Unaltered Secretion of β-Amyloid Precursor Protein in Gelatinase A (Matrix Metalloproteinase 2)-deficient Mice*

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The β-amyloid peptide, which forms extracellular cerebral deposits in Alzheimer's disease, is derived from a large membrane-spanning glycoprotein referred to as the β-amyloid precursor protein (APP). The APP is normally cleaved within the β-amyloid region by a putative protease (α-secretase) to generate large soluble amino-terminal derivatives of APP, and this event prevents the β-amyloid peptide formation. It has been suggested that the gelatinase A (matrix metalloproteinase 2, a 72-kDa type IV collagenase) may act either as α-secretase or as β-secretase. Mice devoid of gelatinase A generated by gene targeting develop normally, except for a subtle delay in their growth, thus providing a useful system to examine the role of gelatinase A in the cleavage and secretion of APP in vivo. We show here that APP is cleaved within the β-amyloid region and secreted into the extracellular milieu of brain and cultured fibroblasts without gelatinase A activity. The data suggest that gelatinase A does not play an essential role in the generation and release of soluble derivatives of APP at physiological conditions.

Amyloid precursor protein (APP) is an integral membrane protein that is produced by most cells (1). Several isoforms ranging from 365 to 770 amino acids are generated by alternative splicing of transcripts from the APP gene on the long arm of chromosome 21 (2). Proteolytic cleavage of APP by enzymes termed α-, β-, and γ-secretases generates various APP fragments that are released from APP expressing cells (3). β-Amyloid peptides (Aβ) of 40–43 amino acids are released by the action of β- and γ-secretases cleaving at or near residues 671 and 713 (numbers refer to APP770), respectively (3). Cleavage of APP at a membrane proximal site by an α-secretase releases larger APP fragments (sAPP) and prevents generation of Aβ.

The absolute and relative amounts of various APP fragments that are released in the brain are thought to be of importance in the formation of first amorphous (diffuse) and then filamentous (amyloid) plaques that are characteristic of Alzheimer's disease. Two larger forms sAPP also known as protease nexin II have a Kunitz type serine protease inhibitor domain (4, 5). In addition all forms of sAPP have in the carboxyl-terminal region a domain that inhibits gelatinase A (matrix metalloproteinase 2, a 72-kDa type IV collagenase) activity (6). On the other hand, gelatinase A has been suggested to act on APP either as α-secretase (6) or as β-secretase (7), although the hypothesis is controversial (8, 9). To clarify the putative role of this enzyme, we studied APP fragmentation and release in gelatinase A knockout mice.

MATERIALS AND METHODS

Generation of Gelatinase A Gene-Deficient Mice—A genomic DNA clone of mouse gelatinase A (Clg4a) was isolated from a 129/Sv genomic library. The fragments used for constructing the targeting vector were a 2.0-kb HindIII fragment of the distal region of the promoter and a 4.5-kb XbaI-Sacl fragment. In the resulting targeting construct, 5.9 kb containing the exon 1 were replaced with the pgh-neo gene cassette (10). A diphtheria toxin A fragment gene cassette was used for negative selection of nonhomologous recombinants (11). The mouse embryonic stem (ES) cell line used was E14 (a gift of M. Hooper). Cell culture, targeting experiment, and microinjection of ES clones into C57BL/6J blastocysts were carried out as described previously (12). The mutation heterozygous mice were obtained by crossing the chimeras to C57BL/6J mice. Heterozygotes were backcrossed to C57BL/6J one to five times and then crossed to obtain the mutation homozygous mice. The genotypes of ES clones and mice were determined by Southern blot analysis as schematically presented in Fig. 1.

Gelatin Zymography—Gelatin zymography was carried out as described (13). The tissues from the newborn mice were homogenized in 50 mM Tris-HCl (pH 7.5) and centrifuged at 15,000 × g for 5 min. 10 μg of supernatant proteins was separated by nonreduced SDS-polyacrylamide gel electrophoresis using 7.5% gels containing 0.1% gelatin.

Antibodies Specific to APP—Used were monoclonal antibody 22C11 (Anti-N), specific for the amino-terminal domain of APP (14) (Boehringer Mannheim); rabbit polyclonal antibodies I-565AHF-28 (Anti-Aβ), specific for residues 1–28 of Aβ (15), a gift from Dr. T. Kunishita; and G-369 (Anti-C), specific to the cysteoplastic domain of APP (16). These antibodies have been shown not to cross-react with amyloid precursor-like proteins under the conditions used.

Western Blot Analysis—The mouse brain soluble and membrane fractions were prepared as described (17). Whole brains were homogenized in 50 mM Tris-HCl (pH 7.4) containing 0.5 mM NaCl, 10 mM MgCl2, 2 mM EDTA, 1 μg/ml leupeptin, and 2 mM phenylmethylsulfonyl fluoride and centrifuged at 100,000 × g for 30 min at 4 °C. The resulting supernatants were used as soluble fractions. The remaining pellets were solubilized in 50 mM Tris-HCl (pH 7.4) with 4 mM urea and 1% SDS and used as membrane fractions. 30 μg of supernatant proteins or 50 μg of the membrane proteins per lane were separated by 7% SDS-polyacrylamide gel electrophoresis and electroblotted onto polyvinylidene difluoride membrane (Millipore) in a buffer containing 10 mM CAPS (pH 11/10%) methanol. After blocking the membranes with 5% skim milk in Tris-buffered saline, the blots were immersed with antibodies mentioned above and peroxidase-conjugated anti-mouse or anti-rabbit antibodies and developed in ECL Western blotting detection reagent (Amersham Corp.). Protein concentration was determined by a protein assay (Bio-Rad).

Embryonic fibroblasts were prepared from embryos of 12.5 days post-coitus. Upon confluence, cells were washed three times with...
FIG. 1. a, schematic representations of the targeting vector and wild-type and mutant mouse Clg4a loci. The targeting vector was designed to replace the promoter and exon 1 of the Clg4a gene by the pgk-neo gene cassette. Diphtheria toxin A fragment gene cassette, indicated by the shaded box, was used for negative selection of nonhomologous recombinants (11). The 5’ and 3’ probes used for the Southern blots in b are indicated by thick bars. b, Sall, SspI, Spel I, b, Southern blot analysis of progeny from a cross of heterozygotes. Genomic DNA was extracted from tail, digested, and probed as described in the figure legends. Lanes 1 and 5 represent mutation homozygotes. Lanes 2, 6, and 7 represent wild-type mice. Others represent heterozygotes. The positions of the wild-type (wt) and the mutant (m) fragments are indicated by arrows. c, gelatin zymography of the proteins of the gelatinase A mutant mice. +/-, heterozygote; +/-, wild type; +/-, homozygote. The pro- and mature gelatinase A (GelA) and gelatinase B (GelB) are indicated by arrows.

secretion of sAPP from the mutant brain, we cultured embryonic fibroblasts from mutant embryos and analyzed the secreted proteins. The results showed that sAPP was secreted from the mutant fibroblasts, indicating that sAPP production is not dependent on the presence of gelatinase A.

To determine whether sAPP is secreted from the mutant brain, we carried out Western blotting of the brain-derived soluble and membrane fractions using various anti-APP antibodies. Anti-N, Anti-Aβ, and Anti-C antibodies were used to detect sAPP in the soluble and membrane fractions of wild-type and mutant mice. The results showed that sAPP production is not dependent on the presence of gelatinase A.

RESULTS AND DISCUSSION

To introduce a null mutation in the mouse gelatinase A gene (Clg4a), we replaced its promoter and first exon with the pgk-neo gene by gene targeting (Fig. 1a). Three of four homologous recombinant ES clones transmitted the mutated allele to the germ line (Fig. 1b). Gelatin zymography revealed a complete lack of gelatinase A activity in the mutation homozygous mice (hereafter simply referred to as mutant mice) (Fig. 1c). Although gelatinolytic bands with molecular masses of 64 and 57 kDa (probably the proenzyme and mature form of the gelatinase A, respectively) were detected in the brain, lung, and kidney of wild-type mice, we detected neither of these forms in tissues from the mutant mice. Reduced levels were found in tissues from mutation homozygous mice, indicating that the mutant allele is nonfunctional. There was no evidence for a compensatory expression of gelatinase B and/or other gelatinolytic enzymes (Fig. 1c).

The mutant mice developed normally and were fertile. Inter-crosses between the heterozygotes backcrossed five times into C57BL/6 background gave rise to 151 wild-type, 318 homozygous, and 143 homozygous mice, nearly proportional to the expected frequency of 1:2:1. Although gelatinase A is expressed ubiquitously and has been thought to be important for the remodeling of tissue architecture during development, the mutant mice did not show any gross anatomical abnormalities. However, it is of interest that the mutant mice show significantly slower growth rate (approximately 15% reduction) as compared with that of wild-type litter mates (Fig. 2) from postnatal day 3 to adulthood, suggesting a role of gelatinase A in development. The mutant mice seem to be smaller even at birth. The reason for slower growth rate is not known. Cultured fibroblasts from mutant embryos grew and migrated normally on collagen- or gelatin-coated and uncoated dishes (data not shown).

To determine whether sAPP is secreted from the mutant brain, we carried out Western blotting of the brain-derived soluble and membrane fractions using various anti-APP antibodies: Anti-N, Anti-Aβ, and Anti-C (Fig. 3a). These antibodies detected 110–125-kDa proteins in the membrane fraction from the wild-type and mutant mice (Fig. 3, b, c, and d, lanes 3 and 4). Anti-N and anti-Aβ antibodies detected 105–110-kDa proteins in the soluble fractions from the brains of wild-type mice (Fig. 3, b and c, lane 1). These proteins did not bind anti-C antibodies (Fig. 3d, lane 1), indicating that they were carboxy-terminal truncated forms of APP, presumably sAPP. The sAPP was also detected in the soluble fraction from mutant mice at a level similar to wild-type mice (Fig. 3b, lane 2, 97.7 ± 9.1%, average ± S.E. (mutants: n = 5) of wild types (n = 5); Fig. 3c, lane 2, 99 ± 10% (mutants: n = 3) of wild types (n = 3)). The relative intensities of bands obtained with anti-N and anti-Aβ antibodies were the same for the soluble fractions from wild-type and mutant mice, suggesting that there is no shift in the choice of α- and β-sites in the mutant mice. These results suggest that APP is normally secreted in the brain of the mutant mice.

To examine more directly the secretion of APP, we cultured fibroblasts from mutant and wild-type embryos and analyzed
secretion of APP in Gelatinase A-deficient Mice

FIG. 3. Synthesis and secretion of APP in the brains of mutant mice. Schematic shows APP with α, β-, and γ-secretase sites (a). The region of Aβ is indicated by the hatched box. The regions recognized by the three antibodies (see “Materials and Methods”) are indicated by thick bars at the bottom of the figure. Western blot analyses of the brain extracts of the mutant mice used anti-N (22C11) (b), anti-Αβ (I-56/Affibody 28) (c), and anti-С (G-369) (d) antibodies. Lanes 1 and 2 represent brain soluble fractions, and lanes 3 and 4 represent brain membrane fractions. Lanes 1–3, wild-type mice; lanes 2 and 4, mutant mice.

FIG. 4. Secretion of sAPP and gelatinase A from cultured embryonic fibroblasts stimulated with and without LPS. Gelatinase A in the serum-free conditioned medium demonstrated by gelatin zymography (a). Western blot analysis of APP in the cell lysates using anti-С antibodies (b). Western blot analyses of sAPP in the conditioned medium (CM) using anti-N (c), anti-Αβ (d), and anti-С (e) antibodies, respectively. Lanes 1 and 4, without LPS; lanes 2 and 5, with 1 μg/ml of LPS; lanes 3 and 6, with 10 μg/ml of LPS. Lanes 1–3, wild-type cells; lanes 4–6, mutant cells.

The conditioned medium for gelatinase A activity by zymography and for APPs by Western blotting. Wild-type fibroblasts produced and secreted large amounts of gelatinase A (Fig. 4a, lane 1). As observed earlier with astrocytes (18) and macrophages (19), LPS enhanced synthesis and secretion of gelatinase A by fibroblasts in a dose-dependent manner (Fig. 4a, lanes 1–3). The activated form of gelatinase A was significantly increased in LPS-stimulated cultures. However, no gelatinase A activity was detected in the medium from mutant fibroblasts (Fig. 4a, lanes 4–6). LPS treatment also enhanced APP synthesis by fibroblasts from both wild-type and mutant mice as shown by Western blotting of cell lysates with anti-С antibody (Fig. 4b). Conditioned medium from wild-type cells contained 120–130-kDa proteins that reacted with anti-N and anti-Αβ antibodies (Fig. 4, c and d, lanes 1–3) but not anti-С antibodies (Fig. 4e). These results indicated that these APP molecules were processed and secreted rather than released from dying cells. sAPP secretion was increased by LPS stimulation (Fig. 4, d and e, lanes 1–3). sAPPs from fibroblasts have slightly larger molecular mass than those detected in the brain. This difference reflects the isofrom distribution in different tissues (20). Western blotting revealed sAPP in the conditioned medium from the mutant cells (Fig. 4, c, d, and e, lanes 4–6) as well as from wild-type cells. The intensity of lane 6 in Fig. 4e was slightly weaker than that of lane 3. However, the difference was inconsistently observed in three independent experiments. In sum, the level of cumulative sAPP from mutant cell cultures seemed to be nearly equivalent to that from wild types.

To analyze the kinetics of maturation and secretion of APP, we carried out a pulse-chase experiment of cultured fibroblasts. As summarized in Fig. 5, we observed insignificant differences between wild-type and mutant cells in the rates of maturation of APP, secretion of sAPP and generation of the 15-kDa cytoplasmic tails (cAPP). Thus, we concluded that the gelatinase A (matrix metalloproteinase 2) does not play a role in the metabolism of APP at physiological conditions. However, we cannot rule out the possibility that during development alternative enzymatic systems replace gelatinase A activity for APP processing, although no abnormal gelatinolytic activities were detected. The analyses under the acutely stressed conditions in vivo and in vitro may help to elucidate the issue. Furthermore, this study does not exclude the possibility that the enzyme hydrolyzes Aβ in vivo (21, 22). This point may be addressed by crossing the mutant mice with Aβ high producer transgenic

FIG. 5. Time course of maturation and secretion of APP in cultured fibroblasts. The fibroblasts from wild-type and mutant embryos were metabolically labeled with [35S] methionine for 20 min, and then chased for times indicated in the figure with serum-free DMEM containing methionine. Immature, mature, and truncated cytoplasmic fragments of APP were immunoprecipitated with anti-N (22C11) antibody from cell lysates. sAPP was immunoprecipitated with anti-N (22C11) antibody from culture supernatants. Radioactivity for each given time point is represented as a ratio of the highest activity of each product. Each point represents an average of duplicate experiments. The variance between the duplicates was less than 15%.

a, immature +/+; b, immature −/−; c, mature +/+; d, mature −/−; Δ, sAPP +/+; ●, sAPP −/−; ○, cAPP +/+; ▼, cAPP −/−.
lines. In any case, the mutant mice might help to elucidate further the relationships between gelatinase A and APP.

It has been suggested that gelatinase A plays an important role in the metastasis of malignant tumors and in angiogenesis associated with tumor growth (23, 24). The mutant mice provide a valuable tool to study these putative functions of gelatinase A.

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