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From differentiation to proliferation: The secretory amyloid precursor protein as a local mediator of growth in thyroid epithelial cells

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ABSTRACT **In various species, thyrotropin (TSH) is known to stimulate both differentiation and proliferation of thyroid follicle cells. This cell type has also been shown to express members of the Alzheimer amyloid precursor (APP) protein family and to release the secretory N-terminal domain of APP (sAPP) in a TSH-dependent fashion. In this study on binding to the cell surfaces, exogenously added recombinant sAPP stimulated phosphorylation mediated by mitogen-activated protein kinase and effectively evoked proliferation in the rat thyroid epithelial cell line FRTL-5. To see whether this proliverative effect of sAPP is of physiological relevance, we used antisense techniques to selectively inhibit the expression of APP and the proteolytic release of sAPP by cells grown in the presence of TSH. The antisense-induced inhibition was detected by immunoblot, immunoprecipitation, and immunocytochemical analyses. After the reduced APP expression and sAPP secretion, we observed a strong suppression of the TSH-induced cell proliferation down to 35%. Recombinant sAPP but not TSH was able to overcome this antisense effect and to completely restore cell proliferation, indicating that sAPP acts downstream of TSH, in that it is released from thyroid epithelial cells during TSH-induced differentiation. We propose that sAPP operates as an autocrine growth factor mediating the proliferative effect of TSH on neighboring thyroid epithelial cells.**

In recent years, observations on a number of proteins involved in the generation of specific diseases have raised wide interest. Despite the detailed knowledge on synthesis, cellular transport, and molecular organization of such pathogenetically important proteins, their normal physiological roles have remained largely unknown. One of these proteins is the Alzheimer amyloid precursor protein (APP), a single-membrane-spanning protein that is expressed in many tissues and highly conserved throughout the evolution of multicellular organisms (1). APP serves as the macromolecular precursor for the amyloid Aβ peptide that is generated by specific proteolytic processing of APP and found in senile plaques and neurofibrillar tangles in the cerebral cortex of patients afflicted with Alzheimer's disease (2, 3). Because of alternative splicing, APP exhibits a remarkable heterogeneity by the generation of at least eight different forms of primary transcription products (4). All known members of the APP family can undergo several types of proteolytic processing, one of which results in the release of the secretory N-terminal portion of APP (sAPP) that carries a number of biologically relevant domains (3, 5, 6).

Several hypotheses have already been put forward to explain the normal physiological role of the APP family. The proposed functions are in part deduced from the domain structure of sAPP, which varies among the different APP forms and which may include a Kunitz protease inhibitor domain and carry binding sites for collagen, laminin, and glycosaminoglycans (7, 8). sAPP has also been shown to stimulate cell division in APP-deficient fibroblasts (9), to enhance neurite outgrowth in neuroblastoma cells (10, 11), or to exert a trophic function on cerebral cortical neurons (12).

We recently observed that members of the APP family are highly expressed in follicle epithelial cells of the thyroid (13, 14) and that their expression and the release of sAPP are regulated by thyrotropin (TSH). sAPP accumulates extracellularly in the thyroid follicle lumen and, after stimulation with TSH, on structural constituents of the extracellular matrix. TSH, which acts by way of the cAMP system, stimulates several differentiated functions of thyroid epithelial cells such as the expression, secretion, and endocytosis of thyroglobulin, iodide uptake and transport (15), and the release of thyroid hormones (16). In addition, in a variety of species, TSH is also known to stimulate the growth of thyroid epithelial cells. This combined role of TSH on differentiation and proliferation is as yet unexplained. However, two contradictory views currently exist that explain this dual role either by a direct effect of TSH in addition to its differentiating activities, suggesting that cAMP may directly operate as a positive signal for both types of cellular responses (16) or operate by the differentiation-dependent release of growth-promoting factors (17). These factors may include insulin-like growth factors I and II (18), basic fibroblast growth factor (19), epidermal growth factor (20), transforming growth factor β (21), and the hepatocyte growth factor/scatter factor (22). As recently observed (23), sAPP in conditioned medium appears to promote epithelial cell proliferation. The function of sAPP as a growth-promoting peptide in thyroid epithelial cells raised the question whether sAPP released by the epithelial cells under the regulatory influence of TSH might be considered as a physiologically relevant growth factor.

In this report, we have addressed this question by specifically inhibiting APP expression through the application of antisense oligonucleotides directed against APP mRNA. We have used FRTL-5 cells, a diploid nontransformed line of rat thyroid

Abbreviations: TSH, thyroid stimulating hormone; APP, amyloid precursor protein; sAPP, secretory N-terminal portion of APP; MAP kinase, mitogen-activated protein kinase; BrdUrd, 5-bromo-2′-deoxyuridine.

Data deposition: The sequence reported in this paper has been deposited in the GenBank database (accession no. X07648).

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epithelial cells (24) whose growth in culture depends on the presence of TSH (18). The results show that antisense oligonucleotides effectively inhibit the expression of APP and the release of sAPP and that this inhibition results in strongly reduced proliferation rates of thyroid epithelial cells. We propose that sAPP acts as a TSH-regulated autocrine growth factor in thyrocytes. A portion of this report has been presented (25).§

MATERIALS AND METHODS

Materials and Cells. FRTL-5 cells were obtained from American Type Culture Collection. Polyclonal antiserum against the 19 amino acids at the C terminus of APP (antiserum 2189) was raised as described (23). The mouse monoclonal antibody 22C11 against the denatured N terminus of APP (26) was provided by K. Beyreuther and G. Multhaup (Center of Molecular Biology, University of Heidelberg, Germany). Recombinant *Escherichia coli* DH5 carrying the pPRO EX-1 expression vector (Life Technologies) and containing sAPP derived from human APP695 (27) was used to prepare recombinant sAPP.

Primers used for PCRs and antisense oligonucleotides (see below) were synthesized by MWG-BIOTECH (Ebersberg, Germany). 5-Bromo-2′-deoxyuridine (BrdUrd), and the monoclonal anti-BrdUrd antibody were purchased from Amersham Buchler (Braunschweig, Germany). Collagen was isolated from rat tails, dried, sterilized by UV-radiation, and solubilized in 0.1% acetic acid as described (28).

Cell Culture. FRTL-5 cells were cultured at 37°C and 5% CO_2/95% air in F-12 medium (Coon's modification, Seromed, Berlin, Germany) supplemented with 5% calf serum, TSH (1 milliunit/ml), insulin (10 μg/ml), 10 μM hydrocortisone, Gly-His-Lys (10 ng/ml), somatostatin (10 ng/ml), transferrin (5 μg/ml), penicillin (50 units/ml), and streptomycin (50 units/ml) (24). Antisense and random oligonucleotides or sAPP were applied at concentrations as indicated.

Purification of His6-sAPP. All steps were carried out at 4°C, unless indicated otherwise. Pellets from bacteria expressing N-terminal His-tagged sAPP were thawed on ice and resuspended in sonication buffer (50 mM Tris·HCl/10 mM 2-mercaptoethanol/1 mM phenylmethylsulfonyl fluoride, pH 8.5) at 4 vol per g (wet weight). Lysozyme (1 mg/ml) was added to this suspension and bacteria were broken by sonification (Branson sonifier B92, Branson). To digest DNA and RNA, DNase I (5 μg/ml) and RNase A (10 μg/ml) were added and the samples were incubated for 15 min. The lysate was centrifuged at 15,000 $\times$ *g* for 20 min. The supernatant of cell extracts was mixed with Ni-nitrilotriacetic acid resin (Qiagen), which was collected by centrifugation, washed with buffer A [20 mM Tris·HCl/100 mM KCl/20 mM imidazole/10 mM 2-mercaptoethanol/10% (vol/vol) glycerol, pH 8.5], and loaded into a column. After washing sequentially with 10 vol of buffer A, 2 vol of buffer B (20 mM Tris·HCl/1 M KCl/10 mM 2-mercaptoethanol/10% glycerol, pH 8.5), and 2 vol of buffer A, sAPP was eluted with 10 vol of buffer C (20 mM Tris·HCl/100 mM KCl/100 mM imidazole/10 mM 2-mercaptoethanol/10% glycerol, pH 8.5). Fractions of 0.5 ml were collected and analyzed by SDS/PAGE and immunoblotting. Fractions containing sAPP were combined and dialyzed overnight against PBS.

Cell Proliferation Assays. The fraction of cells entering the DNA synthesis phase of the cell cycle was used as the criterion for proliferation and assessed by the application of the BrdUrd incorporation assay as described (23).

SDS/PAGE and Immunoblotting. FRTL-5 cells were cultured for 48 hr in serum-free F-12 medium containing antisense or random oligonucleotides. Medium was collected at 4°C and concentrated with a SpeedVac evaporation device (RT 100, Savant). Ten-fold concentrated medium was boiled for 5 min in sample buffer, and proteins were separated on a 12.5% reducing SDS gel (29) and transferred to nitrocellulose. The blots were immersed overnight in blocking solution (6% casein/1% polyvinylpyrrolidone 40/10 mM EDTA in PBS, pH 6.8) and incubated with the mouse anti-APP monoclonal antibody (22C11) and peroxidase-labeled secondary antibody (Dianova, Hamburg, Germany). APP out of cell lysates (15 μg per lane) or recombinant sAPP preparations (5 μg per lane) were analyzed by the use of the mouse anti-APP monoclonal antibody 22C11 and the rabbit anti-sAPP antibody 3329 selectively recognizing the recombinant form of sAPP. Antibody 3329 was raised against the recombinant sAPP in New Zealand White rabbits. Antisera were collected and the specificity and affinity were tested by immunoblot, immunoprecipitation, and immunocytochemical procedures. The APP-specific bands were visualized by chemiluminescence (ECL; Amersham) and documented on XAR-5 films (Kodak).

Assay for Mitogen-Activated Protein (MAP) Kinase Activity. FRTL-5 cells cultured in serum-free medium without TSH were treated with serum-free medium without TSH but supplemented with or without 10 nM sAPP for 10–30 min. MAP kinase activity was determined by using the MAP kinase assay kit (New England BioLabs). In brief, the cells were washed twice with ice-cold PBS and scraped into 1 ml of lysis buffer supplemented with 1 mM phenylmethylsulfonyl fluoride. Lysates were centrifuged to remove cellular debris, normalized to 200 μg of protein, and precipitated for 12 hr at 4°C with 2 μl of phospho-MAP kinase antibody. Immunoprecipitates were washed twice, each with 500 μl of lysis buffer and 500 μl of kinase buffer. The pellet was suspended in 50 μl of kinase buffer supplemented with 100 μM ATP or 5 μCi of [γ-^{32}P]ATP (Amersham; 3,000 Ci/mmol; 1 Ci = 37 GBq) and 1 μg of Elk-1 fusion protein and assayed for kinase activity for 30 min at 30°C. Reactions were terminated by boiling in SDS sample buffer, and products were resolved on 12% polyacrylamide gels and quantitated by PhosphorImager (Fuji BAS-Reader 1000) analysis or visualized by immunoblotting using a phospho-Elk-1-specific antibody and peroxidase-labeled secondary antibody (Dianova).

Oligonucleotides. Expression of APP in FRTL-5 cells was inhibited by the use of antisense oligonucleotides directed against the initiator codon AUG. The antisense oligonucleotide sequence, positions −10 to +10, was 5′-TGGGCAG-CATCGTGATCCTG-3′ and the random sequence was 5′-GTCATGCTGCCTGATGAGCG-3′, lacking any significant homology to other sequences in the EMBL database. Two phosphorothioate modifications were substituted at the 5′ and 3′ ends of the oligonucleotides.

The administration of the oligonucleotides was performed as follows: At 72 hr after plating, when the cells were transferred to serum-free F-12 medium, the oligonucleotides were added at 25 μM. The medium was supplemented with an additional 25 μM of oligonucleotide every 12 hr for 48 hr. Controls were treated with the same concentration of the random oligonucleotide and the same dosage schedule. The uptake of the oligonucleotides by FRTL-5 cells was controlled by incubating the cells with random or antisense oligonucleotides labeled with fluorescein isothiocyanate and analyzed by using a TCS 4D Leica confocal laser scanning microscope (Leica).

Radiometric and Fluorescence Quantitation of the APP Signal. FRTL-5 cells were grown for 3 days in the presence of TSH, supplemented for 48 hr with each oligonucleotides at 25 μM, washed twice in cysteine- and methionine-free F-12 medium containing TSH, and labeled with [^{35}S]methionine/[^{35}S]cysteine (Translabel, ICN; 3.7 MBq/ml) for 5 hr in the presence of the oligonucleotides. FRTL-5 cells were lysed,

§Pietrzik, C. U., Stoeber, K., Hoffman, J., Bauer, C. & Herzog, V., Meeting of the European Cell Biology Organization, March 22–25, 1997, Brighton, U.K., abstr. A-5127, p. 17.

normalized to 40 μg of protein, immunoprecipitated by the use of the anti-APP antibody 2189, and subjected to polyacrylamide gel electrophoretic analysis. To measure the total biosynthetic activity gels were fixed, dried, and exposed to XAR-5 film (Kodak). Gels were analyzed by using a PhosphorImager.

For quantitation of the APP expression, FRTL-5 cells were fixed and stained with an antiserum (antiserum 2189) against the C terminus of APP (see below). To avoid photobleaching, we examined only cells that had not been examined previously and the measurements were made within the linear range of the TCS 4D Leica confocal laser scanning microscope. The fluorescence signals were measured by using a densitometry software for analysis of confocal sections.

Northern Blot Hybridization. Total RNA was isolated by using of the S.N.A.P. total RNA isolation kit (Invitrogen), according to the manufacturer's protocol. Yields and purity of RNA preparations were determined by spectrophotometry and agarose gel electrophoresis (30).

RNA was separated in denaturing 1% agarose formaldehyde gels and transferred to nitrocellulose (Schleicher & Schüll, Dassel, Germany) by Northern blotting. The nitrocellulose was hybridized with ^{32}P-labeled antisense or random oligonucleotides as described (31). As a control, a cDNA fragment of APP_{770} (base pairs 58–484) was used for hybridization. The oligonucleotides and the cDNA fragment were end-labeled by using [γ-^{32}P]ATP (Amersham) and T4 polynucleotide kinase (Boehringer Mannheim). Prehybridization (1 hr) and hybridization were performed in hybridization solution (Sigma) overnight at 65°C, blot was washed three times in 0.2% NaCl/sodium citrate/0.1% SDS at 68°C and exposed to Hyperfilm MP (Amersham).

Light Microscopic Detection of Endogenous APP in FRTL-5 Cells. To localize APP, FRTL-5 cells were seeded on coverslips coated with collagen and grown to subconfluency. Cells were fixed in paraformaldehyde, permeabilized with 0.2% Triton X-100, blocked for 30 min with 3% BSA in PBS, and labeled for 90 min at 37°C with a rabbit serum against the C-terminal region of APP diluted in PBS with 0.3% BSA (PBSA). For immunofluorescence detection, the cells were incubated with 5-[(4,6-dichlorotriazin-2-yl)amino]-fluorescein-labeled goat anti-rabbit IgG (Dianova) diluted 1:50 in PBSA for 60 min at 37°C and viewed with a TCS 4D Leica laser scanning confocal microscope.

Binding of Exogenously Added sAPP to the Surface of FRTL-5 Cells. To analyze the binding properties of sAPP to FRTL-5 cells, monolayers were incubated with recombinant His6-sAPP. For the specific detection of bound recombinant sAPP, a rabbit anti-sAPP antibody (antiserum 3329) exclusively recognizing recombinant sAPP, but not endogenous APP or sAPP, was used. FRTL-5 cells were blocked with 3% BSA in PBS for 60 min at 4°C and afterwards incubated with 1, 10, and 100 nM recombinant sAPP for 20 min. Detection was by immunofluorescence as described above with the exception that the antibody 3329 was applied at 4°C.

Transmission Electron Microscopy. For transmission electron microscopy, cells were seeded on plastic coverslips and incubated with sAPP and primary antibody as described above. As secondary antibody a gold-labeled goat anti-rabbit IgG antibody (Dianova) was used, and the gold label was enhanced by using a silver enhancement kit (IntenSE silver enhancement kit, Amersham). The cells were postfixed for 10 min with 1% osmium tetroxide, block-stained with 2% aqueous uranyl acetate, embedded in Epon 820 (Fluka), and mounted onto the plastic coverslips. Thin sections were stained with lead citrate (10 min) and examined by using a Philips CM120 electron microscope (Philips Electron Optics, Eindhoven, The Netherlands).

Analysis of Quantitative Data. All proliferation assays were repeated at least three times by counting a minimum of 1,000 cells in each experiment. Results were expressed as the mean ± SD. Data were analyzed for statistical differences by Student's unpaired *t* test. $P > 0.05$ was considered statistically significant.

RESULTS

Localization of APP in FRTL-5 Cells and the Effect of Recombinant sAPP on Cell Proliferation. Formaldehyde-fixed monolayers of FRTL-5 cells were permeabilized and prepared for immunocytochemical detection of APP with an antibody against the C-terminal portion of APP. Cells in Fig. 1*A* exhibited a strong perinuclear crescent-shaped staining pattern that has been shown to colocalize to the staining for mannosidase II (14) and to represent, therefore, specific staining of the Golgi complex.

Recombinant sAPP was purified by affinity chromatography making use of its His tag. The purification resulted in a single protein band in gel electrophoretic analysis (Fig. 2). An antibody (antiserum 3329) was prepared which specifically

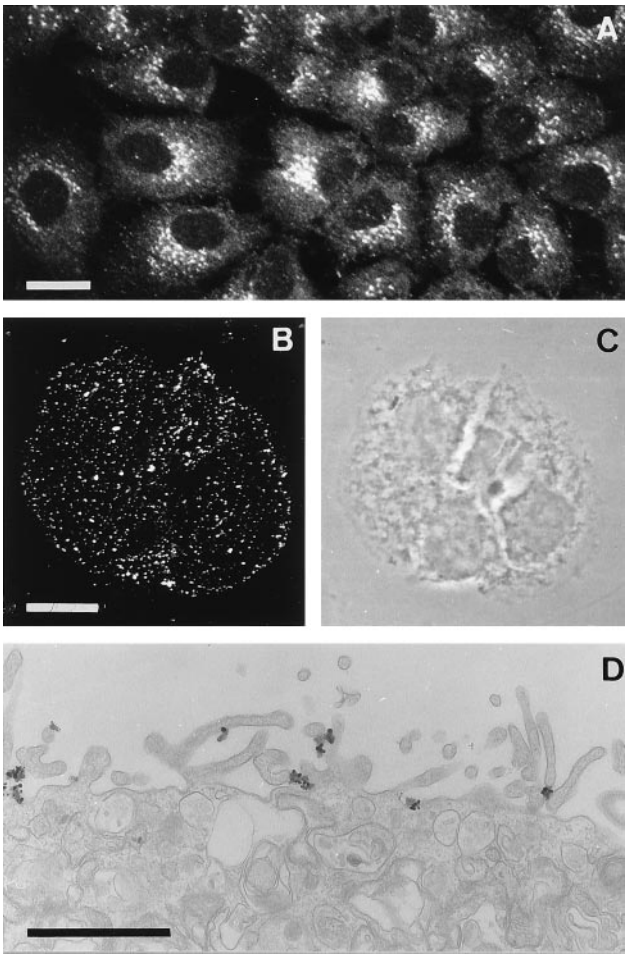

FIG. 1. Immunocytochemical localization of endogenous APP in FRTL-5 cells (*A*) and of recombinant sAPP (*B* and *D*). Visualization of endogenous APP with an antibody directed against the cytoplasmic C-terminal domain (antiserum 2189) resulted in a characteristic crescent perinuclear staining indicative of a Golgi localization of APP (*A*). For visualization of recombinant sAPP, cells were incubated with 100 nM recombinant His-tagged sAPP for 20 min at 4°C, i.e., under conditions that halted membrane flow. The recombinant sAPP was immunolabeled with specific antibodies recognizing only recombinant sAPP, but not endogenous APP (antibody 3329; see Fig. 2), and detected with either fluorescently labeled (*B*) or gold-conjugated antibodies followed by silver enhancement (*D*). The phase-contrast image corresponding to *B* is shown in *C*. [Bars = 10 μm (*A* and *B*) and 2 μm (*D*).]

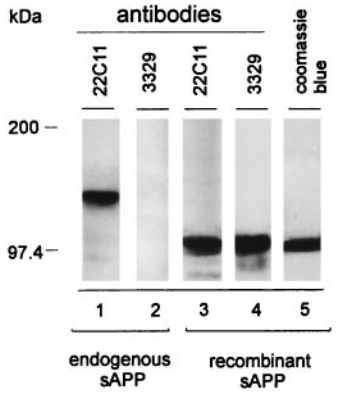

FIG. 2. Antibody 3329 selectively recognizes recombinant sAPP. In immunoblots monoclonal antibody 22C11 reacted with both the secreted sAPP of FRTL-5 cells (lane 1) and the purified recombinant sAPP expressed in *E. coli* (lane 3). Coomassie blue staining of the purified recombinant sAPP is shown in lane 5. The polyclonal rabbit antibody 3329 reacted exclusively with the recombinant sAPP (lane 4) and showed no reaction with endogenous sAPP (lane 2). This antibody was therefore used for binding studies with recombinant sAPP on FRTL-5 cells.

recognized the recombinant form of sAPP and discriminated, therefore, between recombinant and endogenous sAPP (Fig. 2). This purified recombinant sAPP bound avidly to the cell surfaces of FRTL-5 cells as detected immunocytochemically by fluorescence microscopy or by transmission electron microscopy (Fig. 1 *B* and *D*) with the antibody 3329, which did not show any cross-reactivity for endogenous APP (compare with Fig. 1*A*). The molecular basis of this antibody specificity is unknown but current studies showed that it might be related to the glycosylation of eukaryotic sAPP (data not shown). The binding of recombinant sAPP was saturable and specific (data not shown) and resulted in increased MAP kinase-mediated phosphorylation of ELK-1, as detected by immunoblot or radiometric analysis (Fig. 3). This observation was taken as a strong indication of existence of an sAPP-induced signal transduction pathway that, however, was not further characterized in this study. As shown in Fig. 4, these events were followed by increased rates of proliferation as detected by the application of the BrdUrd assay. Note that maximum proliferation appeared to be reached at 10 nM sAPP. Remarkably,

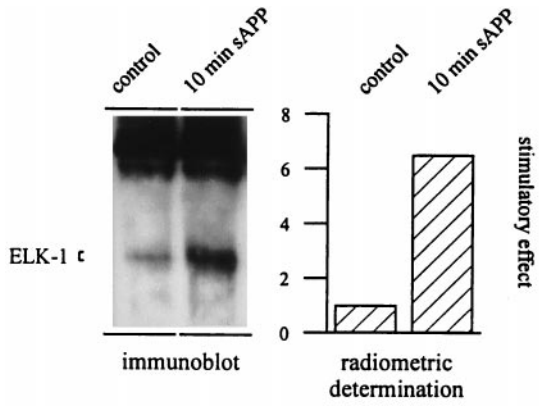

FIG. 3. Effect of 10 nM recombinant sAPP on the stimulation of MAP kinase activity in the absence of TSH. Detection of ELK-1 fusion protein by immunoblot analysis using a phospho-ELK-1-specific antibody showed a significant increase in Elk-1 phosphorylation after the addition of 10 nM sAPP for 10 min as compared with untreated control cells. The stimulatory effect of 10 nM sAPP was determined radiometrically by the use of [γ-^{32}P]ATP and radiolabeled ELK-1 fusion protein. The control value was set as 1.

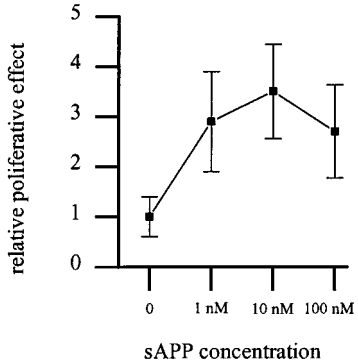

FIG. 4. Effect of recombinant sAPP on BrdUrd incorporation in TSH-stimulated FRTL-5 cells. To investigate the contribution of sAPP to the proliferative effect, FRTL-5 cells were incubated with the indicated concentrations of sAPP. Note that addition of 10 nM sAPP resulted in a 3.5-fold proliferative increase compared with controls. Higher concentrations (100 nM) of sAPP resulted in a decreased stimulatory effect.

higher concentrations resulted in an as yet unexplained decrease in the proliferation rate.

Inhibition of APP Expression and sAPP Release in FRTL-5 Cells. By Northern blot analysis, the antisense oligonucleotide used for the inhibition of APP expression in this study was shown to strongly hybridize with the 3.4-kb RNA (Fig. 5*A*) characteristic in size of the APP transcript (32). An uptake of fluorescent antisense oligonucleotides by FRTL-5 cells could be detected after 12 hr at concentrations of 1 μM (data not shown). Incubation of the cells in the presence of 25 μM antisense oligonucleotides resulted in a strongly reduced expression of APP. This was shown by the quantitation of the emission signal derived from immunofluorescence preparations by using confocal laser scanning microscopy (Fig. 5*B*), from immunoprecipitation of cell lysates after biosynthetic labeling with [^{35}S]methionine and [^{35}S]cysteine (Fig. 5*C*) or from immunoblots of the supernatant of FRTL-5 cell cultures (Fig. 5*C*). The observations show that the antisense-inhibited expression of APP resulted in the reduced release of sAPP into the culture medium. It should be pointed out that total protein synthesis remained unaltered during treatment of cells with random or antisense oligonucleotides (data not shown). The reduced expression of APP and the concomitant reduction in the release of sAPP were accompanied by a decrease in cell proliferation by 65% as compared with untreated cells or cells incubated in the presence of random oligonucleotides (Fig. 6). This proliferative block caused by antisense oligonucleotides was overcome by the addition of 10 nM recombinant sAPP, which resulted in even higher values of the mitotic index as compared with controls (Fig. 6). In contrast, TSH added up to concentrations as high as 50 milliunits/ml was unable to compensate for the antisense-induced inhibition of growth (Fig. 6).

DISCUSSION

In this study, the expression of APP and the release of sAPP in thyrocytes were inhibited by antisense techniques resulting in an efficient block of cell proliferation. Our results provide evidence for the view that endogenously released sAPP may operate as a factor that is able to evoke coordinated responses in specific target cells (11, 33, 34). In thyrocytes, sAPP is a key factor in the TSH-induced cascade leading from epithelial differentiation to proliferation.

Proliferative Effect of sAPP. A recombinant full-length sAPP showed a high density of cell surface labeling as visualized by the use of an antibody specific for the detection of the recombinant form of sAPP. This antibody proved to be useful

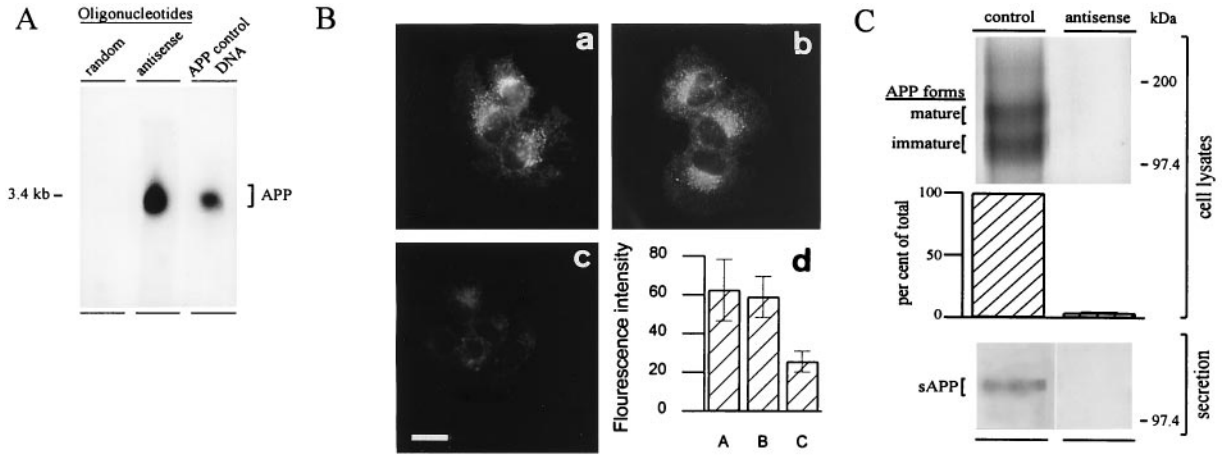

FIG. 5. (*A*) Binding and effect of antisense oligonucleotides on the expression of APP and the release of sAPP. Northern blot analysis of total RNA preparations from FRTL-5 cells using [γ-^{32}P]ATP-radiolabeled oligonucleotides. The antisense oligonucleotides bound to the RNA (lane antisense), whereas the random oligonucleotides did not (lane random). A PCR fragment from APP (lane APP control DNA) was used as a standard, indicating the size of APP mRNA (3.4 kb) in the total RNA preparation. (*B*) Immunofluorescence localization of endogenous APP using an antibody directed against the C terminus of APP (*a*). Effect of 25 μM random oligonucleotide-treated cells (*b*) and 25 μM antisense oligonucleotide-treated cells (*c*) on the expression of APP. (Bar = 10 μm.) The total fluorescence intensity of cells was measured by the use of a laser scanning microscope (*d*). Total protein synthesis in FRTL-5 cells remained unaltered during treatment with oligonucleotides (data not shown), whereas the expression of APP was selectively reduced after antisense treatment (*c*). (*C*) Effect of antisense oligonucleotides on the synthesis of APP and the secretory release of sAPP. Cells grown in the presence or absence of oligonucleotides for 48 hr were biosynthetically radiolabeled with [^{35}S]methionine/[^{35}S]cysteine (3.7 MBq/ml) for 3 hr. APP was immunoprecipitated from cell lysates with an antibody directed against the C terminus of APP, analyzed by SDS/PAGE, and quantified radiometrically (*Top*). Immunoblot analysis showed the effect of antisense oligonucleotides on the secretion of sAPP by using an antibody directed against the N terminus of APP (*Bottom*). Note that the expression of APP and the release of sAPP in antisense-treated cells was significantly decreased as compared with control cells.

for selectively recognizing this peptide in ruling out that endogenous APP caused any interference with the recording of bound exogenous sAPP. sAPP binding to cell surfaces at high density evokes a pronounced proliferative effect on thyroid epithelial cells. It should be pointed out that this proliferation effect of recombinant sAPP requires the presence of TSH. In its absence, cells rapidly lose their ability to respond to sAPP with proliferation (data not shown). Apparently, TSH maintains the cells in a differentiated state in which cells remain competent for proliferation, indicating that sAPP is unable to mimick fully the proliferative action of TSH in its absence.

We point out that the previously described proliferative effect of sAPP in fibroblasts has been shown to be detectable only in APP-deficient fibroblasts (9), whereas the proliferative effect on thyroid epithelial cells was observed in cells that were fully competent to express APP and to release sAPP. Despite the presence of endogenous APP, the exogenously added sAPP is able to induce an additional proliferative effect. These findings clearly show that thyroid epithelial cells are particularly susceptible to the proliferative action of sAPP as compared with other cell types, such as fibroblasts.

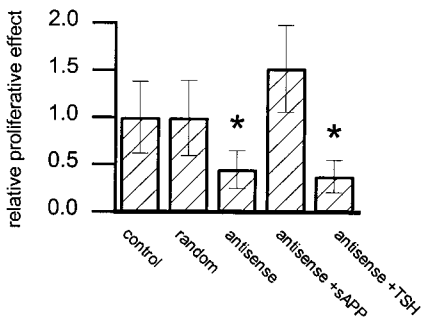

FIG. 6. Effect of antisense and random oligonucleotides on the proliferation of FRTL-5 cells. Note that the proliferative effect was reduced to 35% (*, $P < 0.05$) when cells were cultured in medium containing 25 μM antisense oligonucleotides (bar antisense) compared with the proliferative effect of cells cultured in the presence or absence of 25 μM random oligonucleotides (bars control or random). The inhibitory effect of antisense oligonucleotides was overcome by the addition of 10 nM sAPP (bar antisense + sAPP). Note that increased concentrations of TSH (50 milliunits/ml) to FRTL-5 cells did not compensate for the inhibitory effect of 25 μM antisense oligonucleotides (bar antisense + TSH).

As a practical consequence of these results, a new medium for the culture of thyroid epithelial cells has been developed that contains recombinant sAPP (unpublished results). Whether this culture medium is of general use for other epithelial cell types remains to be determined. It has already been proven particularly useful for the culture of thyroid epithelial cells from species in which the growth of thyrocytes depends on TSH.

sAPP as a Growth Factor Acting Downstream of TSH. We have observed an efficient uptake of antisense oligonucleotides as detected by fluorescence microscopy and recorded their strong binding to RNA preparations from FRTL-5 cells as monitored by Northern blot analysis. In the cells this binding results in the inhibition of endogenous APP expression and sAPP secretion. The mechanism of transit of negatively charged macromolecules such as DNA is still unknown, but the results of this study or those reported in a large variety of experimental applications (35, 36) clearly indicate that a considerable proportion of the applied antisense constructs reach the cytoplasm in a functionally intact state. After application of random oligonucleotides used as nonblocking controls or antisense oligonucleotides, the cells remained viable, showed unaltered rates of total protein synthesis, and exhibited a normal phenotype. All control oligonucleotides showed neither binding to the RNA of FRTL-5 cells nor effects on the expression of sAPP.

In this study, we report that the application of antisense oligonucleotides to block the expression of APP interferes with the release of sAPP in the TSH-induced cascade. Exogenously added sAPP has a pronounced stimulatory effect on cell proliferation, which cannot be achieved when TSH located at the starting point of the proposed cascade is added in excess.

This suggests that sAPP acts at a later stage of the cascade and leads finally to cell proliferation but that TSH appears primarily able to induce cell differentiation. Our study shows a marked reduction of cell proliferation down to 35% after antisense inhibition of APP expression and sAPP secretion. In this context it is of interest that down-regulation of TSH-induced cell growth to approximately the same level has also been reported by inhibition of the thyroid transcription factors TTF-1 and Pax 8 (37).

The binding of sAPP to the cell surface of FRTL-5 cells resulted in an increase of the specific phosphorylation catalyzed by the MAP kinase. A similar effect has been reported in PC-12 cells (33), whereas in other neuronal cells, the formation of inositol triphosphate was increased (11). Prolonged stimulation with sAPP has been shown to stimulate a cGMP-activated kinase (34). Hence, most likely sAPP-dependent events are receptor-mediated and include the activation of second messenger pathways. Our results on thyrocytes involving MAP kinase activation also point to a cAMP-independent signal transduction pathway (see Fig. 3).

In summary, the suppression of sAPP release results in the disruption of a cascade in which the TSH-dependent release of sAPP is followed by increased rates of proliferation. Characteristically, cell proliferation can be restored by the addition of exogenously added sAPP, whereas TSH even at unphysiologically high concentrations has no such effect. This indicates that sAPP exerts its function as an autocrine growth factor downstream to TSH and as a major interface able to shift thyroid epithelial cells from the state of differentiation to cell proliferation.

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