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Intracellular Generation of Amyloid β-Protein from Amyloid β-Protein Precursor Fragment by Direct Cleavage with β- and γ-Secretase

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Two amyloid β protein precursor (βAPP) fragments involving Met and 103 amino acids of C-terminus of βAPP (ΔNOR-β) and its KM-NL substitution (ΔNL-β) were expressed in COS-7 cells to clarify the proteolytic cleavages to generate amyloid β protein (Aβ). The 4.5-kD protein, Aβ with additional N-terminal amino acids, and 4-kD Aβ were directly produced and released from 12.5-kD expression proteins without any production of 11.4-kD C-terminal fragment starting at N-terminus of Aβ and 3-kD "p3" Aβ derivative. Intracellular 4-kD Aβ was also detected. The substitution of KM-NL of βAPP found in Swedish familial Alzheimer's disease (AD) promoted the production of intracellular Aβ and its release with no increase in level of 11.4-kD C-terminal fragment. These results suggested the presence of a distinct pathway in which Aβ is directly cleaved at both N- and C-termini from βAPP fragment intracellularly to release Aβ. Since KM-NL substitution enhanced intracellular Aβ generation, this pathway may be associated with amyloidogenesis in AD.

The deposition of amyloid β protein (Aβ) is an important early event in the development of Alzheimer's disease (AD). The Aβ, a 40–43 amino acid peptide, is derived from its precursor protein (βAPP) by proteolytic cleavage (1–3). The precise mechanism of Aβ generation from its precursor has been the focus of recent studies. In the secretory pathway, some βAPPs are cleaved at Aβ Lys16 and Aβ Asp1 by α- and β-secretase to release C-terminally truncated βAPP (secretory form βAPPα and β) (4,5), and subsequently processed at the C-terminus of Aβ by γ-secretase to release the 3-kD "p3" Aβ derivative and Aβ (6,7). The alternative pathway is the endosomal-lysosomal system, in which some cell-surface βAPPs are reinternalized (8) and cleaved at the N-terminus of the Aβ sequence by β-secretase to produce potentially amyloidogenic C-terminal fragments (CTF) (9,10,11) and subsequently cleaved by γ-secretase to release soluble 4-kD Aβ (12). In addition to this reinternalized pathway, a pathway that targets βAPP from the trans-Golgi network directly to endosomes and lysosomes could exist (13). Thus, multiple pathways for Aβ generation have been postulated. Chemical treatment of cultured cells has shown that Aβ is produced in acidic compartments (14,15). However, intracellular Aβ has not been detected except in neuronal cells (16,17) or non-neuronal cells derived from AD patients (18) and aged animals (19). Although mutant βAPP found in Swedish familial AD (20) is processed to release increased amounts of Aβ (21–23), which is closely associated with a pathogenesis of AD, it remains to be clarified whether the increased release of Aβ is caused by activation of secretase for amino acid substitution of mutant βAPP or by abnormal processing of mutant βAPP in distinct compartments. Here, we report a distinct mechanism of Aβ generation that occurs intracellularly, suggestive of a distinct pathway. The KM-NL double mutation found in Swedish familial AD promoted intracellular Aβ generation, indicating that this pathway may be associated with amyloidogenesis in AD.

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MATERIALS AND METHODS

Two cDNA constructs encoding βAPP fragments were prepared. 1) Δ NOR-β was designed to encode C-terminal 103 amino acids of βAPP with Met at the N-terminus, and 2) ΔNL-β was designed to encode Met and C-terminal 103 amino acids of βAPP with KM-NL substitution found in Swedish AD families (20). These constructs were prepared from βAPP695 cDNA using PCR with primer set 1: βAPP 13 (5′-GGCTCTAGAGATGGAAGTGAAGATGGATGCAGAATT-3′) and βAPP 12 (5′-GGCTCTAGAGCTAGTTCTGCATCTGCTCAA-3′) or primer set 2: βAPP 14 (5′-GGCTCTAGAGATGGAAGTGAATCTGGATGCAGAATTCC-3′) and βAPP 12, and resulting ΔNOR-β or ΔNL-β fragments were cloned into the Xbal site of pCDM8 (Invitrogen). The NOR-β (24) construct encoding the 17 amino acid signal sequence and C-terminal 99 amino acids of βAPP, and βAPP695 (14) construct in pCEP4 (Invitrogen) encoding βAPP695 were also used.

Approximately 5×10^6 COS-7 cells in 10 cm dishes were transfected with 5 μg of each construct (NOR-β, ΔNOR-β and ΔNL-β) in 50 μl of DOTAP (Boehringer) as described by the manufacturer. After 16 hours, medium was changed to D-MEM (Gibco) for 24 hours, then the cells were trypsinized and transferred into 600 ml culture bottles. After 12–24 hours incubation with Opti-MEM (Gibco), conditioned medium and cells were collected for analysis.

For chemical treatment experiments, leupeptin (20 μg/ml), ammonium chloride (10mM), or methylamine (30mM) were added to culture media. SKN-MC cells stably transfected with βAPP695/pCEP4 were selected according to the method previously reported (9).

Antibody G42C (25) raised against Aβ1-40 was used for captured antibody in immunoprecipitation of Aβ. The following antibodies were used for detection of Aβ; 4G8 against Aβ17-24, S40 (26,27) specific to Aβ ending at Val 40, S42 (26,27) specific to Aβ ending at Ala 42, Ab9204 (28) specific to Asp1 of Aβ, and SO against the 5 amino acids immediately preceding Aβ1. Saeko (26) directed against 30 amino acids of the C-terminus of βAPP was used for detection.

Twenty-five ml aliquots of conditioned medium from each dish were centrifuged at 100,000 × g. The supernatants were mixed with 6.3 ml of 5× RIPA-LPT (9), and then incubated with 20 μl of G42C and 20 μl of protein A-agarose for 24 hours at 4°C. Approximately 3×10^7 cells in each bottle were trypsinized and washed twice with Hanks' balanced salt solution (Gibco). Then, pellets were lysed in 5.0 ml RIPA-LPT. After centrifugation at 100,000 × g, 4.0 ml of each supernatant was incubated with 20 μl of G42C and 20 μl of protein A-agarose for 24 hours at 4°C. The immunoprecipitates were washed 3 times with 1× RIPA, once with TBS -LTP (9), and then separated by 10 to 16% Tris-tricine PAGE (14) and transferred electrophoretically onto Immobilon-P membranes (Millipore). To detect CTFs, 25 μl aliquots of cell lysates were directly applied. Blots were labeled with the above antibodies, followed by visualization using an ECL system (Amersham).

RESULTS AND DISCUSSION

CTFs of ∼8.7- to 11.4 kD from endogenous βAPP (9,10,29) were detected in mock-transfected COS-7 cells with the anti-C-terminus antibody Saeko (Fig. 1; lane1). The NOR-β gene showed augmented expression of 11.4-kD protein in cell lysate (Fig. 1;lane2), indicating the presence of the carboxy-99 residues of βAPP after removal of signal sequence encoded by the NOR-β construct. Both ΔNOR-β- and ΔNL-β-transfected cells showed expression of 12.5-kD CTF, but no increase in levels of 8.7-to 11.4-kD CTFs (Fig. 1;lane3,4). The 12.5-kD CTF in ΔNOR-β-transfected cells was labeled with S0 antibody against the 5 amino acids immediately preceding Aβ (Fig. 1;lane5), suggesting the expression of Met and 103 amino acids of βAPP encoded by ΔNOR-β or ΔNL-β constructs.

No 4-kD signal was detected in the media from mock-transfected cells (Fig. 2; lane1). In the conditioned media of ΔNOR-β-transfected cells, 4-kD Aβ and 4.5-kD protein were detected with

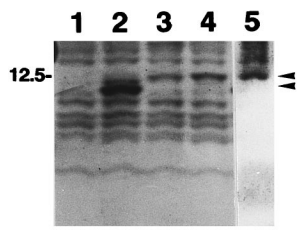

FIG. 1. C-terminal fragments of βAPP. Lanes: 1, mock-transfected COS-7 cells; 2, COS-7 cells transfected with ΔNOR-β;; 3,5, COS-7 cells transfected with ΔNOR-β; 4, COS-7 cells transfected with ΔNL-β. Cell extracts were separated by 10–16% Tris–tricine SDS–PAGE and immunoblotted with Saeko (1–4) directed against the C-terminus of βAPP and S0 (5) against the 5 amino acids immediately preceding Aβ1.

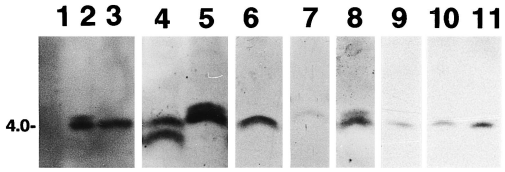

FIG. 2. Secreted and cellular Aβ. Lanes: 1, mock-transfected COS-7 cells; 2, 5, 6, 7, 8, 9, 10, COS-7 cells transfected with ΔNOR-β; 3, 11, COS-7 cells transfected with ΔNL-β; 4, SKN-MC cells transfected with βAPP695. Conditioned media (1–9) and cell lysates (10,11) were immunoprecipitated with G42C. Precipitates were separated by 10–16% Tris–tricine SDS–PAGE and immunoblotted with 4G8 (1–5,10,11), Ab9204 (6), S0 (7), S40 (8), and S42 (9).

antibody 4G8 (Fig. 2;lane2). Although the amount of 4kD Aβ was increased in the conditioned media of ΔNL-β transfected cells to a greater extent than that of ΔNOR-β-transfected cells, 4.5-kD protein was not detected in the media of ΔNL-β-transfected cells (Fig. 2;lane3). No p3 was detected in the media of either cell line. Since 4-kD Aβ and p3 were recognized by our G42C/4G8 detection system in the media of SKN-MC cells transfected with βAPP695/pCEP4 (Fig. 2;lane4), both βAPP fragments by ΔNOR-β and ΔNL-β constructs escaped cleavage processing at the α-secretase site and subsequent processing to release p3. The 4.5-kD protein was labeled with S0 (Fig. 2;lane7), but not with Ab9204 (Fig. 2;lane6). On the other hand, 4-kD Aβ was labeled with Ab9204 (Fig. 2;lane6), but not by S0. Both 4.5-kD protein and 4-kD Aβ were labeled by S40 (Fig. 2;lane8), and 4-kD Aβ was also labeled by S42 (Fig. 2;lane9). These immunological epitope mapping studies showed that the 4-kD Aβ released from transfected cells with ΔNOR-β was Aβ1-40/42. The 4.5-kD protein detected in the media of ΔNOR-β cells was considered to be the entire Aβ ending at 40 with an additional stretch of N-terminal amino acids. Intracellular 4-kD Aβ was detected in ΔNOR-β cells (Fig. 2;lane10), and a greater amount of which was found in ΔNL-β cells (Fig. 2;lane11).

These findings indicated; that 1) the 4-kD Aβ 1-40/42 was produced from 12.5-kD expression protein intracellularly by both β- and γ-secretase, 2) the 4.5-kD N-terminally long Aβ ending at 40 is produced by only γ-secretase cleavage from 12.5-kD expression protein, and 3) KM-NL double mutation of βAPP released increased amounts of 4-kD Aβ, but did not release 4.5-kD protein with no increase in level of 11.4-kD CTF.

Since the amount of 11.4-kD CTF was correlated with the amount of released Aβ (22,30), 11.4-kD CTF is considered to be an intermediate precursor in the generation of Aβ. In other words, β-secretase should work before γ-secretase cleavage to release Aβ. Moreover, recent studies have shown that KM-NL substitution of βAPP increases the production of 11.4-kD CTF (22,23) and subsequent release of increased amounts of Aβ (21–23), suggesting that the this substitution may be an effective signal for β-secretase cleavage. However, ΔNOR-β and even ΔNL-β constructs did not generate 11.4-kD CTF as an intermediate precursor of Aβ. These findings suggested that cleavage by both β- and γ-secretase occurs almost simultaneously.

In this case, the β-secretase and the γ-secretase may be an identical secretase, such as cathepsinD that can cleave both N- and C-termini of Aβ (31). Furthermore, because increased production of 11.4-kD CTF from Swedish type mutant βAPP occurs in the secretory pathway (23), Aβ generation from our βAPP fragments may occur in another distinct pathway. Interestingly, the KM-NL substitution promoted the Aβ generation also in this distinct pathway, suggesting that this pathway may contribute to amyloidogenesis in AD.

In this study, 4-kD Aβ was detected intracellularly from ΔNOR-β, and ΔNL-β cells produced more amount of intracellular Aβ than ΔNOR-β. These findings suggested that the 4-kD Aβ may be generated intracellularly by direct cleavages of 12.5-kD CTF by β- and γ-secretase. Intracellular Aβ has not been detected except in neuronal cells (16,17) or non-neuronal cells derived from AD patients (18) and aged animals (19). A recent report indicated that intracellular Aβ accumulation

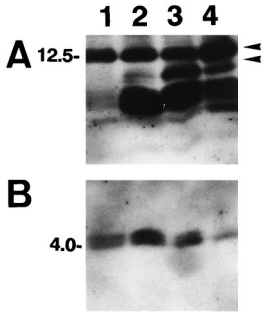

FIG. 3. Effects of chemical treatments. **(A)** C-terminal fragments in cell lysates. **(B)** 4-kD Aβ and 4.5-kD protein in conditioned media. COS-7 cells expressing ΔNOR-β were incubated for 12 hours in the absence (control; lane 1) or presence of leupeptin (20μg/ml; lane 2), ammonium chloride (10mM; lane 3), or methylamine (30mM; lane4). 12.5-kD protein is indicated by the upper arrowhead and 11.4–kD protein by the lower arrowhead.

precedes extracellular Aβ deposition in the aged monkey brain (32). Therefore, intracellular Aβ seems to be associated with pathological processes in the AD brain. In this context, the pathway by which intracellular Aβ is generated from βAPP fragments may contribute to intracellular accumulation of Aβ in the AD brain.

To examine this pathway further, ΔNOR-β-transfected cells were treated with leupeptin, ammonium chloride, and methylamine. Treatment with 20μg/ml leupeptin increased levels of cellular 8.7 - to 11.4-kD CTFs (Fig. 3A; lane2) with no concomitant inhibition of Aβ release (Fig. 3B;lane2). Treatment with 10mM ammonium chloride caused marked accumulation of 11.4-kD protein (Fig. 3A;lane3), but did not inhibit Aβ secretion (Fig. 3B;lane3), indicating that the 11.4-kD protein was not intermediate precursor of Aβ. Since similar amount of 11.4-kD protein was observed in mock-transfected cells with ammonium chloride treatment (not shown), this protein may be derived from endogenous βAPP. In contrast, treatment with methylamine caused accumulation of 12.5-kD ΔNOR-β protein CTF (Fig. 3A;lane4), and marked inhibition of release of 4.5-kD protein and 4-kD Aβ (Fig. 3B;lane4). In addition, intracellular Aβ disappeared after methylamine treatment (not shown). These findings confirmed that the 12.5-kD ΔNOR-β protein is a direct precursor of these 4.5-kD protein and 4-kD Aβ. Since methylamine inhibits multiple points of βAPP processing including secretory cleavages by α-secretase (33) or γ-secretase (34,35), and endocytosis (36), the precise intracellular regions in which Aβ is produced directly from ΔNOR-β could not be determined. However, present findings suggested that methylamine affects the activities of both β- and γ-secretase, and that the intracellular and secreted Aβ were produced in the same subcellular sites that are affected by methylamine.

In conclusion, we propose the existence of a distinct pathway in which Aβ is directly cleaved at both N- and C-termini from βAPP fragment intracellularly to release Aβ, and the pathway may be associated with amyloidogenesis in AD.

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