Constitutive shedding of the amyloid precursor protein ectodomain is up-regulated by tumour necrosis factor- α converting enzyme

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The amyloid precursor protein (APP) of Alzheimer's disease is a transmembrane protein that is cleaved within its extracellular domain, liberating a soluble N-terminal fragment (sAPP α). Putative mediators of this process include three members of the ADAM (a disintegrin and metalloprotease) family, ADAM9, ADAM10 and ADAM17/TACE (tumour necrosis factor- α converting enzyme). Tumour necrosis factor- α protease inhibitor (TAPI-1), an inhibitor of ADAMs, reduced constitutive and muscarinic receptor-stimulated sAPP α release in HEK-293 cells stably expressing M3 muscarinic receptors. However, the former was less sensitive to TAPI-1 (IC_{50} = 8.09 μ M) than the latter (IC_{50} = 3.61 μ M), suggesting that these processes may be mediated by different metalloproteases. Constitutive sAPP α release was increased several-fold in cells transiently transfected with

TACE, and this increase was proportional to TACE expression. In contrast, muscarinic-receptor-activated sAPP α release was not altered in TACE transfectants. TACE-dependent constitutive release of co-transfected APP₆₉₅ was inhibited by TAPI-1 with an IC_{50} of 0.92 μ M, a value significantly lower than the IC_{50} s for inhibition of either constitutive or receptor-regulated sAPP α shedding mediated by endogenous secretases. The results indicate that TACE is capable of catalysing constitutive α -secretory cleavage of APP, but it is likely that additional members of the ADAM family mediate endogenous constitutive and receptor-coupled release of sAPP α in HEK-293 cells.

Key words: Alzheimer's disease, ADAM proteases, disintegrins, sAPP α , α -secretase.

INTRODUCTION

The extracellular domains of many transmembrane proteins are released from cells following proteolytic cleavage [1]. Proteins processed in this way include certain growth factors, growth factor receptors, adhesion molecules and enzymes, and the amyloid precursor protein (APP) of Alzheimer's disease [2]. In many cell types, shedding of transmembrane protein ectodomains is stimulated by phorbol esters, which are direct activators of protein kinase C (PKC) [3–5]. Shedding may also be evoked by calcium ionophores, by inhibitors of serine/threonine or tyrosine phosphatases, or by ligands of receptors coupled to PKC, tyrosine kinases, or mitogen-activated protein kinases [5–11]. Metalloprotease inhibitors block shedding of a variety of transmembrane proteins, including APP, which lack sequence similarity at the sites of cleavage [12–18]. Despite the structural diversity of proteins subject to ectodomain shedding, recent genetic evidence indicates that a common mechanism might underlie this process; specifically, a mutant Chinese hamster ovary cell line that exhibited a defect in phorbol-ester-evoked shedding of transforming growth factor- α was also unable to shed a variety of other unrelated membrane proteins, including L-selectin, the interleukin-6 receptor α subunit and APP, in response to stimulation [14,19]. Three recently identified sheddases are members of the ADAM (a disintegrin and metalloprotease) family [20,21]: tumour necrosis factor- α converting enzyme (TACE)/ADAM17, which cleaves tumour necrosis factor- α [22,23]; Kuzbanian/ADAM10, responsible for the cleavage of Notch [24] and for the release of a soluble form of the Notch ligand Delta [25]; and meltrin- γ /ADAM9, which mediates shedding of the ectodomain of the heparin-binding

epidermal-growth-factor-like growth factor in response to phorbol esters [26].

The amyloid β peptide that aggregates to form amyloid plaques in the brain in Alzheimer's disease is a proteolytic degradation product of APP (for a review see [27]). Cleavage of APP within the amyloid- β peptide domain by an enzyme or family of enzymes, known collectively as α -secretase, releases a large soluble N-terminal fragment of APP (sAPP α) into the extracellular space. The α -cleavage event is of considerable physiological interest because it may preclude the formation of amyloidogenic peptides (for a review see [5]), and because the secreted derivative sAPP α has putative neurotrophic, neuroprotective and cell adhesive properties (for a review see [28]). Importantly, sAPP α and the full-length cell-associated form of APP appear to exert different effects on neurite outgrowth and neuronal cell survival [29,30]; hence the biological properties of APP may be modulated by its conversion from the full-length into the secreted form. The demonstration that stimulation of muscarinic receptor subtypes coupled to phosphoinositide turnover (subtypes M1, M3 and M5) increases sAPP α release [31,32] was the first indication that APP ectodomain shedding is a receptor-regulated process. Since then, many additional receptor families have been shown to regulate α -secretory cleavage of APP [5,33], and the signalling mechanisms that govern proteolytic cleavage of transmembrane proteins and the physiological consequences of this process continue to be intensively investigated.

The ability of the ADAM protease TACE to mediate constitutive and receptor-regulated sAPP α release was examined in HEK-293 cells stably transfected with M3 muscarinic receptors. The results indicate that although overexpression of TACE results in increased constitutive sAPP α release from these cells,

Abbreviations used: ADAM, a disintegrin and metalloprotease; APP, amyloid precursor protein; DMEM, Dulbecco's modified Eagle's medium; PKC, protein kinase C; sAPP α , soluble APP ectodomain released by α -secretase; TACE, tumour necrosis factor- α converting enzyme; TAPI-1, tumour necrosis factor- α protease inhibitor.

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endogenous α -secretase activity is likely to be due to the presence of one or more additional metalloproteases.

EXPERIMENTAL

Materials

Cell culture media, fetal calf serum, and LIPOFECTAMINE PlusTM reagent were from Life Technologies (Rockville, MD, U.S.A.). Carbamylcholine chloride (carbachol), DMSO, PMSF, leupeptin, 4-(2-aminoethyl)benzenesulphonyl fluoride, 1,10-phenanthroline, and deoxycholic acid were from Sigma (St. Louis, MO, U.S.A.). Tumour necrosis factor- α protease inhibitor (TAPI-1) was from Peptides International (Louisville, KY, U.S.A.). Anti-APP antibodies (clone 22C11), and antibodies to ADAM9 and ADAM10 were from Chemicon International Inc. (Temecula, CA, U.S.A.). 6E10 antibodies, which recognize residues 1 to 16 of the amyloid- β peptide domain of full-length APP, were from Senetek (Maryland Heights, MO, U.S.A.). Goat polyclonal antibodies to TACE were from Santa Cruz Biotechnology Inc. (Santa Cruz, CA, U.S.A.). Peroxidase-linked anti-mouse secondary antibodies were from BD Transduction Laboratories (San Diego, CA, U.S.A.), anti-goat/sheep secondary antibodies were from Sigma, and anti-rabbit secondary antibodies were from Bio-Rad (Hercules, CA, U.S.A.). Biotinylated standards (Cell Signaling Technology, Beverly, MA, U.S.A.) were used to estimate molecular masses. The vector encoding APP₆₉₅ [34] was a gift from Dr Carmela Abraham (Boston University School of Medicine, Boston, MA, U.S.A.). A pcDNA3 vector encoding murine TACE [22] was a gift from Dr Roy Black (Immunex Corporation, Seattle, WA, U.S.A.).

Cell culture and transfection

HEK-293 cells stably transfected with M3 muscarinic receptors (HEK-293-M3 cells) were cultured in Dulbecco's modified Eagle's medium (DMEM)/Ham's F-12 bicarbonate medium supplemented with 10% (v/v) fetal-calf serum, and grown in an atmosphere of air/CO₂ (19:1 v/v). Cells were transfected using LIPOFECTAMINE PlusTM reagent. Experiments were usually carried out 48 h after transfection.

Immunoblot analysis

Test incubations were carried out in serum-free DMEM. Stock solutions of carbachol were dissolved in distilled water. Stock solutions of TAPI-1 were dissolved in DMSO, and subsequently diluted with DMEM. Control cultures received equivalent concentrations of DMSO in DMEM. Final concentrations of DMSO did not exceed 0.2% (v/v). Following incubation the medium was collected, treated with PMSF, cleared by centrifugation, and desalted on Sephadex G-25 columns (Pharmacia Biotech, Piscataway, NJ, U.S.A.). The eluates were freeze-dried and suspended in SDS/PAGE sample buffer. Cells were lysed in a RIPA buffer [50 mM Tris/HCl (pH 7.5), 150 mM NaCl, 2 mM 4-(2-aminoethyl)benzenesulphonyl fluoride, 10 mM 1,10-phenanthroline containing 1% (v/v) Nonidet P-40, 0.05% (w/v) SDS, 0.5% (w/v) deoxycholate and 1 μ g/ml leupeptin]. Lysates were centrifuged to remove detergent-insoluble material and diluted in 2 $\times$ SDS/PAGE sample buffer. Samples were boiled, and size fractionated on polyacrylamide mini-gels (Bio-Rad). Proteins were electroblotted on to PVDF membranes, which were then blocked with 5% (w/v) powdered milk (for mouse or rabbit antibodies) or 3% (w/v) gelatin (for goat antibodies) in Tris-buffered saline/0.15% (v/v) Tween 20 (TBST). Membranes

were probed with antibodies to APP or TACE, washed in TBST, then incubated with peroxidase-linked secondary antibodies and washed again. Bands were visualized on X-ray film using enhanced chemiluminescence reagent (DuPont NEN, Boston, MA, U.S.A.). Films were scanned with a flat-bed scanner (UMAX PowerLook III) equipped with a transparent media adapter, and the resulting images were quantified using NIH Image software.

Statistical analysis

Values in the text and Figures are expressed as means $\pm$ S.E. Statistical comparisons among groups were made using one-way ANOVA. Significance testing was performed using Fisher's least significant difference (LSD) test; differences were taken to be statistically significant at *P* values of less than 0.05. IC₅₀ values were estimated using curve-fitting software from Kaleidagraph.

RESULTS

The zinc metalloprotease inhibitor TAPI-1 reduces M3 receptor-coupled release of the APP ectodomain

A number of reports have implicated disintegrin metalloproteases, including TACE/ADAM17, ADAM10 and meltrin γ /ADAM9, in the shedding of the ectodomain of APP, and of other transmembrane proteins. In the present study, the ability of disintegrin metalloproteases to regulate basal and receptor-coupled release of sAPP α was examined using as a model system HEK-293 cells stably expressing M3 muscarinic receptors. As shown previously [31], the acetylcholine analogue carbachol caused a concentration-dependent increase in the release of endogenous sAPP α (sAPP α_{endo}) from HEK-293-M3 cells (Figure 1A). The cells were pretreated with various concentrations of TAPI-1 and challenged with carbachol for 20 min. TAPI-1 caused a concentration-dependent inhibition of carbachol-evoked release with an estimated mean IC₅₀ of 3.61 ± 0.71 μ M (*n* = 4) (Figures 1B and 1C). At a concentration of 20 μ M, the response was decreased by more than 80%.

Overexpression of the ADAM protease TACE does not affect M3 receptor-coupled release of the APP ectodomain

The ability of TAPI-1 to inhibit carbachol-evoked shedding of sAPP α suggested the involvement of a zinc metalloprotease in the response. A co-transfection strategy was therefore employed to determine if the ADAM protease TACE exhibited α -secretase activity. HEK-293 cells, like other non-neuronal cells, predominantly express isoforms of APP containing a Kunitz-type protease inhibitor domain (APP_{751/770}) [27]. An additional isoform, APP₆₉₅, lacks the Kunitz protease inhibitor domain and is predominantly expressed in neurons [27]. HEK-293-M3 cells were transfected with a cDNA construct encoding APP₆₉₅, and the conditioned medium was analysed for sAPP α content. Immunoblot analysis of the medium using 6E10 antibodies detected two proteins (Figure 2A). The faster migrating band representing sAPP α_{695} was not present in the medium of non-transfected cells, and was not recognized by an antibody against the Kunitz protease inhibitor domain (results not shown). The muscarinic agonist carbachol caused a concentration-dependent release of both sAPP α_{endo} and sAPP α_{695} over a similar range of concentrations (Figure 2A). Because similar amounts of APP₆₉₅ and APP α_{endo} were released by transfected cultures, this construct could be used to assess the effects of TACE overexpression on shedding of both endogenous and transfected APP simultaneously.

Figure 1 Inhibition of M3 receptor-evoked sAPP α release by TAPI-1

(A) Cells were treated for 20 min with various concentrations of carbachol (carb; 0–100 μ M) in serum-free DMEM. The medium was collected and analysed for sAPP α content (see the Experimental section). (B) Cells were pretreated for 30 min with serum-free DMEM containing DMSO or TAPI-1 (1–20 μ M), and then treated for an additional 20 min with fresh DMEM containing DMSO or TAPI-1 in the presence or absence of carbachol (100 μ M). The final concentration of DMSO was 0.1% in all experimental solutions. Medium was collected and sAPP α content was determined by immunoblotting. (C) Results from (B) were quantified by densitometry. Values represent means $\pm$ S.E. of four experiments.

HEK-293-M3 cells were co-transfected with APP₆₉₅ and TACE or an empty vector, and sAPP α release was stimulated by the addition of carbachol to the culture medium. In cells transfected with the metalloprotease (0.5 μ g of TACE cDNA/dish) there was a slight increase in basal release of sAPP α ₆₉₅. However, TACE overexpression did not significantly affect carbachol-stimulated release of either endogenous sAPP α , or transfected sAPP α ₆₉₅ (Figures 2B and 2C) at maximally effective concentrations of the agonist. Although, in two experiments, the effect of submaximal concentrations of carbachol was potentiated in TACE transfectants, this response was not consistently observed and, when the results of six independent experiments were combined, no significant effect of TACE transfection on carbachol-evoked release was noted (Figure 2C). When the amount of TACE transfected into the cells was increased to 1 μ g cDNA/dish, a slight decrease in carbachol-evoked sAPP α release was observed (results not shown). Immunoblot analysis of cell lysates confirmed that TACE transfectants overexpressed the metalloprotease (Figure 2D). An antibody to the C-terminal of TACE detected two bands of approx. 125 and 100 kDa re-

Figure 2 Effect of TACE overexpression on M3 receptor-coupled sAPP α release

(A) Cells transfected with an empty vector or APP₆₉₅ were stimulated for 20 min with various concentrations of carbachol. (B) Cells were co-transfected with APP₆₉₅ together with TACE or an empty vector; 48 h after transfection, cells were pre-incubated for 1 h in serum-free DMEM then treated for 20 min with various concentrations of carbachol. The medium was collected and sAPP α content was determined by immunoblotting. (C) The results from (B) were quantified by densitometry. Values represent the means $\pm$ S.E. of 6 experiments. (D) TACE expression was assessed by immunoblot analysis of cell lysates. Full-length TACE is indicated by an arrow. A faster migrating band, possibly representing a form of TACE that lacks the prodomain (see text), is indicated by an arrowhead.

spectively, when proteins were separated on 7.5% polyacrylamide gels under reducing conditions. Two bands of similar sizes have been described by others [35], and are thought to represent immature full-length TACE (Figure 2D, arrow) and a smaller, activated form that lacks the prodomain (Figure 2D, arrowhead). Two proteins of virtually identical sizes, although much less abundant, were present in non-transfectants, suggesting that TACE is also expressed endogenously by these cells.

Overexpression of the ADAM protease TACE potentiates constitutive shedding of the APP ectodomain

Although TACE did not alter carbachol-evoked sAPP α release, an increase in basal sAPP α release was observed in TACE transfectants (Figures 2B and 2C). However, in these experiments, the collection period (20 min) was too brief to allow accurate assessment of constitutive sAPP α release. Therefore, in the next series of experiments, a longer collection period was employed. HEK-293-M3 cells were co-transfected with APP₆₉₅

Figure 3 Effects of TACE overexpression on constitutive sAPPα release

(A) Cells were co-transfected with APP₆₉₅ and various amounts of TACE or empty vector; 48 h after transfection, the medium was replaced with serum-free DMEM for 2.5 h. The medium was collected and analysed for sAPPα content (upper panel). Cell lysates were probed with antibodies to TACE (lower panel). (B) Immunoblots of medium sAPPα₆₉₅ were analysed by densitometry. Values represent the means ± S.E. of 3–6 experiments. Statistical significance was estimated by ANOVA followed by Fisher's least significant difference test (*, $P < 0.05$). (C) Linear regression analysis of sAPPα₆₉₅ release versus TACE expression. Values represent means ± S.E. of three or four experiments.

and various amounts of TACE; 48 h after transfection the cells were incubated in serum-free medium for 2.5 h, the medium was collected and analysed for sAPPα content (Figure 3A, upper panel). Expression of TACE, assessed by immunoblotting, was increased in a near linear fashion by transfection of cells with increasing amounts of TACE cDNA over a range of 0.25–1.0 μg/dish (Figure 3A, lower panel). Basal sAPPα release from cells transfected with APP₆₉₅ increased in a dose-dependent fashion with increasing expression of TACE cDNA (Figure 3A, upper panel; Figure 3B). When basal sAPPα₆₉₅ release was plotted as a function of TACE expression and the values subjected to linear regression analysis, a positive correlation of $r = 0.999$ was obtained (Figure 3C). TACE overexpression also signifi-

Figure 4 Inhibition of constitutive sAPPα release by TAPI-1

(A) Cell cultures were transiently transfected with APP₆₉₅ and, 26 h after transfection, the growth medium was replaced with serum-free DMEM containing DMSO or various concentrations of TAPI-1 for 30 min. The medium was then replaced with fresh medium containing DMSO or the inhibitor. After 18 h, the medium was collected and sAPPα content was determined by immunoblotting with 6E10 antibodies (upper panel). APP in cell lysates was detected with 22C11 antibodies (lower panel). The diffuse band indicated by the arrowhead represents the mature, fully glycosylated form of APP. (B) The sAPPα content of the medium was quantified by densitometry and expressed as a percentage of the maximum release. The values are the means ± S.E. of four experiments (●), and the results of one experiment in which cells were transfected with APP₆₉₅ (○).

cantly increased the release of sAPPα_{endo}, although to a lesser extent (Figure 3A). The smaller effect is not surprising because the average transfection efficiency in these experiments was approx. 50%. These results demonstrate that TACE overexpression markedly potentiates constitutive shedding of the APP ectodomain.

Release of sAPPα from cells overexpressing TACE is more sensitive to the inhibitor TAPI-1

Although the results indicate that TACE overexpression increased constitutive sAPPα release, they do not prove that TACE is the endogenous α-secretase in HEK-293 cells. If this were true, then it might be expected that both endogenous basal sAPPα release and the augmented release observed in cells overexpressing TACE would exhibit similar sensitivities to the inhibitor TAPI-1. In order to assess the effect of TAPI-1 on constitutive release of APP, cells were incubated in serum-free medium for 18 h in the presence of various concentrations of TAPI-1, and the medium was analysed for sAPPα content. The inhibitor caused a concentration-dependent reduction in the release of sAPPα₆₉₅ and sAPPα_{endo} (Figure 4A). Similar results were obtained when a collection period of 4 h was used, and in

Table 1 Inhibition of sAPP α release by TAPI-1

IC₅₀s for individual experiments were estimated by curve-fit analysis, and results from multiple experiments were averaged. Data are shown as means $\pm$ S.E. The number of experiments is indicated in parentheses. Statistical significance was estimated by ANOVA followed by Fisher's least significant difference test. * $P < 0.001$ compared with constitutive release of sAPP α _{endo}; † $P < 0.02$ compared with carbachol-evoked release of sAPP α _{endo}.

Response	IC ₅₀
Constitutive release of sAPP α _{endo}	8.09 $\pm$ 1.01 (4)
Carbachol-evoked release of sAPP α _{endo}	3.61 $\pm$ 0.71 (4)*
TACE-dependent constitutive release of sAPP α _{endo}	1.20 $\pm$ 0.12 (4)*†
TACE-dependent constitutive release of sAPP α ₆₉₅	0.92 $\pm$ 0.08 (4)*†

Figure 5 Inhibition of TACE-dependent constitutive sAPP α release by TAPI-1

Cells were co-transfected with APP₆₉₅ and TACE, or an empty vector; 48 h after transfection, cells were preincubated with serum-free DMEM containing DMSO or various concentrations of TAPI-1 for 30 min. The medium was then replaced with fresh DMEM containing DMSO or the inhibitor for 2.5 h. (A) The medium was collected and sAPP α content was determined by immunoblotting with 6E10 antibodies. (B) sAPP α content was quantified by densitometry. The results are the means $\pm$ S.E. of four experiments. Closed symbols, sAPP α _{endo}; open symbols, sAPP α ₆₉₅. Squares represent sAPP α release from cells transfected with APP₆₉₅ and an empty vector; circles represent sAPP α release from cells co-transfected with APP₆₉₅ and TACE (1 μ g/dish).

cells expressing only endogenous APP. When the results of four experiments were taken, a mean IC₅₀ of 8.09 $\pm$ 1.01 μ M for inhibition of constitutive release of sAPP α _{endo} by TAPI-1 was obtained (Figure 4B and Table 1). The constitutive release of sAPP α ₆₉₅ from cells transfected with APP₆₉₅ (Figure 4B, open circles) showed a similar sensitivity to TAPI-1 (IC₅₀ = 10.3 μ M).

As shown in Figure 3, TACE overexpression caused a marked increase in constitutive sAPP α release. To determine the sensitivity of this response to TAPI-1, basal release of sAPP α from TACE transfectants was measured in the presence of increasing concentrations of the antagonist TAPI-1 (Figure 5). In four

Figure 6 HEK-293-M3 cells express ADAM10

Cell lysates from TACE transfectants (lane 1) or from untransfected cultures (lanes 2–4) were immunoblotted with antibodies to TACE (lane 1) or subjected to immunoprecipitation followed by immunoblotting with antibodies to ADAM10 (lanes 2 and 3). As a control, immunoprecipitates prepared with antibodies to ADAM9 were probed with anti-ADAM10 (lane 4). Arrows indicate full-length TACE (left panel), or full-length and partially processed forms of ADAM10 (right panel).

experiments, the mean IC₅₀ for inhibition of TACE-dependent sAPP α ₆₉₅ release by TAPI-1 was 0.92 $\pm$ 0.08 μ M. The mean estimated IC₅₀ for inhibition of TACE-dependent sAPP α _{endo} release was 1.20 $\pm$ 0.12 μ M. These values were significantly lower than the IC₅₀s for inhibition of constitutive and carbachol-evoked sAPP α release from cultures that were not transfected with TACE (Table 1).

These results suggested that a metalloprotease other than TACE might be the endogenous mediator of sAPP α release from HEK-293 cells. Lysates from HEK-293-M3 cells were therefore examined for the presence of the metalloprotease ADAM10, which has been shown to have α -secretase activity [36,37]. Rabbit polyclonal antibodies raised against the C-terminus of human ADAM10 (Chemicon International Inc.) were used for successive immunoprecipitation and immunoblot analysis of cell lysates. Two bands with approx. molecular masses of 92 000 and 74 000 Da were detected (Figure 6, lanes 2 and 3). These bands were not detected in immunoprecipitates prepared with anti-ADAM9 antibodies and immunoblotted with antibodies to ADAM10 (Figure 6, lane 4). The observed bands were similar in size to the predicted molecular mass of 85 000 Da of full-length ADAM10 [38] and the apparent molecular mass of 62 000 Da of the mature form of ADAM10 lacking the prodomain [38,39].

DISCUSSION

The results presented in this report demonstrate that TACE, when overexpressed in HEK-293-M3 cells, markedly elevates constitutive shedding of the APP ectodomain, and thus may be an α -secretase. However, because constitutive sAPP α release from TACE-overexpressing cells was more sensitive to TAPI-1 than release from non-overexpressing cells, it seems unlikely that TACE is the principal endogenous mediator of constitutive sAPP α release from HEK-293 cells. Alternatively, endogenous TACE may be localized to a compartment that is less accessible to the inhibitor. TACE overexpression did not affect receptor-evoked sAPP α release, suggesting that this process may be mediated by a different protease.

TAPI-1 and TAPI-2 are hydroxamic acid-based inhibitors of metalloproteases [22,40] that block shedding of a number of transmembrane proteins [14], including APP [14,41,42]. In HEK-

293–M3 cells that were not transfected with TACE, the IC₅₀ for inhibition of carbachol-evoked release of sAPP α by TAPI-1 was consistently lower than that for constitutive sAPP α release (Table 1), suggesting that distinct endogenous α -secretases might mediate these two processes. Moreover, even at high concentrations of TAPI-1, some residual release of sAPP α was apparent. This observation has also been made by others [37,42], and suggests the presence of a TAPI-insensitive α -secretase.

Pharmacological evidence from other laboratories points to the presence of multiple α -secretases, distinct from TACE, in some cell types. Thus the hydroxamic acid-based inhibitor compound 4, although a potent inhibitor of TACE, only weakly inhibited sAPP α release from the neuronal IMR-32 and SH-SY5Y cell lines [16,17,43]. A second inhibitor of this class, batimastat, inhibited TACE-induced shedding of a tumour-necrosis-factor- α -related protein from intact cells at lower concentrations [44] than were required to block sAPP α release [43]. Moreover, although batimastat inhibited basal and carbachol-stimulated sAPP α release from SH-SY5Y cells with similar potency [43], the hydroxamic acid-based compound KD-IX-73-4 inhibited receptor-coupled sAPP α shedding in several cell types without affecting constitutive release [18]. Overall, the available evidence suggests that sAPP α shedding is mediated by a number of distinct α -secretases in a cell-type-specific fashion. Importantly, fibroblasts from mice lacking a functional *TACE* gene [22] were shown to be deficient in phorbol-ester-evoked sAPP α release, although exhibiting normal levels of constitutive release [41,45], suggesting that, at least in mouse fibroblasts, TACE is the principal endogenous mediator of receptor-coupled, but not constitutive, α -secretase cleavage.

Although the results of the present study do not indicate whether the effects of TACE overexpression are due to direct cleavage of sAPP α or to activation of another protease with α -secretase activity, the recent demonstration that the recombinant TACE catalytic domain cleaves a peptide derived from the APP α -secretase cleavage site [41] is consistent with the possibility that TACE itself is an α -secretase. In this regard, it should be noted that two additional members of the ADAM family, ADAM9 and ADAM10, have been implicated as possible α -secretases [36,37,46]. Overexpression of ADAM9 in COS cells and ADAM10 in HEK-293 cells increased basal and phorbol-ester-stimulated sAPP α release, and a dominant-negative form of ADAM10, mutated in the zinc-binding site, inhibited endogenous α -secretase activity in HEK-293 cells [36,46]. The latter observation suggests that ADAM10 may be the endogenous α -secretase in HEK-293 cells and, indeed, these cells express detectable amounts of ADAM10 mRNA [36] and protein (see Figure 6). The results of Lammich et al. [36] differ from those described in the present paper, in that regulated, as well as constitutive, release was increased by protease overexpression. Moreover, evidence given in the present study (Figure 1) and elsewhere [12,14,16,17,41,42], clearly indicates that receptor-coupled and PKC-dependent sAPP α release is sensitive to metalloprotease inhibitors. Again, the available evidence points to the involvement of several distinct ADAMs or ADAM-like proteases in constitutive and receptor-coupled sAPP α release.

As yet, it is unclear how the activity of ADAM proteins is influenced by signal transduction pathways. It is possible that ADAM activity is increased following phosphorylation by receptor-activated kinases. Indeed, it has been shown that ADAM9 is phosphorylated *in vitro* by purified PKC δ [26], and in intact cells treated with phorbol esters [47]. Moreover, a recent report that appeared in abstract form indicates that phorbol esters stimulate TACE phosphorylation [48]. However, transfection into TACE-deficient cells of a TACE construct lacking

the cytoplasmic domain was sufficient to reconstitute phorbol-ester-induced upregulation of ectodomain shedding of several TACE substrates [49]. Thus it appears that the stimulatory effect of phorbol esters on TACE activity does not require phosphorylation of the TACE cytoplasmic domain.

TACE, and other members of the ADAM family, are characterized by the presence of several distinct domains including a prodomain, a metalloprotease domain, a disintegrin domain, a cysteine-rich region, a transmembrane domain and a cytoplasmic tail [50,51]. Removal of the prodomain by a furin-like protease appears to be necessary to generate an active enzyme [47,52]. This mode of regulation raises the possibility that the proprotein convertase PC7, which upregulates constitutive sAPP α release when overexpressed in cultured cells, mediates its effect by cleaving the prodomain of an endogenous ADAM protease [53,54]. Of importance in this regard is a recent report that overexpression of ADAM10 in a cell line deficient in the proprotein convertase furin increased constitutive sAPP α release, whereas overexpression of TACE did not, suggesting that furin is an upstream regulator of TACE but not of ADAM10 [37].

Immunoblot analysis of TACE expression in TACE-transfectants and in controls detected two protein bands, of approx. 125 and 100 kDa respectively (see Figures 2 and 3), confirming an earlier report [35]. The faster migrating band is not recognized by antibodies raised against the prodomain of TACE, suggesting that this species represents the mature active form of TACE lacking the prodomain [35]. Although mature TACE can be detected on the cell surface, much of it appears to be localized to an intracellular compartment [35,42]. Using an APP mutant targeted to the *trans*-Golgi network, it was shown that a phorbol-ester-activated α -secretase activity, which is inhibited by TAPI, resides in the *trans*-Golgi compartment [42]. However, PKC-regulated sAPP release from the cell surface is also sensitive to TAPI [55]. Thus it is likely that TACE, or other ADAM family members, may cleave APP within an intracellular compartment, as well as at the cell surface.

The implications of these results with respect to the pathophysiology of Alzheimer's disease have yet to be determined. Northern-blot analysis detected high levels of TACE mRNA in human fetal brain, with lower levels in adult brain [22]. A detailed analysis of the regional expression of ADAM10 and TACE/ADAM17 in adult mouse brain by Northern blot and *in situ* hybridization showed that ADAM10 is more widely expressed than TACE, which is confined mainly to the hippocampus, the inferior colliculus, the cerebellum and the pontine nuclei [56]. Another study reported extensive co-expression of APP and ADAM10 in developing and adult mouse brain, whereas little overlap of APP and TACE was observed during development [57]. Based on these findings, it was suggested that ADAM10 may be the relevant secretase in brain [57]. In Alzheimer's disease, ADAM1 and 2 proteins are found in the cytoplasm of pyramidal neurons and are associated with neurofibrillary tangles. These proteins were not detected in brains from young controls, but were found in neurons from aging control brains that showed some age-related pathology, as indicated by increased tau immunoreactivity [58]. To our knowledge, consistent alterations in expression of TACE in brain from subjects with Alzheimer's disease have not been described. However, even if ADAMs are upregulated in Alzheimer's disease, this would not be likely to be a proximate cause of amyloid deposition, given that stimulation of α -secretase activity tends to reduce amyloid- β peptide formation (for a review see [5]), and metalloprotease inhibitors block this effect [41,42]. On the other hand, impairments in function of metalloproteases might be expected to increase amyloid formation, and potential therapeutic strategies

for the treatment of Alzheimer's disease might be directed toward increasing the activity of these enzymes. To that end, it will be important to determine the mechanisms regulating activation of these proteases.

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