavage of βAPP has recently been shown to be responsible for the release of the Notch intracellular cytoplasmic domain (NICD), a key molecule required for cellular differentiation [5]. Similar to βAPP, Notch is cleaved within or close to its transmembrane domain [6,7]. Interestingly, the AD associated presenilins (PS) [8] have been shown to promote both the γ-secretase cleavage of βAPP and the proteolytic release of NICD by the Notch protease ([9,10], for review see [11,12]).

The purification of these proteolytic activities by conventional methods using soluble substrates is very difficult if not even impossible due to the fact that these proteases appear to require membrane bound substrates. Indeed, the γ-secretase strictly requires membrane bound βAPP to recognize its substrate and to cleave at the C-terminus of the amyloid domain [13,14]. Previously described genetic methods may also not be suitable for the identification of proteases that cleave membrane associated substrates [15,16] since these methods require soluble substrates. We therefore attempted to develop a novel in vivo assay in the yeast *Saccharomyces cerevisiae* allowing the identification of proteases, which specifically cleave membrane bound substrates (like the γ-secretase and the Notch protease). We chose yeast since many proteases typical for the specialized tasks of higher eukaryotic cells, like for example caspases, are absent in this organism [17]. In addition, yeast does not contain homologues of Notch or AD associated genes, like βAPP or the PS [17]. Thus, yeast may be a highly suitable organism for the functional reconstitution of proteolytic cleavage for which the respective proteases are absent.

Here we show that intracellular proteolytic cleavage of a membrane associated substrate resembling that of the γ-secretase or the Notch protease can be monitored with a suitable reporter gene assay in yeast cells.

2. Materials and methods

2.1. Plasmids

All GAL4 constructs used in this study are derived from pCL1 [18] carrying an expression cassette of the *GAL4* gene [19] under control of the *ADH1* promoter and terminator sequences. This cassette was cloned into YCplac22 [20] creating the parental plasmid for ssTM-GAL4 and derivatives thereof. The fusion protein ssTM-GAL4 consists of the signal sequence of yeast invertase (amino acids 1–19) [21] fused to the N-terminus of human PS1 [22] up to the first transmembrane domain (amino acids 3–101) followed by a three amino acid (RPH) spacer region containing unique *Stu*I and *Nde*I restriction sites and yeast GAL4 (amino acids 1–881). ssTM-GAL4 was constructed by PCR amplification of the corresponding cDNA coding regions using standard cloning techniques [23]. The constructs ssTM-poly-

*Corresponding author. Fax: (49)-89-5996 415.
E-mail: chaass@pbm.med.uni-muenchen.de

Abbreviations: Aβ, amyloid β-peptide; βAPP, β-amyloid precursor protein; AD, Alzheimer's disease; β-gal, β-galactosidase; NICD, Notch intracellular cytoplasmic domain; PARP, poly(ADP-ribose) polymerase; PS, presenilin

PII: S0014-5793(99)01627-0

(ADP-ribose) polymerase $(PARP)_D$-GAL4 and ssTM-$PARP_N$-GAL4 were generated by cloning PCR fragments encoding a domain corresponding to amino acids 155–219 of human PARP [24,25] that contained either the wild-type (DEVD) or mutant (DEVN) cleavage site of caspase-3 into the *Stu*I and *Nde*I cut ssTM-GAL4 construct. Fusion proteins ssTM-D-GAL4 and ssTM-N-Gal4 were constructed by inserting annealed oligonucleotides encoding DEVDG or DEVNG into the *Stu*I and *Nde*I restriction sites of the ssTM-GAL4 construct. All cDNA constructs were verified by DNA sequencing. To express caspase-3 in yeast, a full length cDNA of human caspase-3 [26–28] was cloned into a derivative of pVT100-U [29] in which the *URA3* gene had been replaced by the *LEU2* gene. In this construct caspase-3 expression is under control of the *ADH1* promoter and terminator sequences.

2.2. Yeast strains and methods

Standard methods [23] were used to introduce plasmids into yeast strain SFY526 (MATa, *ura3-52, his3-200, ade2-101, lys2-801, trp1-901, leu2-3,112, can*^{R}, *gal4-542, gal80-538, URA3::Gal1-lacZ*) [30] and for the preparation of protein extracts and β-galactosidase (β-gal) assays.

2.3. Antibodies and immunoblotting

Antibodies against the C-terminus of GAL4 or the C-terminus of human caspase-3 were obtained from Clontech or from Santa Cruz Biotechnology, respectively. For analysis by immunoblotting 100 μg of total lysate protein was loaded and separated on 10–20% Tris–Tricine gels (Novex), blotted onto nitrocellulose and probed with the respective antibodies.

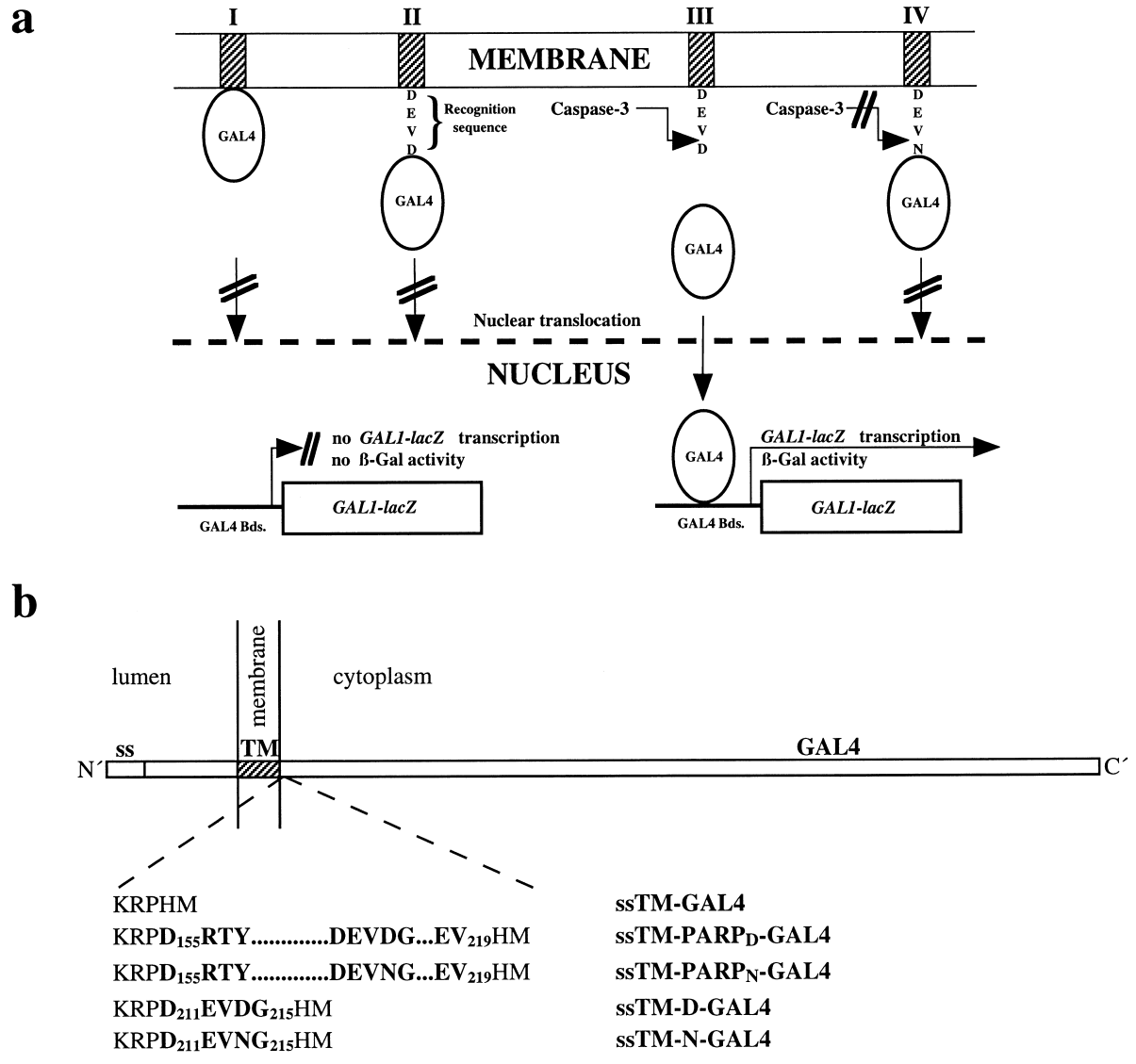

Fig. 1. a: Schematic representation of the assay system to monitor intracellular proteolytic cleavage of membrane bound substrates using the *GAL1-lacZ* gene as a reporter gene. GAL1-lacZ expression can be followed by monitoring β-gal activity. (I) Targeting of the yeast transcriptional activator GAL4 to intracellular membranes leads to inactivation of its activity in transcription of the reporter gene placed under control of a GAL4 responsive element (GAL4 binding site). (II and III) Insertion of a caspase-3 recognition site between the transmembrane domain and GAL4. Upon expression of caspase-3, GAL4 is released from the membrane by proteolytic cleavage, translocates to the nucleus and activates *GAL1-lacZ* transcription resulting in β-gal activity. (IV) Caspase-3 cleavage does not occur when a cleavage site mutant is expressed as a substrate. Consequently, no β-gal activity is detected. b: Schematic representation of the constructs used as substrates for proteolytic cleavage in our assay system. ss: signal sequence, TM: transmembrane domain. The amino acid sequences at the junction between the TM and GAL4 are shown. The parental construct ssTM-GAL4 contains a three amino acid spacer (RPH) harboring unique restriction sites. The lysine residue (K) is the last amino acid of the PS1 transmembrane region; the methionine residue (M) is the first amino acid of GAL4. The derivatives of ssTM-GAL4 contain insertions of human PARP of different length which contain the caspase-3 recognition sequence DEVD or the non-cleavable mutant variant (DEVN) thereof.

3. Results

3.1. Site specific proteolysis of a membrane bound reporter substrate

We developed an in vivo assay for site specific proteolytic cleavage of membrane bound substrates by co-expressing human caspase-3 as model protease with membrane bound GAL4 fusion proteins containing the caspase-3 recognition sequence DEVD of human PARP (see below) in various constructs as a substrate (Fig. 1a,b).

We first constructed a fusion protein (ssTM-GAL4) where the transcriptional activator GAL4 was C-terminally fused to the N-terminal portion of human PS1 including the first transmembrane (TM) domain. In order to target this recombinant protein to membranes we added a signal sequence to its N-terminus. Upon expression in the yeast strain SFY526 harboring a *GAL1-lacZ* reporter gene under GAL4 control [30] no β-gal activity was detected indicating that GAL4 was indeed completely inactive as long as it is membrane bound. In contrast, expression of soluble non-membrane bound GAL4 resulted in a strong β-gal activity indicating its efficient translocation to the nucleus (Fig. 2a). Co-expression of human caspase-3 did not lead to β-gal activity excluding the possibility that ssTM-GAL4 is cleaved unspecifically by caspase-3. Immunoblotting using antibodies to GAL4 revealed the ssTM-GAL4 protein as well as the soluble GAL4 (Fig. 2a). Proteolytic activity of caspase-3 was confirmed by immunoblotting using an antibody directed against the C-terminus of caspase-3 (Fig. 2a). In addition to the unprocessed full-length precursor, the p11 subunit of caspase-3 characteristic for the proteolytically active enzyme was detected [26–28]. Since yeast does not contain the enzymes necessary for apoptosis, caspase-3 activation may be due to autoproteolysis.

To prove if the membrane bound fusion protein can be specifically cleaved by caspase-3, we inserted a domain of human PARP (amino acid residues 155–219) including its caspase-3 recognition sequence DEVD [31] into unique restriction sites generated within the linker between the TM domain and the domain encoding GAL4 (see Fig. 1b). As expected, we detected no β-gal activity upon expression of this construct (ssTM-$PARP_D$-GAL4) in SFY526 suggesting that yeast does not contain an endogenous caspase-3 like activity (Fig. 2b). In order to prove if site specific cleavage could release the transcriptional activator and thus allow its translocation to the nucleus we co-expressed human caspase-3. When caspase-3 was transformed, strong β-gal activity was obtained indicating that GAL4 was released from the membrane by proteolytic cleavage and translocated to the nucleus where it activated *GAL1-lacZ* transcription (Fig. 2b). Immunoblotting using anti GAL4 antibodies revealed that ssTM-$PARP_D$-GAL4 remained as an uncleaved membrane bound precursor in the absence of caspase-3 expression (Fig. 2b). However, when we co-expressed caspase-3, we obtained robust levels of soluble GAL4, which co-migrated with native soluble GAL4 (Fig. 2b). Therefore, these experiments show that caspase-3 expression results in the liberation of the transcriptional activator and this can be efficiently monitored by the blue color of the protease expressing colonies (Fig. 2b). To further prove the specificity of the observed cleavage, we introduced a mutation into the caspase-3 recognition sequence. For that purpose we exchanged the essential aspartate residue at the P_1 position to an asparagine. Mutation of the P_1 site is

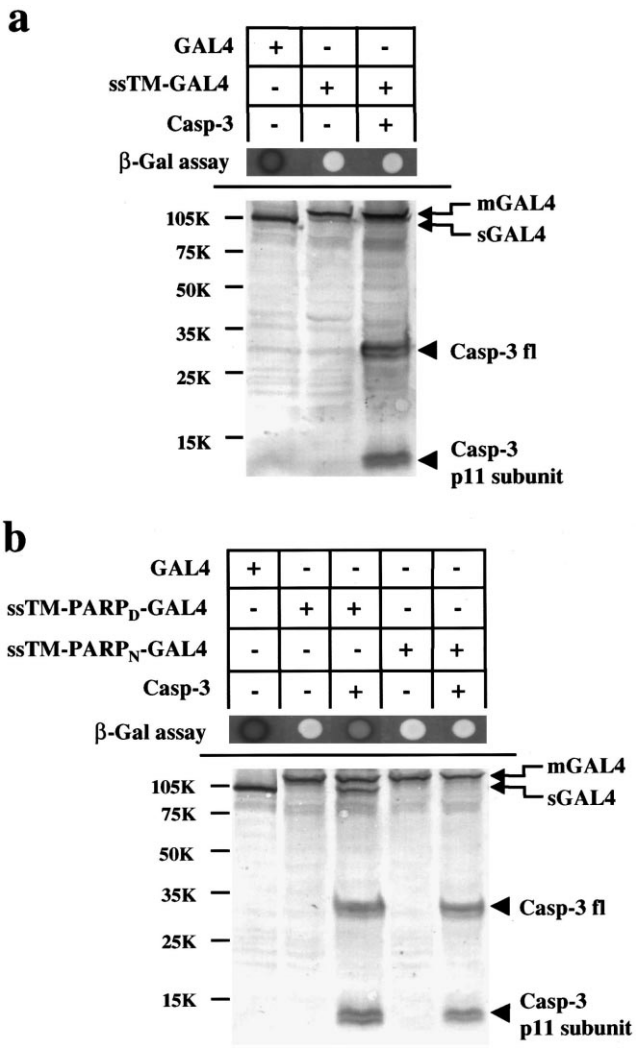

Fig. 2. Membrane bound GAL4 constructs containing the caspase-3 cleavage site (DEVD) of PARP are specifically cleaved upon co-expression of caspase-3 and strongly activate *GAL1-lacZ* transcription. a: In contrast to soluble GAL4, membrane bound GAL4 does not activate *GAL1-lacZ* transcription. Upper panel: Cells were assayed for β-gal activity. Yeast cells expressing ssTM-GAL4 do not activate *GAL1-lacZ* transcription neither in the presence nor in the absence of co-expressed caspase-3. Lower panel: Protein extracts were prepared from yeast strain SYF526 expressing either GAL4 or ssTM-GAL4 in the presence or absence of co-expressed human caspase-3. Soluble (sGAL4) and membrane bound GAL4 (mGAL4) proteins as well as caspase-3 were detected by simultaneous immunoblotting using antibodies to the C-terminus of GAL4 or caspase-3, respectively. Functional expression of caspase-3 was confirmed by the identification of the proteolytically active p11 subunit, that is detected in addition to the unprocessed full-length (fl) precursor protein. b: ssTM-$PARP_D$-GAL4 is specifically cleaved upon caspase-3 co-expression. Upper panel: Cells were assayed for β-gal activity. Co-expression of caspase-3 leads to strong activation of β-gal activity in cells expressing ssTM-$PARP_D$-GAL4, but not in cells expressing the cleavage site mutant ssTM-$PARP_N$-GAL4. Lower panel: Protein extracts were prepared from yeast strain SFY526 expressing either ssTM-$PARP_D$-GAL4 or ssTM-$PARP_N$-GAL4. Soluble (sGAL4) and membrane bound GAL4 (mGAL4) proteins as well as caspase-3 were detected by simultaneous immunoblotting using antibodies to the C-terminus of GAL4 or caspase-3, respectively. Functional expression of caspase-3 was confirmed by the identification of the proteolytically active p11 subunit that is detected in addition to the unprocessed full-length (fl) precursor protein. Note the specific liberation of GAL4 that co-migrates with native GAL4 in cells co-expressing ssTM-$PARP_D$-GAL4 and caspase-3.

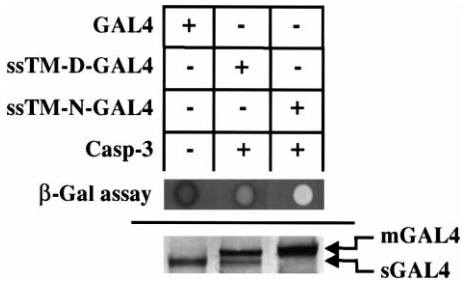

Fig. 3. Insertion of the minimal caspase-3 cleavage site of PARP into ssTM-GAL4 is sufficient for specific cleavage. Upper panel: Cells were assayed for β-gal activity. Co-expression of caspase-3 leads to strong activation of β-gal activity in cells expressing ssTM-D-GAL4, but not in cells expressing the cleavage site mutant ssTM-N-GAL4. Lower panel: Protein extracts were prepared from yeast strain SFY526 co-expressing caspase-3 with the indicated GAL4 proteins. Soluble (sGAL4) and membrane bound GAL4 (mGAL4) proteins were detected by immunoblotting using antibodies to the C-terminus of GAL4. Note that GAL4 is proteolytically released in cells expressing ssTM-D-GAL4, but not in cells expressing the cleavage site mutant ssTM-N-GAL4.

known to efficiently block caspase mediated cleavage of numerous 'death substrates' [31] including PARP [32]. Upon expression of this construct (ssTM-$PARP_N$-GAL4) no change in the color of the caspase-3 overexpressing cells was observed in the β-gal assay (Fig. 2b). Concomitant to the lack of β-Gal activity, no liberation of soluble GAL4 from its recombinant membrane bound precursor in the presence of caspase-3 was observed (Fig. 2b). Again, proteolytic activity of caspase-3 was confirmed by immunoblotting using an antibody directed against the C-terminus of caspase-3 as above (Fig. 2b). Taken together, these data demonstrate that sequence specific cleavage of a membrane bound recombinant reporter protein can be specifically monitored under in vivo conditions in a highly sensitive assay system.

3.2. Site specific proteolysis of the minimal recognition sequence

We next investigated whether the introduction of the smallest possible cleavage site immediately at the membrane would still allow site specific cleavage by caspase-3. We inserted the amino acid sequence DEVDG of PARP into the linker region of ssTM-GAL4. Co-expression of this construct (ssTM-D-GAL4) together with caspase-3 resulted in β-gal activity indicating its proteolytic cleavage (Fig. 3). In contrast, when the control construct ssTM-N-GAL4 containing the cleavage site mutation DEVNG was used, no β-gal activity was detected. Immunoblotting with anti GAL4 antibodies revealed soluble GAL4, which co-migrated with native GAL4 when ssTM-D-GAL4 was co-expressed with caspase-3, whereas ssTM-N-GAL4 remained as an uncleaved precursor (Fig. 3). Thus, insertion of the minimal cleavage site immediately at the membrane still allows the sensitive and specific monitoring of proteolytic cleavage.

4. Discussion

We have developed an assay, which could allow the identification of almost any intracellular protease since recognition sites of interest could easily be inserted into a membrane bound GAL4 construct. Given the absence of the target protease in yeast cells, cleavage may be reconstituted by functional expression of the protease gene upon transformation of an expression library. In case of the presence of a corresponding endogenous yeast protease, deletion of the putative protease encoding genes in the yeast genome, which is completely sequenced [17], may allow its specific and rapid identification. In contrast to previous screening systems [15,16,33] our system was particularly designed to allow the identification of target proteases, which cleave membrane bound substrates within, at, or close to the membrane. Our test construct ssTM-D-GAL4, which mimics such a membrane bound substrate contained, apart from three linker residues, only the sequence DEVDG between its transmembrane domain and GAL4, yet it was specifically cleaved by caspase-3. This demonstrates that the fusion of even a very short recognition sequence with the TM domain and GAL4 allows site specific cleavage of proteases and that the large GAL4 domain does not sterically hinder proteolysis.

As mentioned above potential target proteases include the Notch protease, which cleaves Notch within or close to the membrane [6,7]. Moreover, our system may be particularly valuable for the identification of the γ-secretase. This protease is strictly dependent on membrane bound substrates and can not be isolated using conventional screening methods. Since the γ-secretase is involved in the generation of the amyloidogenic Aβ it represents one of the key targets for therapeutic treatment of AD.

Acknowledgements: We thank H. Jacobsen for the caspase-3 and PARP cDNA, K. Dietmeier for yeast strain SFY526 and E. Hurt for helpful suggestions. This work was supported by the Boehringer Ingelheim K.G. and a Grant from the Deutsche Forschungsgemeinschaft (1737/5-1) to C.H.

References

[1] Thornberry, N.Y. and Lazebnik, Y. (1998) Science 281, 1312–1316.
[2] Wellington, C.L., Ellerby, L.M., Hackam, A.S., Margolis, R.L., Trifiro, M.A., Singaraja, R., McCutcheon, K., Salvesen, G.S., Propp, S.S., Bromm, M., Rowland, K.J., Zhang, T., Rasper, D., Roy, S., Thornberry, N., Pinsky, L., Kakizuka, A., Ross, C.A., Nicholson, D.W., Bredesen, D.E. and Hayden, M.R. (1996) J. Biol. Chem. 273, 9158–9167.
[3] Haass, C. and Selkoe, D.J. (1993) Cell 75, 1039–1042.
[4] Selkoe, D.J. (1999) Nature Suppl. 399, A23–A31.
[5] Chan, Y.M. and Yan, Y.N. (1998) Cell 94, 423–426.
[6] Schroeter, E.H., Kisslinger, J.A. and Kopan, R. (1998) Nature 393, 382–385.
[7] Struhl, G. and Adachi, A. (1998) Cell 93, 649–660.
[8] Haass, C. (1997) Neuron 18, 687–690.
[9] De Strooper, B., Annaert, W., Cupers, P., Saftig, P., Craessaerts, K., Mumm, J.S., Schroeter, E.H., Schrijvers, V., Wolfe, M.S., Ray, W.J., Goate, A. and Kopan, R. (1999) Nature 398, 518–522.
[10] Steiner, H., Duff, K., Capell, A., Romig, H., Grim, M.G., Lincoln, S., Hardy, J., Yu, X., Picciano, M., Fechteler, K., Citron, M., Kopan, R., Pesold, B., Keck, S., Baader, M., Tomita, T., Iwatsubo, T. and Haass, C. (1999) J. Biol. Chem. 274, 28669–28673.
[11] Yee-Ming, C. and Yuh, N.J. (1999) Neuron 23, 201–204.
[12] Haass, C. and De Strooper, B. (1999) Science 286, 1916–1919.
[13] Citron, M., Teplow, D. and Selkoe, D.J. (1995) Neuron 14, 661–670.
[14] Tischer, E. and Cordell, B. (1996) J. Biol. Chem. 271, 21914–21919.
[15] Smith, T.A. and Kohorn, B.D. (1991) Proc. Natl. Acad. Sci. USA 88, 5159–5162.

[16] Sices, H.J. and Kristie, T.M. (1998) Proc. Natl. Acad. Sci. USA 95, 2828–2833.
[17] Mewes, H.W., Albermann, K., Bähr, M., Frishman, D., Gleissner, A., Hani, J., Heumann, K., Kleine, K., Maierl, A., Oliver, S.G., Pfeiffer, F. and Zollner, A. (1997) Nature Suppl. 387, 7–65.
[18] Fields, S. and Song, O. (1989) Nature 340, 245–246.
[19] Laughon, A. and Gesteland, R. (1984) Mol. Cell. Biol. 4, 260–267.
[20] Gietz, R.D. and Sugino, A. (1988) Gene 74, 527–534.
[21] Moir, D.T. and Dumais, D.R. (1987) Gene 56, 209–217.
[22] Sherrington, R., Rogaev, E.I., Liang, Y., Rogaeva, E.A., Levesque, G., Ikeda, M., Chi, H., Lin, C., Li, G., Holman, K., Tsuda, T., Mar, L., Foncin, J.-F., Bruni, A.C., Montesi, M.P., Sorbi, S., Rainero, I., Pinessi, L., Nee, L., Chumakov, I., Pollen, D., Brookes, A., Sanseau, P., Polinsky, R.J., Wasco, W., da Silva, H.A.R., Haines, J.L., Pericak-Vance, M.A., Tanzi, R.E., Roses, A.D., Fraser, P.E., Rommens, J.M. and St. George-Hyslop, P.H. (1995) Nature 375, 754–760.
[23] Ausubel, F.M., Brent, R., Kingston, R.E., Moore, D.D., Seidman, J.G., Smith, J.A. and Struhl, K. (1994) Current protocols in Molecular Biology. John Wiley and Sons, New York.
[24] Cherney, B.W., McBride, O.W., Chen, D.F., Alkhatib, H., Bhatia, K., Hensley, P. and Smulson, M.E. (1987) Proc. Natl. Acad. Sci. USA 84, 8370–8374.
[25] Uchida, K., Morita, T., Sato, T., Ogura, T., Yamashita, R., Noguchi, S., Suzuki, H., Nyunoya, H., Miwa, M. and Sugimura, T. (1987) Biochem. Biophys. Res. Commun. 148, 617–622.
[26] Fernandes-Alnemri, T., Litwack, G. and Alnemri, E.S. (1994) J. Biol. Chem. 269, 30761–30764.
[27] Tewari, M., Quan, L.T., O'Rourke, K., Desnoyers, S., Zheng, Z., Beidler, D.R., Poirier, G.G., Salvesen, G.S. and Dixit, V.M. (1995) Cell 81, 801–809.
[28] Nicholson, D.W., Ambereen, A., Thornberry, N.A., Vaillancourt, J.P., Ding, C.K., Gallant, M., Gareau, Y., Griffin, P.R., Labelle, M., Lazebnik, Y.A., Munday, N.A., Rayu, S.M., Smulson, M.E., Yamin, T., Yu, V.L. and Miller, D.K. (1995) Nature 376, 37–43.
[29] Vernet, T., Dignard, D. and Thomas, D.Y. (1987) Gene 52, 225–233.
[30] Bartel, P.L., Chien, C.T., Sternglanz, R. and Fields, S. (1993) BioTechniques 14, 920–924.
[31] Nicholson, D.W. and Thornberry, N.A. (1997) Trends Biochem. Sci. 22, 299–306.
[32] Lazebnik, Y.A., Kaufmann, S.H., Desnoyers, S., Poirier, G.G. and Earnshaw, W.C. (1994) Nature 371, 346–347.
[33] Hawkins, C.J., Wang, S.L. and Hay, B.A. (1999) Proc. Natl. Acad. Sci. USA 96, 2885–2890.