Communication

β-Amyloid Peptide and a 3-kDa Fragment Are Derived by Distinct Cellular Mechanisms*

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We have analyzed the cellular processing pathways which produce the 4-kDa amyloid β-peptide (Aβ) and a 3-kDa derivative (p3) of the β-amyloid precursor protein (βAPP) found in conditioned media of tissue culture cells and in cerebrospinal fluid. Pulse-chase experiments reveal that both peptides are secreted in parallel with soluble βAPP (APP); no precursor-product relation between Aβ and p3 was found. The protease inhibitor leupeptin did not influence the production of either peptide. In contrast, the weak base ammonium chloride (NH4Cl) showed a dose-dependent inhibition of Aβ production with less decrease in p3. A similar effect was observed using the monovalent ionophore monensin. Brefeldin A completely inhibited the production of both peptides, indicating that proteases located in the endoplasmic reticulum or early Golgi are not sufficient for the production of the small peptides. Deletion of the βAPP cytoplasmic domain, which removes a consensus sequence that probably mediates reinternalization, caused an increase in secretion of both APP, and p3 and did not abolish Aβ production. These observations suggest that completely mature βAPP within the late Golgi and/or at the cell surface is a prerequisite for Aβ production but processing within the lysosome might not be directly required. p3 appears to derive from the 10-kDa C-terminal stub of βAPP following secretion of APP.

Alzheimer’s disease is characterized by the formation in the brain of insoluble amyloid plaques and vascular deposits consisting of amyloid β-peptide (Aβ).1 Aβ is derived from the membrane-spanning βAPP (Kang et al., 1987). Four major isoforms of βAPP have been described which are all derived by alternative splicing. In addition to the 695-amino acid form (Kang et al., 1987), three other major forms have been reported which contain an additional exon encoding a protease inhibition domain (βAPP 563, 751, and 770) (Kogauchi et al., 1988; Ponte et al., 1988; Tanzi et al., 1988; DeSouvage and Octave, 1989). Upon maturation of βAPP within the endoplasmic reticulum (ER) and Golgi, the precursor is cleaved by an as yet unidentified protease designated βAPP secretase to create the secreted form of βAPP (APP) and a 10-kDa C-terminal fragment that remains membrane-bound (Weidemann et al., 1988; Oltersdorf et al., 1990; Esch et al., 1990; Wang et al., 1991). Because this cleavage occurs within the Aβ domain, this processing pathway inhibits the formation of Aβ. In contrast, some βAPP molecules are reinternalized from the cell surface and targeted to late endosomes/lysosomes (Haass et al., 1992a), where Aβ-containing C-terminal fragments of βAPP accumulate (Golde et al., 1992; Estus et al., 1992; Haass et al., 1992a). These fragments could potentially give rise to the formation of Aβ. Recently, we and others found that Aβ is normally present in the media of cultured cells (Haass et al., 1992b; Shoji et al., 1992) and cerebrospinal fluid (Seubert et al., 1992; Shoji et al., 1992), indicating that the production and release of Aβ is a normal physiological event. In addition to the 4-kDa Aβ, we detected a 3-kDa peptide (p3) corresponding to a truncated fragment of Aβ as well as a number of minor Aβ-related peptides (Haass et al., 1992b). To characterize the cellular pathways that produce the two peptides, we analyzed the formation of the peptides in pulse-chase experiments and after treatment of cultured cells with a variety of agents that interfere with cellular processing pathways. In addition, the effect of a C-terminal deletion of βAPP on the formation of Aβ and p3 was studied.

EXPERIMENTAL PROCEDURES

Drug Treatments—Colchicine was made as a 1 mM stock solution in media. Monensin was made as a 10 mM stock solution in ethanol. Leupeptin was used as described previously (Haass et al., 1992a). NH4Cl was added from a 5 mM stock solution. Brefeldin A (BFA) was added from a 5 mg/ml stock solution in ethanol. For control experiments in the absence of drugs, the appropriate carrier was added. All drugs and carriers were diluted into the media before the mixture was applied to the cells. Human embryonic kidney 293 cells stably transfected with βAPP 695 (Selkoe et al., 1988) were incubated during a 16-h labeling period in methionine-free media containing 10% fetal calf serum with the corresponding drugs (colchicine, leupeptin, monensin, NH4Cl). Identical results were obtained during a 3-h pulse label in the presence of the corresponding drug. Experiments using BFA were carried out only in a 3-h pulse-labeling experiment. All experiments were repeated three to nine times. Metabolic labeling and immunoprecipitations from cell extracts and media were performed as described (Haass et al., 1991, 1992b). Immunoprecipitated APP, was separated on 10% SDS-polyacrylamide gel, whereas Aβ and p3 were separated on a 10–20% Tris-Tricine gel (Haass et al., 1992b). Autoradiography was carried out as described (Haass et al., 1991). The inhibitory effect of drugs on the formation of Aβ and p3 was quantified by densitometry.

Antibodies Used for Immunoprecipitation—The polyclonal antibody C7 (Podlisny et al., 1991) is directed against the last 20 amino acids of the cytoplasmic tail of βAPP. This antibody immunoprecipitates N-’ and N-’ plus O’-glycosylated full-length βAPP, the 10-kDa and a variety of potentially amyloidogenic C-terminal fragments (Haass et al., 1992a). The affinity-purified polyclonal antibody B5 (Oltersdorf et al., 1990) was raised to a recombinantly expressed protein of βAPP 1-752 (numbering of βAPP 695; Kang et al., 1987))
and immunoprecipitates APP, and N'- and N' plus O'-glycosylated full-length βAPP. The polyclonal antibody R1280 (Tamaoka et al., 1992) was raised to synthetic Aβ24-40. This antibody immunoprecipitates Aβ, p3, and small amounts of APP, from media of tissue-culture cells (Haass et al., 1992b).

**Construction of a βAPP C-terminal Deletion Construct and Transfection**—A cDNA construct was designed to encode a truncated species of βAPP by introducing a stop codon after amino acid 653 of full-length PAPP. The polyclonal antibody R1280 (Tamaoka et al., 1992) was raised to synthetic Aβ24-40. This antibody immunoprecipitates Aβ, p3, and small amounts of APP, from media of tissue-culture cells (Haass et al., 1992b).

To analyze the cellular mechanisms involved in the formation of the two peptides in greater detail, we studied the effect of a variety of drugs on the production of Aβ and p3 by 293 cells stably transfected with βAPP 695. Since microtubule depolymerizing agents are known to inhibit tubulin vesicles from fusing with prelysosomal compartments (Kelly, 1990), we analyzed the formation of Aβ and p3 upon treatment of cells with colchicine and nocodazole. Colchicine (Fig. 2A) and nocodazole (data not shown) had no significant effect on the production of Aβ and p3, despite the fact that immunocytochemical experiments using anti-tubulin antibodies revealed a complete depolymerization of the microtubular cytoskeleton (data not shown).

To determine whether lysosomal proteases may be involved in the production of Aβ and p3, 293 cells were treated with leupeptin. Increasing amounts of leupeptin had no influence on the production of both peptides (Fig. 2B), despite the fact that this drug causes a substantial accumulation of Aβ-containing C-terminal fragments of βAPP in the lysosomes of these cells (Haass et al., 1992a). In contrast, increasing amounts of NH4Cl clearly inhibited the formation of Aβ (~74% at the maximum concentration) with less inhibition of p3 formation (~67% at the maximum concentration; Fig. 2C), indicating that an acidic compartment is involved in the generation of Aβ. The monovalent ionophore monensin, which is known to abolish H+, Na+, and K+ gradients and thus inhibit late Golgi and lysosomal functions (Tartakoff, 1983), had a strong dose-dependent inhibitory effect on the formation of Aβ (~96% at the maximum concentration), with less effect on p3 formation (~67% at the maximum concentration; Fig. 2D). In parallel, monensin also down-regulated APP, secretion (Fig. 2D) and inhibited the maturation of intracellular βAPP, resulting in a marked accumulation of an incompletely mature form of βAPP within the cell (Fig. 2E) and low level secretion of incompletely mature APPs, (see down-shift in apparent molecular mass of APP, upon monensin treatment in Fig. 2D). The small amount of C-terminal 10-kDa fragment seemed to be stabilized by monensin (Fig. 2E), suggesting an inhibition of the lysosomal degradation of this fragment. The effect of monensin on the maturation of intracellular βAPP is similar to that reported by Caporaso et al. (1992). The drug experiments described above were carried out by incubating the cells with drugs during a 16-h period of metabolic labeling. Repetition using a 3-h pulse-labeling experiment gave essentially the same results (data not shown). The latter experiments rule against the possibility that a 16-h treatment with these drugs leads to reduced viability of the tissue culture cells.

The possibility of Aβ formation within the ER or early Golgi was excluded by the use of brefeldin A (BFA), which causes a redistribution of Golgi into ER (Pelham, 1991). Under these conditions, Aβ and p3 could not be detected either in the media (Fig. 2F) or inside the cell. As expected, maturation of full-length βAPP was inhibited, resulting in an
incompletely glycosylated βAPP molecule (Fig. 2G). These experiments also exclude the possibility that Aβ might be formed within the cytoplasm after incomplete translocation of βAPP into the ER, since such a process would be unaffected by BFA. Interestingly, we could not detect any intracellular Aβ in the extracts of the cells treated with each of the different drugs described above (data not shown), a finding consistent with previous data (Shoji et al., 1992; Haass et al., 1992b).

To determine the influence of the cytoplasmic domain of βAPP, which contains a potential consensus sequence for coated pit-mediated reinternalization of cell surface proteins (NPXY; Chen et al. (1990)), on the formation of Aβ and p3, a stop codon was inserted after position 653 (numbering according to βAPP 696; Kang et al. (1987)), giving rise to C-terminal truncated βAPP. This construct (named CMV695ΔC) and the wild type cDNA (CMV695) were transfected into 293 cells, followed by metabolic labeling and immunoprecipitation by R1280 and B5 (Fig. 3). Immunoprecipitation of APP, by antibody B5 revealed that deletion of the cytoplasmic tail resulted in increased secretion of APP. Kidney 293 cells were transfected with CMV695 (lane 1) or CMV695ΔC (lane 2), and the media from the metabolically labeled cells were immunoprecipitated with B5. B, p3 and Aβ are detected in the medium of cells transfected with CMV695ΔC. Kidney 293 cells were transfected with CMV695 (lane 1) or CMV695ΔC (lane 2), and the media from the metabolically labeled cells were immunoprecipitated with R1280. Cotransfection with a plasmid encoding the human growth hormone (Nichols Institute) confirmed similar transfection efficiencies with CMV695 and CMV695ΔC.

| Drug | Concentration | APPp3 | APPs |
|------|---------------|-------|------|
| Colchicine (μM) | 0 | 2 | | |
| | 1 | | | |
| Leupeptin (μg/ml) | 0 | | | |
| | 1 | | | |
| NH4Cl (mM) | 0 | | | |
| | 20 | | | |
| Monensin (μg/ml) | 0 | | | |
| | 20 | | | |

**FIG. 2.** The effect of colchicine, leupeptin, NH4Cl, monensin, and brefeldin A on the production of Aβ and p3. A. colchicine does not inhibit the formation of Aβ and p3. Kidney 293 cells were treated with no colchicine (lane 1) or 1 μM colchicine (lane 2). B, increasing amounts of leupeptin had no influence on the formation of Aβ and p3. Lane 1, control; lane 2, 5 μg/ml; lane 3, 50 μg/ml C, increasing amounts of NH4Cl inhibit the formation of Aβ, with less effect on p3. Lane 1, control; lane 2, 5 mM; lane 3, 10 mM; lane 4, 20 mM D, increasing amounts of monensin inhibit the formation of Aβ, with less effect on p3. Lane 1, control; lane 2, 10 μg/ml; lane 3, 25 μg/ml. Asterisks indicate immature form of APP. E, monensin inhibits the normal maturation of βAPP. Cell extracts from the experiment shown in panel D were immunoprecipitated with an antibody (C7) raised against the C terminus of BAPP (Podlisny et al., 1990). Asterisks indicate N’- and N”- plus O’-glycosylated βAPP in untreated control cells. Lane 1, control; lane 2, 10 μM; lane 3, 25 μM F, BFA completely inhibits the production of p3 and Aβ. Lane 1, control; lane 2, 10 μg/ml BFA. G, BFA inhibits the maturation of cellular βAPP. Cell extracts from the experiment shown in panel F were immunoprecipitated with antibody C7. Lane 1, control; lane 2, 10 μg/ml BFA. Asterisks indicate N’- and N”- plus O’-glycosylated βAPP in untreated control cells. Lower arrow, 10-kDa C-terminal fragment. The 200-kDa proteins detected in all immunoprecipitations of βAPP, which contains a potential consensus sequence for coated pit-mediated reinternalization of cell surface proteins (NPXY; Chen et al. (1990)), on the formation of Aβ and p3, a stop codon was inserted after position 653 (numbering according to βAPP 696; Kang et al. (1987)), giving rise to C-terminal truncated βAPP. This construct (named CMV695ΔC) and the wild type cDNA (CMV695) were transfected into 293 cells, followed by metabolic labeling and immunoprecipitation by R1280 and B5 (Fig. 3). Immunoprecipitation of APP, by antibody B5 revealed that deletion of the cytoplasmic tail resulted in increased secretion of APP. Kidney 293 cells were transfected with CMV695 (lane 1) or CMV695ΔC (lane 2), and the media from the metabolically labeled cells were immunoprecipitated with B5. B, p3 and Aβ are detected in the medium of cells transfected with CMV695ΔC. Kidney 293 cells were transfected with CMV695 (lane 1) or CMV695ΔC (lane 2), and the media from the metabolically labeled cells were immunoprecipitated with R1280. Cotransfection with a plasmid encoding the human growth hormone (Nichols Institute) confirmed similar transfection efficiencies with CMV695 and CMV695ΔC.

**FIG. 3.** Deletion of the cytoplasmic domain of βAPP causes an increase in APP, secretion and production of p3. A, deletion of the C terminus of βAPP causes an increased secretion of APP. Kidney 293 cells were transfected with CMV695 (lane 1) or CMV695ΔC (lane 2), and the media from the metabolically labeled cells were immunoprecipitated with B5. B, p3 and Aβ are detected in the medium of cells transfected with CMV695ΔC. Kidney 293 cells were transfected with CMV695 (lane 1) or CMV695ΔC (lane 2), and the media from the metabolically labeled cells were immunoprecipitated with R1280. Cotransfection with a plasmid encoding the human growth hormone (Nichols Institute) confirmed similar transfection efficiencies with CMV695 and CMV695ΔC.

2 E. Koo, personal communication.
be involved in the generation of Aβ. This is based on the observation that NH₄Cl decreases the amounts of Aβ. However, leupeptin did not have any effect on the formation of Aβ (Shoji et al., 1992). The fact that leupeptin and colchicine/nocodazole do not inhibit the formation of both peptides in the present experiments indicates that lysosomes may not be directly involved in the proteolytic processing that results in the formation of either p3 or Aβ. This conclusion is further supported by the fact that we were unable to detect the two peptides within isolated lysosomes purified from metabolically labeled 293 cells (data not shown). Furthermore, leupeptin strongly increases the amounts of the 10-kDa C-terminal fragment and larger potentially amyloidogenic fragments within the lysosome (Caporaso et al., 1992; Golde et al., 1992; Haass et al., 1992a). Despite this accumulation of very high amounts of the 10-kDa C-terminal fragment and slightly larger Aβ-containing fragments in the lysosome, we do not observe higher amounts of p3 or Aβ in the media. We hypothesize that only prelysosomal 10-kDa fragments (most likely on or near the cell surface) and larger Aβ-containing precursor fragments can give rise to p3 and Aβ, respectively. One could argue that leupeptin treatment could inhibit the formation of both peptides within the lysosome, but this should result in reduced amounts of the two peptides upon leupeptin treatment, a finding which is clearly not the case (see Fig. 2B).

The hypothesis that lysosomal degradation of βAPP is not an absolute prerequisite for Aβ production is further supported by the finding that cultured fibroblasts from patients with 1-cell disease, which are unable to target proteases directly to lysosomes, still produce Aβ.² Our data do not exclude the possibility that full-length βAPP or Aβ-bearing fragments thereof might be reinternalized from the cell surface, giving rise to Aβ and p3 within an early endocytic vesicle that subsequently recycles to the cell surface and releases the peptides into the medium.

As a working model, we propose the possibility that the cleavage producing the N terminus of Aβ may be mediated by alternative cleavage of mature βAPP by the secretase or a closely related enzyme within late Golgi and/or cell surface. This event could take place either on the cell surface or within the acidic compartments of late Golgi or transport vesicles derived from a reinternalization pathway carrying βAPP or fragments thereof. Indeed, evidence has recently been presented that alternative secretory cleavage of βAPP may potentially create the N terminus of Aβ (Seubert et al., 1993).