Communication

Evidence for a Nonsecretory, Acidic Degradation Pathway for Amyloid Precursor Protein in 293 Cells

IDENTIFICATION OF A NOVEL, 22-kDa, β-PEPTIDE-CONTAINING INTERMEDIATE*

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We have analyzed the metabolic pathway of maturation of APP751 in stably transfected 293 cells, in the presence of either of the cysteine protease inhibitors leupeptin or E-64. Metabolic labeling, followed by immunoprecipitation at various times in the chase with a rabbit polyclonal antibody (anti-BX6) specific to the carboxyl-terminal end of amyloid precursor protein (APP), revealed the accumulation of a novel ~22-kDa carboxyl-terminal fragment (22-CTF) in the inhibitor-treated cells. This fragment, which was not detectable in untreated cells, was immunoprecipitated by four separate antibodies to the carboxyl-terminal region of APP as well as by polyclonal and monoclonal antibodies specific to the N-terminal amino acids of the β-peptide domain. Antibodies to the carboxy-terminal end of APP do not, however, recognize the fragment. Co-treatment of the inhibitor-treated cells with either of the lysosomotropic agents chloroquine or ammonium chloride completely blocked the generation of this fragment but did not significantly affect APP maturation or secretion. All, however, slowed the intracellular turnover of the cell-associated, ~9-kDa carboxyl-terminal fragment (c-CTF) produced during constitutive secretion. Denaturing analyses of these results suggest that this non-secretory pathway of APP degradation, mediated by cysteine proteases in an intracellular acidic compartment, accounts for ~70% of total APP metabolism and that a key processing intermediate in this pathway is a 22-kDa, β-peptide-containing APP carboxyl-terminal fragment. It is possible that inefficient degradation of such an intermediate leads to the formation of aggregating β-peptide.

The major pathway of cellular maturation of APP results in the secretion of the bulk of the considerable extracellular domain of the transmembrane precursor protein, mediated by a proteolytic processing step which cleaves inside the β-peptide region (Esch et al., 1990). This pathway, first established in HeLa (Weidemann et al., 1989) and in 293 cells (Olterdsdorf et al., 1990), has since been shown to be operative in many cell culture and expression systems (Anderson et al., 1991; Wang et al., 1991; Lowery et al., 1991). A clue to a possible targeting of APP into a separate metabolic pathway is present in the protein itself, since its short cytoplasmic tail contains a NPXY sequence, recently shown to be a key functional motif recognized as an internalization signal for a variety of cell-surface protein receptors, including the low density lipoprotein receptor (Chen et al., 1990). Internalization is followed by the delivery of the ligand, or ligand-receptor complex, into the acidic endosomal-lysosomal pathway (Mellman et al., 1986) for degradation. By analogy, it is possible that a subpopulation of APP, perhaps one that has not undergone the secretory cleavage, is indeed targeted to an acidic degradation pathway, possibly by endocytosis from the cell surface. In support of this, clathrin-costed vesicles isolated from human brain have been found to contain mature full-length APP. Indeed, it has recently been reported (Golde et al., 1992) that full-length APP is intracellularly processed, apparently by the endosomal-lysosomal pathway, into a family of potentially amyloidogenic 8-12-kDa carboxyl-terminal derivatives. In this report, we have used inhibitors of lysosomal cysteine proteases, in conjunction with acidotropic amines, to obtain direct experimental evidence for a novel, ~22-kDa preamyloid APP intermediate, generated from full-length APP in an acidic compartment, in 293 cells stably transfected with either APP751 or -695.

EXPERIMENTAL PROCEDURES

Leupeptin, E-64, and chloroquine were obtained from Sigma. 293 cells overexpressing APP751 were obtained and grown as described previously (Olterdsdorf et al., 1990).

Antibodies—As described earlier (Olterdsdorf et al., 1990) the polyclonal antibodies anti-BX6 and anti-BX5 were raised to fusion proteins comprising, respectively, APP592-695 and APP444-592. Antibodies 1G5 was also raised to synthetic 01-28, and antibodies c7, c8, and R1280 were obtained from Dr. Dennis Selkoe (Brigham & Women's Hospital, Boston). The anti-β-peptide monoclonals 6C6 and 10D5 were raised to synthetic β1-28.

Metabolic Labeling and Immunoprecipitation—These were carried out as described previously (Olterdsdorf et al., 1990) with the following modifications. Cells were incubated with either leupeptin (0.1 mM) or E-64 (0.1 mM) for 4-16 h prior to labeling with 0.1 Ci/ml 35S methionine for 2 h. Chase times were from 1 to 24 h. In the experimental cells, the inhibitor was present during the chase periods. Control cells were treated exactly as described, except no inhibitor was added at any time. At each chase period, medium (supernatants) was gently removed, the cells lysed in 1 ml of ice-cold 50 mM Tris, pH 8.0, 15 mM NaCl, 20 mM Na-EDTA, 1% sodium deoxycholate, 1% Triton X-100, and 0.1% SDS, and the crude lysates centrifuged at 100,000 × g for 4 °C for 5 min to obtain cell extracts. Approximately 40 μg of total protein equivalent (determined as described by Smith et al. (1985)) from each extract was immunoprecipitated with 5 μg of each antibody.

Treatment with Acidotropic Bases—Ammonium chloride (10 mM)

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The abbreviations used are: APP, amyloid precursor protein; c-CTF, cell-associated, ~9-kDa carboxyl-terminal fragment.

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or chloroquine (0.1 mM) was added immediately prior to the label and maintained through the label and chase periods (0–4 h).

**Quantitation of Autoradiograms**—Immunoprecipitated bands were individually quantitated using a Molecular Dynamics laser densitometer (Palo Alto, CA). Raw data were corrected for the predicted methionine content for each fragment.

**RESULTS**

Immunoprecipitation of cell extracts with the APP carboxyl-terminal-specific antibody anti-BX6 revealed the presence of a novel, ~22-kDa APP fragment in cells treated with either leupeptin (Fig. 1, *Cell Extracts, panel B*) or E-64 (panel C), but not in control cells (panel A). This 22-kDa carboxyl-terminal fragment (22-CTF) is readily detectable at the end of the 2-h label period (lane 1), rapidly accumulates between 2 and 4 h (lanes 2 and 3), and decays between 4 and 8 h (lanes 4 and 5). Treatment with either of the inhibitors also leads to the accumulation, and relatively little degradation, of the c-CTF arising out of the constitutive secretory pathway. Neither inhibitor has any effect on the time course of appearance and extent of accumulation of secreted APP in the medium (Fig. 1, *Supernatants, panels B and C*) or on the turnover of full-length APP (Fig. 1, *Cell Extracts, panels B and C, top arrow*) when compared with untreated cells (panel A).

These qualitative conclusions are strengthened by the densitometric evaluation of these same autoradiograms, as shown in Fig. 2. At the peak, twice as much 22-CTF is immunoprecipitated from the inhibitor-treated cells, as is c-CTF. Analysis of the secreted APP accumulating in the medium over the same time period also suggests that only ~30% of total cell-associated APP is secreted into the medium during the chase, and this fraction does not significantly change in either the absence or the presence of the inhibitors (data not shown). Taken together, these data strongly suggest that only about one-third of total APP is processed by the constitutive secretory pathway; the remaining two-thirds is normally degraded by an alternate, cysteine protease inhibitor-sensitive pathway.

Although the time course of appearance of the 22-CTF parallels that of the c-CTF, we wished to determine whether both of them were arising out of the same secretory pathway. Co-treatment of the cells with leupeptin and either chloroquine (Fig. 3, lanes 5A and 5B) or ammonium chloride (lanes 6A and 6B) blocks the appearance of the 22-CTF induced by leupeptin alone (lanes 4A and 4B) but not of c-CTF. Compared with untreated cells (lanes 1A and 1B), treatment with chloroquine alone (lanes 2A and 2B) inhibits the time course of appearance of c-CTF slightly, whereas treatment with ammonium chloride (lanes 3A and 3B) in fact leads to an intensification of this band. None of these treatments significantly affected secretion (data not shown). Thus, acidotropic weak bases, present concurrently with leupeptin, block the appearance of the 22-CTF but do not significantly affect secretion or the appearance of c-CTF.

Finally, we wished to verify that the 22-CTF is indeed a true APP-derived fragment and that, as indicated by its apparent Mr, it does contain the entire β-peptide domain. Fig. 4 shows that, in addition to the carboxyl-terminal-specific antibody anti-BX6 (lane 1), the 22-CTF is immunoprecipitated by two other polyclonal carboxyl-terminal antibodies, c7 (lane 2) and c8 (lane 3), and the monoclonal 13G8 (lane 6), but not by either anti-BX3 (lane 4) or 1G5 (lane 5), antibodies specific to the extracellular domain of APP. Three separate antibodies, the monoclonals 10D5 and 6C6 and the polyclonal R1280, all specific for the amino-terminal end of the β-peptide region, also immunoprecipitated 22-CTF (lanes 7–9, respectively) but, as expected, did not immunoprecipitate c-CTF. Thus, 22-CTF does appear to be a true APP fragment.

**FIG. 1.** Immunoprecipitation of cell-associated and secreted APP in the presence or absence of protease inhibitors. 293 cells overexpressing APP751 were metabolically labeled with [35S]methionine for 2 h, then chased for 2, 4, 8, and 24 h, as described under "Experimental Procedures." At each time point, cell extracts were immunoprecipitated with anti-BX6, whereas supernatants were immunoprecipitated with anti-BX5. In each case, panel A represents control cells, panel B, treatment with leupeptin, and panel C, treatment with E-64; lanes 1–5 represent chase times of 0, 2, 4, 8, and 24 h, respectively.

**FIG. 2.** Densitometric analyses of cell-associated CTFs in the presence or absence of protease inhibitors. Autoradiograph of the experiment described in Fig. 1 was scanned by a laser densitometer (Molecular Dynamics), and c-CTF and 22-CTF amounts at each time point were separately quantitated (arbitrary units). The numbers obtained were plotted as a function of time, after correction for relative methionine content.

**FIG. 3.** Immunoprecipitation of cell-associated APP following treatment with ammonium chloride or chloroquine, in the presence or absence of leupeptin. Cell extracts were made from cells treated with control (1), 0.1 mM chloroquine (2), 10 mM ammonium chloride (3), 0.1 mM leupeptin (4), 0.1 mM chloroquine + 0.1 mM leupeptin (5), and 10 mM ammonium chloride + 0.1 mM leupeptin (6). In each case, cells were metabolically labeled for 2 h, and extracts made at chase time 0 (A) and chase time 4 h (B) were immunoprecipitated with anti-BX6.
were immunoprecipitated with anti-BX6 antibodies. Leupeptin-treated cells were metabolically labeled for 1 h, and cell extracts were prepared after a 4-h chase. Equal aliquots were immunoprecipitated with anti-BX6 (1), c7 (2), c8 (3), anti-BX3 (4), 1G5 (5), 13G8 (6), 10D5 (7), 6C6 (8), and R1280 (9). Epitopes recognized by these antibodies are described under “Experimental Procedures.”

containing the entire β-peptide region but lacking distal extracellular epitopes.

DISCUSSION

The constitutive secretory pathway of APP maturation, which would preclude the formation of the β-peptide, is a virtually ubiquitous pathway, evident in many cell types, expression systems, and species. However, the quantitative role of this pathway in APP metabolism, especially in neuronal cells, has been the subject of debate and, recently, has been suggested to play a relatively minor role (Haas et al., 1991) in microglia and astrocytes. In their early report on APP metabolism in HeLa cells, Weidemann et al. (1989) also recovered only ~30% of the initial label in the form of secreted APP in the conditioned medium and suggested that the rest is rapidly degraded in the cells. Our results strongly suggest that even in 293 cells, in which the secretory pathway was originally firmly established, the quantitative role of the pathway in total APP turnover appears to be modest.

Quantitatively, as much as ~70% of the total APP in 293 cells may be normally metabolized via this alternate, non-secretory pathway. Since both leupeptin and E-64 are potent inhibitors of many of the lysosomal cysteine pro tease and since the appearance of the 22-CTF is exquisitely sensitive to weak bases which are known to disrupt competent lysosomal function (Mellman et al., 1986), it is most likely that this intermediate is being generated in an endosomal-lysosomal compartment, distinct from the secretory pathway. The results obtained further establish that the cellular turnover of the predominant CTF being generated in the secretory pathway, the ~9-kDa c-CTF, is also mediated by cysteine pro tease in an acidic compartment.

The notion of lysosomal processing of APP is not new. Indirect evidence for lysosomal processing of APP was first presented by Cole et al. (1989), who showed that increased amounts of the mature forms of full-length APP are recoverable from various cell lines treated with leupeptin. Very recently, other investigators (Estus et al., 1992; Golde et al., 1992) have described a family of CTFs, with molecular masses between 8 and 12 kDa, that are apparently generated during normal processing of APP in 293 cells, and these authors concluded that such fragments were being generated in an endosomal-lysosomal compartment, in distinction from the secretory pathway. Moreover, they report that a family of such short carboxyl-terminal fragments are also detected in human brain, suggesting that the same pathway may be operating in vivo as well. In addition to the prominent 9-kDa c-CTF produced in the secretory pathway, we also observe such a family of CTFs, with molecular masses between 10 and 13 kDa, which accumulate in the leupeptin-treated cells, in agreement with the results obtained by these investigators.

An ~22-kDa, stable fragment of APP, containing the β-amidoid region, has been recently described in human brain microvessels (Tamaoka et al., 1992). At first glance, the 22-kDa intermediate described in this report would appear to be identical with the microvesSEL-associated fragment. However, anti-BX5 (raised to APP444-592), which was one of the polyclonal antibodies used to identify the microvesSEL-associated fragment, failed to recognize the 22-CTF described in this report, in either immunoprecipitations or on Western blots. It is possible, of course, that specific glycosylation, or other post-translational differences, between human brain APP and kidney cell-derived APP, leads to the formation of slightly different intermediates of approximately the same size but with different antigenic recognition.

A bulk of the APP is therefore normally degraded by what appears to be a lysosomal pathway in 293 cells. In 293 cells, the 22-CTF appears not to be detectable in the absence of the protease inhibitors used in this report; this suggests that it is a transitory intermediate, normally rapidly degraded in the protease-rich lysosomal milieu. It is therefore tempting to speculate that inefficient degradation of either the 22-CTF, or some other metabolic intermediate, is responsible for the production and accumulation of β-peptide in Alzheimer’s disease. It is unlikely that APP degradation by the lysosomal pathway simply occurs because of overexpression of APP in these stably transfected cells, since we have observed the same pattern on treating either non-transfected 293 cells or rat PC12 cells with either leupeptin or E-64 (data not shown). More likely, the proportion of APP that does not undergo the secretory cleavage may end up at the cell surface and then is rapidly endocytosed and degraded; this would be consistent with the presence of the possibly functional NPXY internalization signal in the cytoplasmic tail. This cell-surface endocytic “shuttling” may reflect an aspect of the physiological role of APP, which is poorly understood. Nonetheless, considering the pivotal role of APP in Alzheimer’s disease, a clear understanding of all the metabolic pathways traveled by this protein is probably critical to the understanding of the pathogenesis of amyloid deposition in this neurodegenerative disease.

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FIG. 4. Immunoprecipitation of 22-CTF with various APP antibodies. Leupeptin-treated cells were metabolically labeled for 1 h, and cell extracts were prepared after a 4-h chase. Equal aliquots were immunoprecipitated with anti-BX6 (1), c7 (2), c8 (3), anti-BX3 (4), 1G5 (5), 13G8 (6), 10D5 (7), 6C6 (8), and R1280 (9). Epitopes recognized by these antibodies are described under “Experimental Procedures.”

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