Histone Deacetylase Inhibitor Valproic Acid Inhibits Cancer Cell Proliferation via Down-regulation of the Alzheimer Amyloid Precursor Protein

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The β-amylloid precursor protein (APP) represents a type I transmembrane glycoprotein that is ubiquitously expressed. In the brain, it is a key player in the molecular pathogenesis of Alzheimer disease. Its physiological function is however less well understood. Previous studies showed that APP is up-regulated in prostate, colon, pancreatic tumor, and oral squamous cell carcinoma. In this study, we show that APP has an essential role in growth control of pancreatic and colon cancer. Abundant APP staining was found in human pancreatic adenocarcinoma and colon cancer tissue. Interestingly, treating pancreatic and colon cancer cells with valproic acid (VPA, 2-propylpentanoic acid), a known histone deacetylase (HDAC) inhibitor, leads to down-regulation of APP and increased GRP78 levels. In contrast, treating cells with valpromide, a VPA derivative lacking HDAC inhibitory properties, had no effect on APP levels. VPA did not modify the level of epidermal growth factor receptor, another type I transmembrane protein, and APLP2, a member of the APP family, demonstrating the specificity of the VPA effect on APP. Small interfering RNA-mediated knockdown of APP also resulted in significantly decreased cell growth. Based on these observations, the data suggest that APP down-regulation via HDAC inhibition provides a novel mechanism for pancreatic and colon cancer therapy.

β-Amyloid precursor protein (APP)! is a highly conserved single transmembrane protein (type I) with a receptor-like structure and consists of a heterogeneous group of proteins migrating between 110 and 135 kDa (1, 2). The heterogeneity is due to alternative splicing, leading to eight distinct isoforms (namely APP677, APP695, APP696, APP714, APP733, APP751, APP752, and APP770), as well as by a variety of post-translational modifications, including O- and N-glycosylation, sulfation, and phosphorylation. APP isoforms exist as immature (N-glycosylated) and mature (N- and O-glycosylated, tyrosyl-sulfated) species. Immature APP localizes in the endoplasmic reticulum and cis-Golgi, and the mature APP form preferentially localizes in the trans-Golgi network, secretory and endocytic vesicles, and at the plasma membrane (3, 4).

APP695 is the most common isoform in the central nervous system, whereas APP751 and APP770 are predominantly expressed in non-neuronal cells (5). The key event in the pathogenic cascade in Alzheimer disease is the amyloidogenic pathway characterized by subsequent cleavage of APP by the enzyme β-secretase and further processing by γ-secretase, which finally leads to the generation of Aβ peptides. However, the predominant route of APP processing consists of successive cleavages by α- and γ-secretases in non-neuronal cells (6, 7). The cleavage of APP at Lys16-Leu17 bond by α-secretase within the Aβ sequence liberates the sAPPα and the nonamyloidogenic C-terminal APP fragment (8–10). APP is one of three members of a small gene family, which includes amyloid β precursor-like protein 1 (APLP1) and amyloid β precursor-like protein 2 (APLP2). All encode type I transmembrane proteins and share similar domain structures, with a large extracellular N-terminal domain and a short cytoplasmic region that undergo similar processing. In contrast to APLP1, which preferentially is expressed in neuronal tissues, APLP2 is expressed also in peripheral non-neuronal tissue (11). The biological activity of APP is still not well understood, especially in non-neural and cancer cells. Several studies showed that APP and its secreted forms promote adhesion, migration, neurite outgrowth, and general growth-promoting properties (reviewed in Refs. 2, 11). In a previous study, we demonstrated that SH-SY5Y neuroblastoma cells transfected with APP695 showed an increase in cell proliferation compared with mock-transfected controls (12).

Recent evidence supports the observation of an inverse link between cancer and Alzheimer disease (13). The authors demonstrated that Alzheimer disease was longitudinally associated with a reduced risk of cancer, and a history of cancer was associated with a reduced risk of Alzheimer disease suggesting a
common mechanism linking both diseases. In this study, we could demonstrate that APP is selectively overexpressed in pancreatic and colon carcinoma, but not in healthy tissues. Because APP seems to have an important function as a growth factor, it has received considerable attention in the oncology field. Studies showed that patients with increased APP levels have a significantly lower survival rate and has therefore been suggested as a potential biomarker to evaluate cancer prognosis (14–17). For decades, valproic acid (VPA, 2-propylpentanoic acid) has been the drug of choice for the treatment of epilepsy and bipolar disorder and represents one of the most important therapeutic agents in psychiatry (18, 19), although the underlying mechanisms for brain activity are controversial. Furthermore, VPA is a well established histone deacetylase (HDAC) inhibitor and affects cell growth in different types of cancer in vitro and in vivo (19–24). We examined VPA-induced alterations in the processing of endogenous APP. We further focused on the molecular mechanism responsible for the highly specific impairment in the maturation of APP and the reduction of secreted sAPPα caused by VPA in the cancer cell lines. The binding immunoglobulin protein (Bip) (also called glucose-regulated protein 78, GRP78) is a molecular chaperone that uses ATP/ADP cycling to regulate protein folding. GRP78 is a 78-kDa heat shock protein induced by VPA (25), and it is involved in maturation of APP (26). The aim of this report was to study the potential impact of APP on prominent gastrointestinal tumor growth and to elucidate the underlying molecular mechanism.

**EXPERIMENTAL PROCEDURES**

**Reagents and Antibodies**—The following antibodies were used: monoclonal APP/αβ antibody W0-2 (1:5000, The Genetics Co.), APP (1:250 of monoclonal antibody 22C11, Chemicon; 1:500 of polyclonal antibody 23850, generous gift from Gerd Multhaup), polyclonal APP antibody 5313 (27), anti-actetyl histone H4 (1:2000, Millipore), EGFR (1:200, Santa Cruz Biotechnology), APLP2 (1:5000, Calbiochem), GRP78 (1:1000, Cell Signaling Technology), and monoclonal mouse anti-actin (1:5000, Sigma). VPA (Sigma) was prepared in sterile water as concentrated stock solution and added to the final concentrations as indicated. Trichostatin A stock solution (5 mM in DMSO) was purchased from Sigma. Valproamide (VPM), a kind gift from Katwijk Chemie B.V., was dissolved in DMSO and added to final concentrations as indicated.

**Human Specimens**—Histological classification (tumor type, grade of malignancy) was carried out according to the current World Health Organization and International Union Against Cancer criteria. All slides were re-evaluated again, and diagnosis was approved by an experienced pathologist. All tumor specimens (n = 3 of each tumor type) were obtained from the Department of Pathology, University Medicine, Goettingen, Germany.

**Cell Culture and Transfection**—Stably expressing cell lines were obtained by transfecting the mammalian expression vector pCEP4 (Invitrogen) alone (mock) or with the APP695wt or SPA4CT constructs into SH-SYSY cells using Lipofectin 2000 (Invitrogen). 300 μg/ml hygromycin (Invitrogen) was added to maintain stable integration of the constructs in the transfected cells. APP695-transfected and mock-transfected SH-SYSY control cells have been in culture for an identical period of time with a similar number of passages. All transfected cell lines were cultured in Dulbecco’s modified Eagle’s medium/F-12 (Pan Biotech GmbH), supplemented with 10% fetal calf serum, 2 mM l-glutamine, and 1% nonessential amino acids. Three pancreatic cancer cell lines (BxPC3, PANC-1, and CFAPC-1) and four colon cancer cell lines (SW480, LoVo, CaCo-2, and T84) were used in this study (kindly provided by Prof. Ghadimi, University of Göttigen) and were cultured in RPMI 1640 medium (Pan Biotech GmbH) containing 10% fetal calf serum and 2 mM l-glutamine. All cell cultures were incubated at 37 °C in a humidified atmosphere of 5% CO2. Data are presented only with the BxPC3 and SW480 cell lines.

**Immunohistochemistry on Paraffin Sections**—Paraffin-embedded colon and pancreas tissue sections (4 μm) were deparaffinized in xylene and rehydrated in a series of ethanol concentrations. Primary antibodies 22C11 and 23850 were incubated overnight in a humid chamber at room temperature. Sections were subsequently incubated with a horseradish peroxidase-conjugated polymer, which carries antibodies to rabbit and mouse immunoglobulins (EnVision/HRP™, Dako, Hamburg, Germany), and signals were visualized with 3,3’-diaminobenzidine (Dako). Counterstaining was carried out with Meyer’s hematoxylin, mounted in Super Mount medium, and equipped with an Olympus DP-50 camera using the software Viewfinder Lite version 1.0.134 (Pixera Co.). All tissue sections were analyzed with the monoclonal antibody 22C11 and polyclonal antibody 23850. For negative controls, blocking solution was used in place of the primary antibody.

**Cell Growth Assay**—Cell growth was measured by a colorimetric cell proliferation assay (CellTiter 96 AQ assay, Promega) using the reduction of 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (Owens’s reagent) to formazan according to the protocol of the supplier as reported previously (12). In brief, BxPC3 and SW480 cancer cell lines were seeded at 5 × 103 cells/well in 96-well culture plates and incubated for 24 h with RPMI 1640 medium containing 10% fetal bovine serum. The cells were then treated with medium containing VPA, TSA, and VPH for 24 h. After 24 h, 20 μl of dye solution was added to each well, and the plate was incubated for 2 h at 37 °C, 5% CO2. The absorbance was measured at 490 nm using an enzyme-linked immunosorbent assay microplate reader. Each assay was performed at least six times.

**Western Blot Analysis**—For treatment experiments, 1 × 106 BxPC3 and SW480 cells were seeded per 25-cm2 flask. Cells were treated for 24 h with serum-free media followed by 24 h of treatment with the compounds. Protein concentration was measured using a commercially available kit (Roti-Quant, Roth). Cells were harvested using lysis buffer (50 mM Tris, 150 mM NaCl, 1% Nonidet P-40, 1% Triton X-100, 2 mM EDTA, pH 7.5) supplemented with 1× complete protease inhibitor mixture (Roche Applied Science) for 30 min at 4°C. Cellular debris was removed by centrifugation (5000 × g, 10 min), and equal amounts of soluble proteins (usually 10–15 μg) were separated by electrophoresis on 4–12% Vario-Gels (Anamed) and trans-
ferred to nitrocellulose membranes (GE Healthcare). After washing in Tris-buffered saline, including Tween 20 (TBS-T), the blots were incubated with the primary antibody, followed by horseradish peroxidase-conjugated secondary mouse/rabbit antibodies (1:4000/1:3000, Dako). For detection of sAPPα, conditioned culture media were collected and centrifuged at 14,000 × g for 2 min at 4 °C to remove cell debris. Subsequently, supernatant (2 ml) was concentrated by centrifugal filter devices (Amicon Ultra-4 50K, Millipore) at 4000 × g for 5 min. Protein levels in the supernatant were normalized to the protein concentration in the corresponding cell lysate. For quantification of relative protein levels, x-ray films (Hyperfilm EC, Amersham Biosciences) were scanned, and densitometric analysis was carried out using ImageJ software (version 1.41o, National Institutes of Health). Each immunoblot was done three to six times.

**Knockdown of APP Using siRNA—**BxPC3 and SW480 cells were grown in 6-well plates to ~30–50% confluence in antibiotic-free medium and transfected with siRNA duplexes (Ambion, Silencer® Select validated siRNA, s1500) targeted to APP as follows: sense sequence, CAAGGAAAGCUGUACGGAAAATT; antisense sequence, UUCGGUAACUGAUCCUGGT. Transfection of the cells was carried out using Lipofectamine 2000 and Opti-MEM I medium (Invitrogen) according to the manufacturer’s protocol at a final concentration of 50 nM. Random siRNA (Ambion, Silencer® negative control siRNA) served as negative control targeted to a scrambled sequence. According to the manufacturer, the specificity of the Silencer® select siRNAs was validated by TaqMan gene expression assays. Specific knockdown of APP by Silencer® select siRNA has been shown previously (28). After 48 h, cell lysates and media were collected and analyzed using the protocol for cell lysis described above.

**Statistical Analysis—**Statistical differences were evaluated using one-way ANOVA followed by Bonferroni post hoc test or unpaired t test as indicated. All data are given as means ± S.E. All statistics were calculated using GraphPad Prism version 5.00 software.

**RESULTS**

**Expression of APP in Colon and Pancreas Carcinoma—**Using immunohistochemistry, the expression of APP in neoplastic tissue was studied in colon carcinoma and pancreas carcinoma. Immunohistochemistry revealed that colon carcinoma cells showed a strong expression of APP, whereas no expression was seen in normal epithelial cells of the colon (Fig. 1, A–D). In pancreas carcinoma, an intense expression of APP could be found in tumor cells (Fig. 1, G and H). Interestingly, islet cells of the endocrine pancreas presented a strong expression of APP (Fig. 1, E and F), whereas acinar cells showed a slight staining for APP. In contrast, APP was not detected in the normal epithelial cells of the salivary duct system as well as the exocrine pancreas, respectively (Fig. 1, E and F). To detect APP, we used the monoclonal antibody 22C11, which recognizes residues 66–81 (29) and the polyclonal antibody 23850 which detects residues 18–491 of human APP (30, 31).

**APP Knockdown Results in Inhibition of Tumor Cell Growth—**To further validate the functional relevance of APP in pancreatic and colon cancer, a loss-of-function study using siRNA was performed. Detection of APP in SW480 cells by Western immunoblotting revealed two bands at 110 and 130 kDa. To test whether these two variants represent immature and mature APP, respectively, we performed pulse-chase experiments. After pulse labeling, a major band of 110 kDa was detected in cell lysates. During the chase period an additional band was detected at 130 kDa, indicating that immature APP was converted to its mature form by O-glycosylation. Immunoprecipitation of APP from conditioned media revealed secretion of soluble APP after 20 min that accumulated during the chase. These data indicate that the two bands observed by Western immunoblotting represent immature and mature variants of a single splice variant (supplemental Fig. S1).

The expression of APP was efficiently suppressed by treatment of SW480 cells with APP siRNA but not with random siRNAs (Fig. 2). Cellular and secreted APP were reduced by 56 ± 3.9 and 67 ± 5.7%, respectively. In BxPC3 cells, treatment with APP siRNA also resulted in significantly reduced levels of cellular (62 ± 2.7%) and secreted sAPPα (69 ± 7.7%). Importantly, siRNA-mediated down-regulation of APP significantly reduced cell growth in both cell types. siRNA treatment did not affect cellular morphology either in pancreatic or in colon cancer cells (data not shown). APP siRNA treatment had no effect on APLP2 levels (supplemental Fig. S2).

**VPA Treatment Inhibits Cell Growth, Decreases APP Levels, and Reduces Secreted sAPPα in Pancreatic and Colon Cell Lines—**A range of studies have shown that VPA and other HDAC inhibitors alter cellular proliferation and induce programmed cell death in pancreatic and colon cancer in vitro and in vivo (23, 32). Based on these observations, we performed cell proliferation assays on BxPC3 (pancreatic cancer) and SW480 (colon cancer) treated with VPA (Fig. 3), as well as using additional pancreatic (PANC-1 and CFAPC-1) and colon (LoVo, CaCo-2, and T84) cancer cell lines (data not shown). All cells were treated with different concentrations of VPA ranging from 0 to 100 mM and showed similar growth inhibition. After 24 h of incubation, we performed a 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium assay to evaluate drug effect on cellular growth. Results are presented as percentage inhibition compared with untreated control (Fig. 3, A and D). VPA treatment resulted in dose-dependent inhibition in proliferation with both cell lines. To further evaluate the effect of VPA on APP expression, BxPC3 and SW480 were exposed to 0, 1, 2.5, 5, or 10 mM VPA for 24 h. To analyze total APP and the processing product sAPPα, we used the APP/β-specific antibody W0-2 recognizing a region between amino acid 5 and 8 of human Aβ (33). This antibody detects full-length APP and C99 in cell lysates and sAPPα in culture media. All blots were re-probed with a β-actin antibody to ensure equal protein load. Significant down-regulation was seen in BxPC3 cells at 1 mM and in SW480 at 2.5 mM VPA treatment. APP decreased with higher doses of VPA in BxPC3 as follows: 1 mM, 30 ± 2.3%; 2.5 mM, 40 ± 8.9%; 5 mM, 43 ± 6.2%, and 10 mM 80 ± 5.4% (Fig. 3C). APP expression further decreased with higher doses of VPA in SW480 cells.
metabolism by measuring sAPPα in the supernatant of conditioned cells. Exposure to VPA markedly reduced sAPPα corresponding to full-length APP levels. Significant down-regulation of sAPPα was seen in BxPC3 cells at 1 mM and in SW480 at 2.5 mM VPA treatment. sAPPα decreased with higher doses of VPA in BxPC3 (means ± S.E. in percent reduction of sAPPα levels versus untreated control) as follows: 1 mM, 33 ± 2.7%; 2.5 mM, 27 ± 3.5%; 5 mM, 45 ± 1.7%; 10 mM, 87 ± 2.3% (Fig. 3E). sAPPα decreased with higher doses of VPA in SW480 (means ± S.E. in % reduction of sAPPα levels versus untreated control) as follows: 1 mM, 0 ± 2.7%; 2.5 mM, 40 ± 7.1%; 5 mM, 67 ± 2.1%; 10 mM, 72 ± 4.8% (Fig. 3E).

To evaluate the effect of VPA on mature and immature APP, levels were quantified and indicated as a ratio of mature to immature APP. The quantification of the ratio immature APP to β-actin showed no significant changes (Fig. 4). These results indicate that VPA impairs maturation of APP and consecutively leads to reduced secreted sAPPα. In SW480 cells, mature APP decreased in a concentration-dependent fashion (means ± S.E. in % reduction of mature APP levels versus untreated control) as follows: 1 mM, 30.7 ± 10.7%; 2.5 mM, 52.1 ± 8.9%, 5 mM 73 ± 5.9%. Similar results were obtained with BxPC3 cell lines (data not shown).

**HDAC Inhibitory Properties of VPA Are Specific for APP Reduction and Responsible for the Suppression of Tumor Growth**—Consistent with previous studies, VPA inhibits HDAC isoenzymes in several cell types (19, 22, 23) causing hyperacetylation of N-terminal tails (lysine residues) of histone H4. VPA treatment of SW480 resulted in hyperacetylated histone H4 in a dose-dependent manner using an antibody specific to acetylated histone H4 (H4Ac) (Fig. 5B). The same result was obtained using BxPC3 cell lines (data not shown). To determine whether the APP reducing effect of VPA is specific, epidermal growth factor receptor (EGFR) and the highly homologous family member protein,
amyloid β (A4) precursor-like protein 2 (APLP2) (34), were examined by Western blot. There was no effect of even 5 mM VPA treatment on EGFR or APLP2 protein levels. The same was found in BxPC3 cells (data not shown). GRP78 protein levels, an APP-binding protein, were elevated after VPA treatment (Fig. 5B) suggesting that the VPA-induced impairment of APP maturation is due to up-regulation of GRP78.

VPM (19, 35), in which the carboxyl group is modified to an amide, lacking HDAC inhibitory activity. VPM had no effects on GRP78, H4Ac, EGFR, or APLP2 protein levels. Consistent with our hypotheses, APP protein levels were not affected. VPM is an even more potent anti-convulsive drug than VPA having no anti-neoplastic potency (Fig. 6) (36, 37).

Treatment of SW480 cells with TSA, a potent but to VPA structurally unrelated pan-HDAC inhibitor (38, 39), resulted in a similarly significant reduction of APP and increased hyperacetylation of histone H4. APLP2 and EGFR levels were again unchanged. GRP78 levels were also elevated after TSA treatment (Fig. 7).

As expected, VPA treatment triggered GRP78 mRNA levels and had no effect on APP mRNA levels indicating that the HDAC inhibitory effect was mediated via GRP78 transcriptional regulation (supplemental Fig. S3). Taken together, these findings suggest that the reduction of APP levels by inhibition of class I HDAC enzymes (HDAC1–3 and 8) is highly specific and mediated by GRP78 elevation.

N-terminal Domain of APP/sAPPα Is Essential for Growth-promoting Effects—To further characterize the growth promoting activity of APP, we stably transfected SH-SY5Y neuroblastoma cells with APP695, SPA4CT (N-terminal domain deleted), or the empty vector pCEP4 construct alone (mock) (Fig. 8A). APP695-transfected cells presented a 43 ± 4.8% higher proliferation rate compared
with mock-transfected control cells (Fig. 8B). We hypothesized that the growth regulating activity is localized to the N-terminal part of APP/sAPPα. To test this hypothesis, we used the shorter construct lacking the N-terminal domain (SPA4CT). The SPA4CT construct contains the signal peptide of APP followed by leucine and glutamic acid and the C-terminal fragment (C99) of APP (40). In contrast, the SPA4CT construct that lacks the ectodomain of APP had no effect on cellular growth compared with mock-transfected neuroblastoma cells. This observation reveals that the N-terminal domain is essential for the growth-promoting effect of APP (Fig. 8C).

We next sought to determine whether addition of conditioned media derived from APP-overexpressing cells would increase cellular proliferation. Importantly, conditioned media from APP-overexpressing cells significantly increased proliferation of both mock-transfected control and the SPA4CT-transfected cell. Mock-transfected cells presented a 7.5% increase in cell growth, whereas SPA4CT-transfected cells showed a 40 ± 7.5% increase in cell growth (Fig. 8D).

In addition, a 24-h treatment of SW480 cells with conditioned media from APP-overexpressing SY5Y cells increased proliferation by 55%. The treatment with 5 mM VPA was completely rescued by co-treatment with conditioned media (Fig. 9).

**DISCUSSION**

Growth factors and their receptors make significant and well-documented contributions to the process of tumorigenesis and represent an important hallmark of cancer. Their contribution to tumorigenesis is in part due to their ability to control cell cycle entry, to confer resistance to apoptotic stimuli, and to increase cellular invasion and metastasis (41).

This study shows that the class I HDAC inhibitor VPA is an effective and highly specific repressor of APP maturation and secretion. This pharmacological strategy might therefore have therapeu-
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VPA effect on mature and immature APP levels. A, SW480 cells were treated with increasing concentrations of VPA for 24 h. Cell lysates were analyzed by Western blot for full-length APP. B and C, signals corresponding to the different bands were quantified by densitometric analysis, and the ratios of the mature (130 kDa) to the immature (110 kDa) APP levels were calculated. Results are from an average of at least three experiments. Data were expressed as mean ± S.E. Differences were calculated using one-way ANOVA followed by Bonferroni post hoc analyses (**, p < 0.01; ***, p < 0.001).

FIGURE 4. VPA effect on mature and immature APP levels. A, SW480 cells were treated with increasing concentrations of VPA for 24 h. Cell lysates were analyzed by Western blot for full-length APP. B and C, signals corresponding to the different bands were quantified by densitometric analysis, and the ratios of the mature (130 kDa) to the immature (110 kDa) APP levels were calculated. Results are from an average of at least three experiments. Data were expressed as mean ± S.E. Differences were calculated using one-way ANOVA followed by Bonferroni post hoc analyses (**, p < 0.01; ***, p < 0.001).

HDAC inhibitor VPA specifically up-regulates GRP78 and has no effect on APLP2 and EGFR levels. A, molecular structure of VPA. B, SW480 cells were treated with increasing concentrations of VPA for 24 h. Cell lysates were analyzed by Western blot with antibodies against EGFR, GRP78, and APLP2, H4Ac, and β-actin. Like H4Ac, GRP78 protein levels clearly showed dose-response characteristics. C, quantification of EGFR levels; D, quantification of GRP78; E, quantification of APLP2. Results were from an average of at least three experiments. Data were expressed as mean ± S.E. Differences were calculated using one-way ANOVA followed by Bonferroni post hoc analyses (*, p < 0.05; ***, p < 0.001).

FIGURE 5. HDAC inhibitor VPA specifically up-regulates GRP78 and has no effect on APLP2 and EGFR levels. A, molecular structure of VPA. B, SW480 cells were treated with increasing concentrations of VPA for 24 h. Cell lysates were analyzed by Western blot with antibodies against EGFR, GRP78, and APLP2, H4Ac, and β-actin. Like H4Ac, GRP78 protein levels clearly showed dose-response characteristics. C, quantification of EGFR levels; D, quantification of GRP78; E, quantification of APLP2. Results were from an average of at least three experiments. Data were expressed as mean ± S.E. Differences were calculated using one-way ANOVA followed by Bonferroni post hoc analyses (**, p < 0.01; ***, p < 0.001).
sue, mainly using immunohistochemistry. Interestingly, in prostate and oral squamous cell carcinoma, the rate of cancer-specific survival for patients with APP-positive tumors was significantly lower than those with APP-negative tumors (16, 17). This study is in good agreement with the previous reports as we have demonstrated that APP is highly expressed in only pancreatic and colon carcinoma but not in healthy control tissue.

Moreover, APP is expressed very early during embryogenesis in newborn vessels and could have a role in angiogenesis (55). Interestingly, a recent study using short hairpin RNA silencing in utero could clearly demonstrate that the regulation and amount of full-length APP is essential for the proper migration of neurons in the developing cerebral cortex suggesting that dysregulation might have an impact on cortical development (56). In good agreement with previous studies, we could demonstrate that overexpression of APP induces increased cellular proliferation compared with mock-transfected controls. To determine the growth promoting activity of APP, we used the shorter SPA4CT construct, lacking the N-terminal domain of APP. SPA4CT-transfected cells (40) did not promote increased proliferation rate as compared with mock-transfected controls. Furthermore, we showed that addition of conditioned medium from APP-overproducing cells could stimulate proliferation of SPA4CT- and mock-transfected cells. These results clearly indicate that the secreted N-terminal domain of APP plays an essential regulatory role in promoting cellular growth. Several lines of evidence showed that sAPP\(\alpha\) fulfills criteria of a trophic growth factor (42, 45, 57–59) and that the growth regulating activity of APP is specified to the N-terminal domain of sAPP containing the 5-amino acid sequence RERMS (APP-(328–332)). Peptides containing this pentapeptide sequence could increase cellular proliferation in fibroblasts and induce

### Figure 6

Valpromide has no effect on APP and GRP78 levels. A, molecular structure of VPM. B, SW480 cells were treated for 24 h with 5 mM VPA or equimolar concentrations of VPM. VPM is a carboxamide derivative of VPA lacking HDAC inhibitory activity. Although VPM had no effect, VPA significantly inhibited cell growth. C, SW480 cells were treated with increasing concentrations of VPM for 24 h. Cell lysates were analyzed by Western blot with antibodies against APP, EGFR, GRP78, APLP2, and H4Ac (prolonged exposure), and β-actin, respectively. Representative Western blots show no changes on any protein levels. Results were from an average of at least six experiments. Data were expressed as means ± S.E. Differences were calculated using one-way ANOVA followed by Bonferroni post hoc analyses (***, \(p < 0.001\)).

### Figure 7

Structurally unrelated HDAC inhibitor trichostatin A has the same effects as VPA. A, molecular structure of TSA. B, SW480 cells were treated for 24 h with 250 nM TSA having a potent inhibitory effect on cell growth. C, cell lysates were analyzed by Western blot with antibodies against APP, EGFR, GRP78, APLP2, H4Ac, and β-actin. TSA specifically down-regulates APP and up-regulates GRP78 protein levels. No effect was seen on EGFR and APLP2 levels. Results were from an average of at least six (B) and three (D and E) experiments. Data were expressed as means ± S.E. Differences were calculated using unpaired \(t\) test (**, \(p < 0.01\); ***, \(p < 0.001\)).
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**A**

![Schematic Illustration of APP695 and SPA4CT](image)

**B**

Mock: APP 695; SPA4CT

**C**

Cell growth [%]

**D**

Mock: SPA4CT

FIGURE 8. N-terminal domain is essential for the growth-promoting properties of APP. A, schematic illustration of APP695 and the N-terminal truncated SPA4CT construct used in the study. The Ab domain is shown in black. SH-SY5Y cells were transfected with plasmids coding for APP695, SPA4CT, or with the empty vector pCEP4 alone. For all constructs the signal peptide sequence (SP) of APP was introduced in-frame with the remaining domains. The SPA4CT lacking the entire N-terminal ectodomain precedes the Ab domain. AICD, APP intracellular domain. B, SH-SY5Y cells were stably transfected with APP695, SPA4CT, and empty vector pCEP4 construct alone (mock). C, cell lysates were analyzed by Western blotting for full-length APP and the different APP cleavage products C99 and Ab. C, proliferation analysis showing a significant increased cell growth rate only in APP696-transfected cells. SPA4CT did not stimulate cell growth. **D**, to study the influence of exogenous sAPP, media were replaced with conditioned medium (cond. Medium) from SH-SY5Y APP695-overexpressing cells (hatched bars) or treated with serum-free media (solid bars). Cellular proliferation was significantly elevated in mock-transfected as well as in SPA4CT-transfected cell lines. Results were from an average of at least six experiments. Data were expressed as means ± S.E. Differences were calculated using one-way ANOVA followed by Bonferroni post hoc analyses (C) and unpaired t test (D) (**, p < 0.01; ***, p < 0.001).

To evaluate whether the proliferative effect of APP/sAPPα is of pathophysiological relevance, we used siRNA to selectively knock down APP gene expression. siRNA treatment led to decreased APP and sAPPα protein levels and significantly inhibited cellular growth in both cancer cell lines. This observation is in good agreement with earlier work in N2a cells (63).

An important finding of our study is that APP levels and sAPPα secretion are down-regulated upon HDAC inhibition. Other type I transmembrane proteins like EGFR and the APP family member APLP2 were not affected, demonstrating the specificity of HDAC activity on APP metabolism. VPA, a well tolerated drug with an extensively characterized toxicity profile, emerged as one of the leading candidates for epilepsy and tolerance drug with an extensively characterized toxicity profile, emerged as one of the leading candidates for epilepsy and neuroprotection. It is a member of the HER family of receptor tyrosine kinases and is overexpressed in a broad range of tumors, including pancreatic and colon cancer. Like APP, this cell surface receptor plays important roles in growth control, and increased expression has been associated with increased cellular proliferation (71, 72). Our results demonstrate that EGFR levels were not altered by treatment with HDAC inhibitors.

Moreover, VPA treatment also did not affect amylloid β (A4) precursor-like protein 2 (APLP2) protein levels. With 71% sim-
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In conclusion, our results support previous observations that APP is a crucial mediator of tumor growth in general. The anti-epileptic and well tolerated HDAC inhibitor VPA can be used to modulate endogenous growth-promoting APP levels in gas-
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trointestinal carcinomas and might therefore provide a molecular tool for therapeutic intervention.

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