Evidence That Tumor Necrosis Factor α Converting Enzyme Is Involved in Regulated α-Secretase Cleavage of the Alzheimer Amyloid Protein Precursor*

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The amyloid protein, Aβ, which accumulates in the brains of Alzheimer patients, is derived by proteolysis of the amyloid protein precursor (APP). APP can undergo endoproteolytic processing at three sites, one at the amino terminus of the Aβ domain (β-cleavage), one within the Aβ domain (α-cleavage), and one at the carboxyl terminus of the Aβ domain (γ-cleavage). The enzymes responsible for these activities have not been unambiguously identified. By the use of gene disruption (knockout), we now demonstrate that TACE (tumor necrosis factor α converting enzyme), a member of the ADAM family (a disintegrin and metalloprotease-family) of proteases, plays a central role in regulated α-cleavage of APP. Our data suggest that TACE may be the α-secretase responsible for the majority of regulated α-cleavage in cultured cells. Furthermore, we show that inhibiting this enzyme affects both APP secretion and Aβ formation in cultured cells.

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‡ The abbreviations used are: APP, amyloid protein precursor; APPα-secreted APP; TNF-α, tumor necrosis factor α; TACE, TNF-α converting enzyme; IC3, Immunex compound 3; HPLC, high performance liquid chromatography; PMA, phorbol 12-myristate 13-acetate; PDBu, phorbol 12,13-dibutyrate; Tricine, N-[2-hydroxy-1,1-bis(hydroxymethyl) ethyl]glycine.

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FIG. 1. Regulated secretion of APP in primary embryonic fibroblasts derived from wild-type, but not TACE-deficient, mice. Primary embryonic fibroblasts derived from wild-type mice or from mice in which the TACE gene had been disrupted (knockout), were incubated for 2 h in the absence or presence of PMA and/or IC3. The amounts of secreted APP (APPs) were then determined by immunoblotting 50-μl aliquots of the culture medium with antibody 22C11. Data are representative of three experiments performed in duplicate. Note that all samples were run in duplicate on the same gel to facilitate comparisons (duplicate lanes were excised from the final image for simplicity).

The exact composition of the digests was determined by liquid chromatography/mass spectrometry (LC/MS), using a 1-mm inside diameter Vydac C18 column with a flow rate of 50 μl/min and a linear gradient of 2% acetonitrile/min in 0.1% trifluoroacetic acid. MS was carried out by directing 10% of the HPLC effluent to the electrospray source of a Finnigan TSQ700 triple quadrupole mass spectrometer (San Jose, CA).

RESULTS

As a direct and definitive test for an involvement of TACE in the α-secretase pathway, we examined basal and regulated APP secretion in primary embryonic fibroblasts derived from control mice or mice in which the TACE gene had been disrupted by homologous recombination (knockouts) (28) (Fig. 1). In cells derived from control mice, regulated secretion was stimulated 300–500% by activation of protein kinase C by phorbol 12-myristate 13-acetate (PMA). Inhibition of protein phosphatases 1 and 2A by okadaic acid had similar effects (not shown). These effects were blocked by IC3. In contrast, in cells derived from knockout mice, there was no increase in the formation and secretion of APPs caused by the addition of PMA (Fig. 1) or okadaic acid (not shown). The levels of cell-associated, full-length APP were identical in the two cell populations, indicating that TACE knockout cells are not deficient in expressing APP (not shown). As an additional control, basal and PMA-activated protein kinase C activities were determined and were identical between the cell populations (not shown).

The nearly total lack of PMA- and okadaic acid-induced cleavage and secretion of APP indicates that TACE (or a substrate of TACE) is the predominant α-secretase for regulated secretion in this system. Note that basal formation and secretion of APPs was unaffected in cells derived from knockout mice supporting the existence of two classes of secretases.

To begin to distinguish whether it is TACE itself that acts on APP or whether TACE acts via an intermediary, we tested whether recombinant TACE catalytic domain (35) could cleave a synthetic peptide, acetyl-VHHQKLVFFA-amide, encompassing the sequence of the α-secretase site of APP. TACE was able to cleave the synthetic peptide between Lys and Leu, at the APP α-secretase site (37–39) (Fig. 2), indicating that TACE has the potential to appropriately cleave APP. It should be noted that the α-secretase activity responsible for basal secretion of APP (which may not be TACE, see Fig. 1) acts at a site largely, but not completely, determined by the distance of the site from the membrane (40, 41). The amino acid requirements for sub-

FIG. 2. Cleavage by TACE of a peptide encompassing the α-cleavage site of APP. Acetyl-VHHQKLVFFA-amide was incubated with buffer alone (upper panel) or with the catalytic domain of TACE (lower panel), followed by HPLC to separate the reaction products, as described under “Methods.” Peak 1 is the intact peptide, peak 2 is LVFFA-amide, and peak 3 is acetyl-VHHQK. The very minor UV-absorbing species generated were not detected by the alternate LC/MS procedure (see “Methods”). Data are representative of three experiments.

FIG. 3. Regulated formation and secretion of Aβ and its modulation by IC3. CHO cells, stably expressing human APP751, were metabolically labeled for 2 h with [35S]methionine followed by a 2-h chase period in which the cells were incubated in the presence of excess unlabeled methionine. During the chase period, cells were incubated in the absence or presence of PDBu and/or IC3. After incubation, secreted APP (APPs) and Aβ were immunoprecipitated using antibodies 6E10 and 4G8 (which reacts with Aβ). Upper panel, the precipitates were resolved on 10–20% Tris-Tricine gels, dried, and subjected to quantitative storage phosphorautoradiography. Lower panel, means ± S.E. of three independent experiments performed in duplicate are shown. *, p < 0.005; **, p < 0.05.

strate cleavage by TACE have not been fully determined although it is clear that TACE functions most effectively when it and its substrate are appropriately oriented in the membrane. In addition to their effects on the formation and secretion of APPs, second messenger cascades are also able to regulate the formation and secretion of Aβ. For example, activation of protein kinase C and/or inhibition of protein phosphatase 1 leads to increased formation and secretion of APP, together with a decrease in the formation and secretion of Aβ (18–20). It has been hypothesized that the effects of these compounds are due to limiting amounts of substrate (APP) (7, 18), such that by
stimulating the formation and secretion of APPs there is per force less Aβ formed. To test this hypothesis, cells stably expressing APP751 were metabolically (pulse-chase) labeled and examined for effects of phorbol 12,13-dibutyrate (PDBu) and/or IC3 on APPs and Aβ formation and secretion, as determined by quantitative immunoprecipitation (Fig. 3). Importantly, the effects of PDBu on Aβ formation were completely blocked by IC3. This paralleled the effects of IC3 on regulated secretion of APPs consistent with the hypothesis detailed above. Interestingly, not only were the effects of PDBu on Aβ formation blocked by IC3 but there was a significant increase in Aβ formation in the presence of PDBu and IC3. This indicates that there is a herefore masked pathway in which activation of protein kinase C can lead to increased formation and secretion of Aβ. At a final point, the antibodies used for immunoprecipitation of APPs (6E10) recognize the portion of APP between the β- and α-cleavage sites. Thus, it is the α-secretase-derived species of APP, that is formed in the presence of PDBu and blocked by IC3. Similar results were observed with cells expressing mutant (NL) APP or when PMA (1 μM) was used in place of PDBu (not shown).

**DISCUSSION**

In this study, we have examined the role of TACE in regulated α-secretion. Pharmacological manipulation of TACE leads to altered α-cleavage of APP and altered Aβ formation. More importantly, disruption of the TACE gene completely abolishes regulated α-cleavage in cultured cells. This result is consistent with TACE being an α-secretase, a premise further supported by the ability of TACE to cleave the APP synthetic peptide at the appropriate site. We cannot, however, rule out the possibility that TACE is acting on a separate protein which then affects APP cleavage.

It is important to note that basal formation and secretion of APPs was unaffected in cells derived from knockout mouse. This supports the existence of two classes of secretases, one involved in basal secretion and one (perhaps TACE) involved in regulated secretion. Note, however, that in cultured CHO cells some basal secretion seems to be inhibited by IC3. Finally, by the use of IC3, we provide additional evidence that the effects of protein kinase C activation on Aβ formation are secondary to effects on the formation and secretion of APPs and identify a novel effect of protein kinase C on increasing Aβ formation under restricted conditions.

Alterations in TACE activity during aging may contribute to amyloid formation. In addition, activating TACE by pharmacological manipulation might prove beneficial in Alzheimer’s disease. Furthermore, homologs of TACE might be involved in basal secretion of APPs and in β-cleavage of APP.

**Note Added in Proof**—A distinct molecular mechanism for α-secretase, involving GPI-linked aspartyl proteases, has been identified recently in yeast (Romano, H., Seeger, M., Gandy, S., Wang, G. T., Kraft, G. A., and Fuller, R. S. (1998) J. Biol. Chem. 273, in press).