Late Simian virus 40 transcription factor is a target of the phosphoinositide 3-kinase/Akt pathway in anti-apoptotic Alzheimer's amyloid precursor protein signalling

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The association of familial Alzheimer's disease (FAD) with mutations in Alzheimer's amyloid precursor protein (APP) suggests important functions for APP in the central nervous system. Mutations in APP impair its function to confer resistance to apoptosis in cells under stress, and this may contribute to neurodegeneration in Alzheimer's disease (AD) brain, but the mechanisms involved are unknown. We examined the role of the late Simian virus 40 transcription factor (LSF), in anti-apoptotic APP pathways. We show that in APP-deficient B103 cells, expression of wild-type human APP (hAPPwt), but not of FAD-mutant APP, inhibited staurosporine (STS)-induced apoptosis. This inhibition was further enhanced by expression of LSFwt, although LSFwt alone was not sufficient to inhibit STS-induced apoptosis. In contrast, expression of dominant-negative LSF led to a marked increase in STS-induced cell death that was

significantly blocked by hAPPwt. These effects of APP were accompanied by LSF nuclear translocation and dependent gene transcription. The activation of LSF is dependent on the expression of hAPPwt and is inhibited by the expression of dominant-negative forms of either phosphoinositide 3-kinase or Akt. These results demonstrate that LSF activation is required for the neuroprotective effects of APP via phosphoinositide 3-kinase/Akt signalling. Alterations in this pathway by aberrations in APP and/or LSF could promote neuronal loss in AD brain, due to secondary insults. Thus a link is established between APP and LSF and AD.

Key words: amyloid precursor protein, apoptosis, phosphoinositide 3-kinase, phosphorylation, staurosporine.

INTRODUCTION

The conservation of Alzheimer's amyloid precursor proteins (APPs) across species, with high abundance in the brain and the association of APP mutations with autosomal familial Alzheimer's disease (FAD) [1], suggests important roles for APP in the central nervous system. Several studies have shown a complex network of interacting proteins (G_o heterotrimeric protein, APP-binding protein 1, neuron-specific protein XII and Fe65 adapter protein) centred on the last 52 amino acid residues of APP [2,3], which is consistent with the role of APP in signal transduction. These proteins may modulate key steps in apoptosis, leading to neuronal loss in Alzheimer's disease (AD) brain [4,5]. In the presence of apoptosis-inducing stimuli, overexpression of wild-type human APP (hAPPwt), but not FAD-associated mutant APP (hAPPmut), conferred resistance against p53-induced apoptosis in rat neuroblastoma (B103) cells [6], and oxidative stress-induced cell death in pheochromocytoma (PC12) cells [7]. The protective capacity of APP in cells challenged with secondary insults is supported by several observations: the synthesis of both APP and APP-like proteins is up-regulated during trophic factor withdrawal-induced neuronal apoptosis [8], secreted APP counteracts the proapoptotic action of mutant presenilin-1 [9] and protects neurons against excitotoxicity [10]. In contrast, mutations in APP promote apoptosis through constitutive activation of G_o heteromeric proteins that provokes various effects including DNA fragmentation [11]. Knowledge about the molecular mechanisms involved in anti-apoptotic APP

signalling may provide insights into the causes of neurodegeneration in AD brain.

Activated transcription factors function as important regulators deciding between life and death in cells, and they play critical roles in the aberrant induction of apoptosis in largely irreplaceable cells such as neurons. The late Simian virus 40 transcription factor (LSF) is of interest in AD, because apart from influencing global gene expressions in cell growth and mitogenic stimulation [12], it interacts with gene products Fe65 [3], α -2 macroglobulin [13], glycogen synthase kinase (GSK) [14] and viruses [15], which have been suggested to play a role in the pathogenesis of AD [16,17]. Also, some linkage studies have demonstrated that LSF is probably a disease-modifying gene for AD [18,19], and the inhibition of LSF-dependent gene-transcription induced apoptosis [20]. Collectively, these results suggest that LSF may be involved in APP signalling pathways that are linked to the pathogenesis of AD. In the present study, we demonstrate that APP effectively prevents apoptosis in differentiated rat neuroblastoma B103 cells, by signalling the activation of LSF via phosphoinositide 3-kinase (PI 3-kinase) and protein kinase B/Akt serine/threonine kinase activity.

EXPERIMENTAL

Cell culture and treatment

Rat neuroblastoma B103 cells that lacked expression of APP and APP-like proteins [21] were maintained in Dulbecco's modified

Abbreviations used: AD, Alzheimer's disease; APP, Alzheimer's amyloid precursor protein; CAT, chloramphenicol acetyltransferase; DIG, digoxigenin; DTT, dithiothreitol; EMSA, electrophoretic mobility-shift assay; FAD, familial AD; GSK, glycogen synthase kinase; LSF, late Simian virus 40 transcription factor; MTT, 3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyl tetrazolium bromide; PI 3-kinase, phosphoinositide 3-kinase; PKC, protein kinase C; STS, staurosporine; TS, thymidylate synthase.

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Eagle's medium containing 10% (v/v) foetal bovine serum and 5% (v/v) horse serum at 37 °C in an atmosphere containing 5% CO₂. After transfection in the undifferentiated state, cells were washed twice with serum-free Dulbecco's modified Eagle's medium and were cultured in Neurobasal-A medium containing N-2 supplement (Life Technologies, Burlington, ON, Canada) for differentiation as described previously [6]. The N-2 supplement is an optimized serum substitute used for the maintenance of neuronal cells without the need for exogenous growth factors [22]. After 72 h, cytosine arabinoside (10 µM) was also added to limit the growth of undifferentiated cells. To induce apoptosis, differentiated B103 cells were exposed to 5 µM staurosporine (STS; Sigma) for 5 h. In the present study, all experiments were performed on differentiated B103 cells, unless otherwise specified.

Measurement of *in vitro* cytotoxicity

Cell number was measured spectrophotometrically as a function of mitochondrial dehydrogenase activity in living cells. This activity was measured using the 3-(4,5-dimethyl thiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay kit, according to the supplier's instructions (Sigma). Briefly, mitochondrial dehydrogenase reduces MTT to purple formazan. The formazan was solubilized by propanol and its absorbance *A* at 580 nm was measured, from which background absorbance at 690 nm was subtracted to yield values of relative MTT reduction. An increase or decrease in cell number results in a concomitant change in the amount of formazan formed, indicating the degree of cytotoxicity under the different transfection conditions used.

Expression vectors and reporter-gene constructs

The pSVβgal plasmid consists of the β-galactosidase gene under the control of the Simian virus 40 early promoter and enhancer (Promega, Madison, WI, U.S.A.). The wild-type human APP₆₉₅ (hAPPwt) and FAD-associated mutant hAPP₆₉₅ with a V642F substitution (hAPPmut) have been described previously [6]. The Akt-1 cDNA allelic pack (Upstate Biotechnology, New York, U.S.A.) contains the expression vector alone (pUSE amp). Akt-1wt, constitutively active Akt-1 (Akt-1ca) and dominant-negative Akt-1 (Akt-1dn) are tagged with the human *myc*-epitope (*hmyc*) to distinguish the human Akt-1 from endogenous rat Akt-1. LSFwt and LSFdn plasmids (pEF1α-LSFwt and pEF1α-LSFdn respectively) and the thymidylate synthase (TS) promoter region –160 to –142 (–160/–142) chloramphenicol acetyltransferase (CAT) reporter gene have been described previously [20]. The LSFwt-binding site in the TS promoter (–160/–142) is not known to interact with any other nuclear factor.

Stable transfections

Cells (2 × 10⁵) were co-transfected with 10 µg of hAPPwt or hAPPmut plasmids and 1 µg of the neomycin-resistant plasmid (pcDNA 3.1), using the calcium phosphate DNA precipitation method (Sigma). After 3 weeks of selecting Geneticin sulphate (G418)-resistant clones in undifferentiated medium, colonies were pooled and grown as mass cultures. Stable cell lines expressing hAPPwt or hAPPmut were ascertained by Western blots. Cells co-expressing hAPP and LSF were generated by transfecting hAPP cells with 25 µg of pEF1α-LSFwt or pEF1α-LSFdn. Similarly, hAPPwt or hAPPmut cultures were transfected with 25 µg of pUSE amp (vector only), with Akt-1wt, Akt-1ca or Akt-1dn. The hAPPwt- and Akt-1wt-co-expressing stable cell lines were confirmed by Western blots using anti-APP antibody (Sigma) and anti-*hmyc* epitope-tagged antibody (Upstate Biotechnology). Stable cells expressing hAPPwt and p110ca, hAPPwt

and Δp85 and p110ca and Δp85 were also isolated. In control experiments, cells were stably transfected with mock vectors.

Preparation of cell extracts and Western-blot analysis

Cells were harvested, washed in chilled PBS, resuspended in lysis buffer containing 20 mM Hepes (pH 7.4), 100 mM NaCl, 100 mM NaF, 1 mM sodium orthovanadate, 5 mM EDTA, 0.1% Triton X-100 and protease inhibitor cocktail (1 mM PMSF, 5 mM aprotinin, 2.5 mM leupeptin and 1 mM pepstatin). Lysates were centrifuged at 10000 *g* for 5 min at 4 °C. The supernatant was collected and the amount of total protein was determined by using the micro bicinchoninic acid assay (Pierce, Rockford, IL, U.S.A.). Samples were boiled in SDS buffer [62.5 mM Tris/HCl (pH 6.8)/2% (w/v) SDS/10% (v/v) glycerol/50 mM dithiothreitol (DTT)/0.1% (w/v) Bromophenol Blue] and resolved by SDS/PAGE. The protein-blotted membranes were incubated in 20 mM Tris/HCl (pH 7.6), 137 mM NaCl, 0.2% (v/v) Tween 20 with 5% (w/v) non-fat dried milk for 1 h, washed in Tris/NaCl/Tween 20, and incubated for 1 h with primary antibody in Tris/NaCl/Tween 20 containing 1% milk at room temperature (25 °C) for non-phospho-specific antibodies and overnight at 4 °C for phospho-specific antibodies. The antibody dilutions used were the following: APP (1:1000), LSF (1:500; Transduction Laboratories, Lexington, KY, U.S.A.), Fe65 (1:500), Akt (1:100) and phospho-Akt (Ser⁴⁷³) (1:2000; New England Biolabs, Beverly, MA, U.S.A.), Δp85 (1:2000), p110 (1:2000; Upstate Biotechnology), and *hmyc* (1:2000). After further washes, membranes were incubated for 1 h with horseradish peroxidase-linked anti-IgG secondary antibody, and the immunoreactive bands were detected by enhanced chemiluminescence (ECL[®]; Amersham Pharmacia Biotech, Piscataway, NJ, U.S.A.). Quantitative analysis of protein bands was performed by densitometric scanning of the autoradiograms using the Bio-Rad Image software. The results are representative of at least three separate experiments.

Immunostaining

Differentiated cells grown on coverglass slides were washed with cold PBS, fixed for 15 min in 4% (w/v) paraformaldehyde at 4 °C and then blocked with 5% (v/v) goat serum. Fixed cells were washed once with cold PBS and permeabilized in 0.1% Triton X-100 for 15 min at 4 °C. After three washes, cells were incubated with primary antibodies (anti-APP or anti-LSF) for 2 h at room temperature, washed in PBS and incubated with goat anti-mouse or anti-rabbit FITC-conjugated secondary antibody (1:200; Amersham Pharmacia Biotech) for indirect immunofluorescence analysis. Cell nuclei were counterstained with 10 µM Hoechst 33258 and mounted with ProLong anti-fade (Molecular Probes, Eugene, OR, U.S.A.).

Image analysis

Images were collected with a Zeiss epifluorescence microscope camera device and presented in Adobe Photoshop. Image processing and measurement of nuclear fluorescence were performed with the Image Pro Plus software (Media Cybernetics, Silver Spring, MD, U.S.A.). LSF-positive and LSF-negative nuclei (after subtraction of the background) were then counted in several randomly selected fields from several coverslips (> 300 cells). To analyse the images, nuclear area of randomly selected control cells (untreated) was calculated from the segmented Hoechst image. This area with Hoechst image was defined as representing a cell. A segmentation area from the cell with minimal FITC intensity will then be decreased until a pixel

with zero intensity is achieved. This was considered to be the background signal for LSF staining and was subtracted from all FITC images defined within the average nuclear area. An FITC value of 0.0 thus denotes negative LSF fluorescence and values greater than 1 represent positive LSF immunoreactivity. These values were integrated if the LSF image was within the defined area of the Hoechst image to eliminate bias of different intensities of partial or predominantly LSF-immunostained nuclei. Negative FITC values indicate loci located outside of the Hoechst image due to improperly defined cells. To assess the reproducibility of this method, the same set of 50 images was analysed independently. The analysis took into consideration the number of nuclei, LSF-positive nuclei and FITC intensity per nuclei above background levels. One-way ANOVA of the results showed that the variance between the interterritory positions of FITC values in nuclei of transfected cell populations was not significantly greater than the variance between intraterritory positions within each transfected cell type.

Apoptosis assays

STS-induced apoptosis was assessed by two methods: (i) nuclear fragmentation was detected by *in situ* labelling with the TUNEL (terminal transferase deoxynucleotidyl uridine end labelling) assay kit. Briefly, transfected cells in 6-well plates were fixed with 4% (w/v) paraformaldehyde solution in PBS for 30 min at room temperature and washed once with PBS. Cells were resuspended in permeabilization solution (0.1% Triton X-100 in 0.1% sodium citrate) for 2 min at 4 °C, washed with PBS and incubated with fluorescein-labelled dUTP and terminal deoxynucleotidyl transferase for 1 h at 37 °C in a humidified atmosphere in the dark. After three rinses with PBS, cells were analysed by flow cytometry to determine the percentage of apoptosis. (ii) DNA fragments released into the cytoplasm during apoptosis were measured with the ELISA apoptosis kit. Briefly, 1.5×10^4 cells were seeded into a 96-well microtitre plate and incubated overnight. The medium was then replaced with differentiation medium containing bromodeoxyuridine, and the cells were incubated for another 48 h. DNA fragmentation induced by subsequent exposure to STS was quantified in cell lysates with a peroxidase-conjugated anti-bromodeoxyuridine antibody.

Akt kinase activity assay

Briefly, 200 μ g of cell lysates was subjected to immunoprecipitation with 5 μ g of immobilized anti-Akt antibody. *In vitro* kinase reactions were performed in kinase buffer by the incorporation of non-radioactive phosphate into the Akt-specific substrate GSK-3 during a 30 min incubation at 30 °C of Akt-immunoprecipitated pellets in 40 μ l of 1 $\times$ kinase buffer supplemented with 200 μ M ATP and 1 μ g of GSK-3 fusion protein (New England Biolabs). The reaction was terminated with 20 μ l of 3 $\times$ SDS sample buffer, and the solution was vortex-mixed and then microcentrifuged for 2 min. The supernatant (30 μ l) was boiled for 5 min and resolved by SDS/PAGE (12% gel). The phosphorylation of GSK-3 was measured by Western blotting using phospho-GSK-3 α/β antibody.

Preparation of nuclear proteins

Briefly, using precooled buffers, cells were harvested with 0.3% trypsin, centrifuged at 450 *g* for 5 min, and the packed cell volume (PCV) was estimated. Lysis buffer (500 μ l; 5 $\times$ PCV) containing 100 mM Hepes (pH 7.9), 15 mM magnesium chloride, 100 mM potassium chloride, protein inhibitor cocktail (Sigma) and 0.1 mM DTT, was added to 100 μ l of packed cells. The cell

pellet was resuspended in lysis buffer with 36 μ l of 10% IgePal CA-360, incubated on ice for 15 min and vortex-mixed. This was followed by centrifugation for 30 s at 11000 *g*, and the supernatant (cytoplasmic fraction) was transferred to a fresh tube. The nuclear pellet was resuspended in 100 μ l of high salt extraction buffer [20 mM Hepes (pH 7.9)/1.5 mM magnesium chloride/0.42 M NaCl/0.2 mM EDTA/25% (v/v) glycerol/1 mM DTT] (Sigma), agitated at a moderate speed for 30 min and centrifuged for 4 min at 21000 *g*. The supernatants (nuclear extracts) were snap-frozen as aliquots in liquid nitrogen and quantified for protein content by the bicinchoninic acid assay.

Electrophoretic mobility-shift assay (EMSA)

The optimal LSF *cis*-element, wild-type and mutant double-stranded consensus oligonucleotides corresponding to the TS promoter region (−160/−142) [20] were digoxigenin (DIG) 5'-end-labelled according to the supplier's instructions (Boehringer Mannheim, Indianapolis, IN, U.S.A.). A typical binding reaction mixture contained 10 μ g of protein from nuclear extracts and 50 fmol of DIG-labelled probes in binding buffer [75 mM NaCl/0.1 mM EGTA/15 mM Tris (pH 7.5)/0.5 mM DTT/0.05% Nonidet P40/5% (v/v) glycerol] supplemented with 100 μ M dI/dC in a total reaction volume of 25 μ l. The mixture was incubated for 15 min at room temperature. The incubation mixture was electrophoresed in 7% native polyacrylamide with 0.25 $\times$ TBE (90 mM Tris/borate, 1 mM EDTA) at a 30 mA constant current for 2.5 h. The gels were blotted and the membranes were processed for chemiluminescence detection and exposed to X-ray film.

Transient transfections and reporter-gene expression assay

Transfections were performed with 1 μ g of pSV β -gal and 5 μ g of the TS-CAT reporter genes. This 4 $\times$ TS-CAT reporter plasmid consists of the CAT gene driven by a synthetic promoter that contained four copies of the LSF-binding site from the TS promoter (−160/−142), upstream of the adenovirus E1b TATA box. Transfected cells were differentiated and resuspended in reporter-lysis buffer. The activities of β -galactosidase were measured using the β -gal enzyme kit with *o*-nitrophenyl- β -D-galactopyranoside as the substrate (Promega). Before CAT assays, adjusted cell extracts were incubated at 60 °C for 10 min to inactivate endogenous deacetylase activity. CAT assays were quantified by two-phase extraction/liquid-scintillation counting and TLC of the butyrylated and non-butyrylated forms of D-threo-[dichlorobutyryl-1,2-¹⁴C-labelled chloramphenicol] (Amersham Pharmacia Biotech) and normalized to identical β -gal enzyme units.

Statistical analysis

Data were analysed using the Stat View J5.0 software. Non-parametric Friedman ANOVA for related samples was used to evaluate differences between sample repetitions within each transfected cell line. Differences in nuclear LSF level before and after STS treatment for different transfected cells were assessed by χ^2 tests for independent samples, using the average scores for control (mock-transfected) and for each individual transfected cell culture. To correct for multiple comparisons, the experiment-wise error was set at $\alpha = 0.05$ and the comparison-wise error at $\alpha' = 0.006$. Densitometry data of Western blots were analysed by ANOVA, followed by Fisher's *post hoc* tests for comparisons. Results are expressed as means $\pm$ S.E.M., and *n* indicates the number of independent experiments. *P* < 0.05 was considered significant.

Figure 1 Expression of hAPP and LSF in APP-deficient B103 cells

(A) Western blot of APP in human brain homogenate (positive control), in B103 cells transfected with hAPPwt, hAPPmut or mock plasmid. APP (100–110 kDa) was detected with H-43 anti-APP antibody. (B) Distribution of hAPPwt and hAPPmut in B103 cells (epifluorescent images of H-43 immunostaining). Scale bar, 200 μ M. (C) Expression of LSFwt and LSFdn in mock, hAPPwt or hAPPmut cells. The relative level of LSF was calculated as (absorbance $\times$ area of LSF band) divided by the total chemiluminescence signals of LSF and glyceraldehyde-3-phosphate dehydrogenase peaks. (D) LSF consensus binding site in relation to the LSFwt-binding site in TS promoter (–160/–142), and the mutations generated in this site. (E) EMSA of 20 μ g of nuclear extract with the DIG-labelled LSF-binding site. Binding reactions contained no nuclear extract (lane 1), nuclear extract (lane 2), nuclear extracts from hAPPwt and LSFdn cells (lane 3) and nuclear extracts from HeLa cells (lane 4). (F) EMSA with LSF-binding site probe. The probes were incubated with increasing amounts of unlabelled LSF-binding site oligonucleotides. Control (lane 1), 10-fold (lane 2), 20-fold (lane 3) and 30-fold (lane 4) molar excess of unlabelled probe. (G) EMSA of 40 μ g of nuclear extracts with wild-type- or mutant-LSF-binding site probes. Control (lane 1), nuclear extracts (lane 2), nuclear extracts and anti-LSF antibody (lane 3) and nuclear extracts (lane 4). Positions of the LSF–DNA complexes (arrows) and free DIG-labelled probe (arrow heads) are indicated.

RESULTS

We assessed the role of LSF, a 63 kDa protein also known as HIV long terminal repeat binding protein type 1c [23] or α -globin promoter polycytidine-rich binding protein [24], in anti-apoptotic APP signalling in B103 cells. These cells share common neuronal properties with other widely used APP-expressing cell lines [25].

Characterization of the expression of wild-type versus mutant hAPP and LSF

As expected, mock-transfected cells did not show any APP expression, but similar expression levels of wild-type and FAD-mutant APP that migrated with the same molecular mass as the APP₆₉₅ isoform in human brain were observed (Figure 1A). Immunofluorescence analysis showed that wild-type and FAD-mutant APP had a similar intracellular distribution, and APP immunoreactivity was detectable in soma and neurites, but not in the nucleus (Figure 1B). Cell lines co-expressing hAPP and LSFwt or LSFdn were generated. LSFdn, a double amino acid substitution mutant of LSF, initially named 234QL/236KE, is unable to bind the TS promoter region (−160/−142) [23] and inhibits the DNA-binding activity of LSFwt [20,23]. The relative level of LSFwt was 3.3 ± 0.4 in hAPPwt and 3.0 ± 0.2 in hAPPmut, and the relative level of LSFdn was 3.4 ± 0.4 in hAPPwt and 3.2 ± 0.3 in hAPPmut. In contrast, the relative levels of LSFwt and LSFdn in mock-transfected cells were 1.0 ± 0.2 and 1.3 ± 0.5 respectively. Although the relative levels of LSFwt and LSFdn were similar in hAPP-transfected cells, these levels were significantly ($P < 0.001$, $n = 5$) enhanced by at least 3-fold, compared with the relative levels of LSFwt and LSFdn in mock cells (Figure 1C). EMSA showed that LSF bound the anticipated LSF-binding site in the TS promoter (Figure 1E, lane 2). As expected, this binding activity was abolished in cells transfected with LSFdn (Figure 1E, lane 3). The LSF–DNA complex migrated with similar band shifts in B103 and HeLa nuclear extracts (Figure 1E, lane 4), and the LSF-binding activity in B103 cells was gradually eliminated with increasing concentrations of molar excess of unlabelled LSF–DNA-binding site oligonucleotides (Figure 1F). Antibodies directed against LSF in EMSA showed a supershift complex (Figure 1G, lane 3). This complex was not formed with the LSF-binding TS-promoter mutant site probes (Figure 1G, lane 4). These results demonstrate the specificity of the LSF–DNA-binding activity in B103.

Wild-type APP inhibited STS-induced apoptosis and partially blocked LSFdn-enhanced apoptosis

In the absence of STS, expression of hAPPmut showed no significant ($P > 0.05$, $n = 5$) increase in apoptosis compared with hAPPwt and mock transfections (Figures 2A and 2B). The STS-induced apoptosis was $13 \pm 2\%$ in hAPPwt cells, which is significantly ($P < 0.01$, $n = 5$) less compared with 38 ± 3 and $35 \pm 2\%$ in hAPPmut and mock-transfected cells respectively. Expression of LSFwt markedly ($P < 0.001$, $n = 4$) enhanced the capacity of hAPPwt to reduce STS-induced apoptosis from 13 ± 2 to $3 \pm 0.8\%$, compared with 35 ± 3 and $32 \pm 0.4\%$ in hAPPmut- and mock-expressing cells respectively. LSFdn alone did not show any increase in apoptosis ($1.03 \pm 0.3\%$) compared with hAPPwt, hAPPmut and mock transfections (0.95 ± 0.2 , 1.08 ± 0.01 and $0.99 \pm 0.3\%$ respectively). In contrast, expression of LSFdn significantly ($P < 0.03$) increased STS-induced apoptosis to $70 \pm 3\%$ in mock and $68 \pm 2\%$ in hAPPmut, compared with $28 \pm 2\%$ in hAPPwt cells (Figures 2A and 2B). These results

Figure 2 Expression of wild-type but not mutant APP blocked LSFdn-enhanced STS-induced apoptosis

STS-induced apoptosis was measured based on (A) the proportion of fragmented nuclei labelled by TUNEL and (B) DNA fragmentation assessed by ELISA, which was quantified and graphed. Columns and error bars represent the means $\pm$ S.E.M. for five separate experiments. The ELISA results represent average fold increases over baseline levels in untreated cells (arbitrarily defined as 1.0). Differences between mock and transfected cells in the absence of STS treatment were not statistically different ($P > 0.05$, $n = 5$). $P < 0.05$ was determined by Tukey–Kramer *post hoc* tests. (C) Frequency of relative MTT assay.

showed that hAPPwt but not hAPPmut inhibited STS-induced apoptosis; LSFwt inhibited STS-induced apoptosis only in the presence of hAPPwt; and LSFdn-mediated increase in STS-induced apoptosis was partially blocked by hAPPwt. These

Figure 3 Expression of wild-type APP-mediated nuclear translocation of LSF induced by STS

Western-blot analysis of LSF in mock-, hAPPwt- and hAPPmut-transfected B103 cells. (A) Total cellular lysates. (B) Nuclear extracts in the absence of STS treatment and (C) nuclear extracts after STS treatment (5 μ M STS for 5 h). Loading control was confirmed by reprobing with anti-Fe65 and cytidine–thymidine–cytidine-rich zinc finger binding factor (CTCF) antibody [49,50]. (D) The relative absorbance of LSF was calculated as (absorbance $\times$ area of the LSF band) divided by the total chemiluminescence signals for Fe65 or CTCF peaks. Data are expressed as the means $\pm$ S.E.M. ($n = 5$). (E) Western blot of cytoplasmic fractions after STS treatment. The position of the molecular-mass markers LSF, Fe65, CTCF and glyceraldehyde-3-phosphate dehydrogenase immunoreactive bands are indicated in (A)–(C) and (E). (F) The relative level of LSF. Data are expressed as the means $\pm$ S.E.M. ($n = 5$). (G) Expression of wild-type APP in B103 cells mediated LSF nuclear translocation induced by 5 μ M STS for 5 h. Epifluorescence images of anti-LSF immunostaining. Scale bar, 50 μ M. Nuclei lacking LSF (open arrows) and LSF strongly localized in nuclei (filled arrows) are indicated. (H) The frequency of nuclear LSF immunoreactivity was determined by assessing the number of LSF positive nuclei divided by the total number of cells counted. Error bars represent the means $\pm$ S.E.M. ($n = 4$), and > 300 cells were scored per experiment.

Figure 4 Expression of hAPPwt promoted the activation of LSF during STS-induced apoptosis

(A) EMSA of LSF–DNA-binding activity. Nuclear extracts (20 μ g) from mock-, hAPPwt- and hAPPmut-transfected cells after STS treatment were analysed by EMSA of wild-type- and mutant-LSF-binding sites. Positions of the LSF–DNA complexes and free-labelled probe are shown. The relative LSF binding was calculated as (absorbance of LSF–DNA complex $\times$ area of the band shift) divided by the amount of DIG-labelled probe in each binding reaction and normalized to arbitrary units. (B) Relative LSF–DNA-binding activity for an increase in molar excess of unlabelled LSF-binding site oligonucleotides. Data are the means for four independent experiments. (C) Transactivation of the TS promoter region by endogenous LSF. The TS-CAT reporter genes were driven by four copies of either the wild-type- or mutant-LSF-binding site in mouse TS promoter region (–160/–142) upstream of the E1b TATA box. A representative TLC CAT assay autoradiograph is also shown. (D) Frequency of relative CAT activity. The relative CAT activity values obtained for each sample in the absence of STS treatment were subtracted from those obtained in the presence of STS and compared with the arbitrary value set for mock cells at 100%. The error bars represent means $\pm$ S.E.M. ($n = 5$).

effects cannot be attributed to the cytotoxicity of expressing hAPPmut or LSFdn, because comparable levels of relative MTT reduction (and indirectly cell numbers) were observed between the transfected cells (Figure 2C).

Differential effects of wild-type and mutant APP on LSF nuclear translocation

The relative levels of LSF in cytoplasmic or nuclear fractions were similar in mock, hAPPwt and hAPPmut cells (Figures 3A

and 3B). After STS treatment, the relative level of nuclear LSF in hAPPwt was $580 \pm 15\%$, which is significantly ($P < 0.001$, $n = 5$) greater compared with cells transfected with either mock ($30 \pm 10\%$) or hAPPmut ($28 \pm 5\%$) (Figures 3C and 3D). This increase in nuclear LSF was inversely proportional to the relative levels of LSF in the corresponding cytoplasmic fractions (Figures 3E and 3F). No significant difference was observed between the basal levels of LSF-positive nuclei in mock, hAPPwt and hAPPmut cells ($P > 0.05$, $n = 5$) (Figure 3G). However, after STS treatment, hAPPwt cells showed pronounced

Figure 5 Expression of hAPPwt stimulates Akt phosphorylation-dependent serine-threonine kinase activity

(A) Western blot of the total protein extract from mock-, hAPPwt- and hAPPmut-transfected B103 cells was probed with phospho-specific antibodies to phospho-Akt at Ser⁴⁷³ and then reprobed with Akt. Immunoblotting with phospho-specific Akt antibodies was performed at least four times with similar results. (B) Densitometric analyses of phosphorylated Akt in mock-, hAPPmut- or hAPPwt-transfected cells after STS-induced apoptosis. The relative phosphorylated Akt was calculated as (absorbance $\times$ area of phosphorylated Akt band) divided by the chemiluminescence signal of total Akt signal peak. The error bars represent means $\pm$ S.E.M. ($n = 5$). (C) Akt kinase activity in Akt immunocomplex. (D) The Akt-kinase activity was calculated as (absorbance $\times$ area of the phospho-GSK immunoreactive band) divided by the total chemiluminescence signal of the phosphorylated and unphosphorylated Akt peaks. The error bars represent means $\pm$ S.E.M. ($n = 4$).

nuclear translocation of endogenous LSF in $77 \pm 6\%$ of cells. Thus nuclear LSF was found in only $8 \pm 2\%$ of nuclei of mock-transfected cells and in $9.5 \pm 1\%$ of nuclei of hAPPmut-transfected cells [$\chi^2 = 30.1$; 1 d.f. (degree of freedom); $P < 0.001$, $n = 4$ for hAPPwt compared with mock and hAPPmut] (Figures 3G and 3H).

Wild-type but not mutant APP mediated the activation of LSF

We tested whether the nuclear translocation of LSF in hAPPwt cells would influence LSF-DNA-binding activity and dependent gene transcription. As determined by EMSA with the LSF-binding site (AGAAGTGGTTACAGCTTGC) in the TS promoter region ($-160/-142$), expression of wild-type but not FAD-mutant hAPP markedly enhanced LSF-DNA-binding activity (by 4-fold) after STS treatment (Figure 4A). The level of the LSF-DNA binding activity relative to the amount of DIG-labelled probe was 8 ± 2.1 in nuclear extracts from hAPPwt. This value was significantly ($P < 0.001$, $n = 4$) increased compared with the relative levels of 1.2 ± 0.3 and 1.85 ± 0.1 in nuclear extracts from mock and hAPPmut cells respectively. To eliminate bias due to unequal loading of protein-DNA complexes, we also determined the extent of LSF-binding activity in a competition EMSA. The relative binding activity of LSF in hAPPwt was decreased to 50% by a 15-fold molar excess of

unlabelled competitor, compared with a 6-fold molar excess for mock and a 8-fold molar excess for hAPPmut cells (Figure 4B). The LSF-DNA-binding activity was eliminated with mutant probes and nuclear extracts from STS-treated mock, wild-type and FAD-mutant APP-transfected cells. It should be noted that no difference in DNA-binding activity of LSF was observed in the absence of STS treatment, indicating specificity of the LSF-DNA-binding activity. To determine whether LSF could activate transcription from LSF-dependent gene promoters, the $4 \times$ LSF-CAT reporter-gene activity was determined (Figure 4C). After STS treatment, expression of hAPPwt significantly ($P < 0.001$, $n = 5$) increased the relative CAT activity by $400 \pm 30\%$ compared with relative CAT activity of $103 \pm 10\%$ for hAPPmut and 100% for mock-transfected cells (Figure 4D).

Wild-type but not mutant APP activated the Akt serine/threonine kinase pathway

As the PI 3-kinase/Akt pathway plays a critical role in anti-apoptotic signalling events in neuronal cells [26,27], which involves activation of Akt via phosphorylation by an upstream target of PI 3-kinase [28], we assessed the activation of Akt in wild-type and FAD-mutant APP-transfected cells or mock-transfected cells by measuring the relative levels of Akt phospho-Ser⁴⁷³ and the Akt kinase activity. In STS-induced apoptotic

Figure 6 Inhibition of PI 3-kinase blocked Akt serine/threonine kinase activity and LSF nuclear translocation

(A) Measurement of Akt kinase activity in lysates from transfected cells that were treated with 5 μ M STS for 5 h. The relative Akt kinase activity was calculated by dividing the chemiluminescence signal (absorbance $\times$ area of the phospho-GSK band) by the total chemiluminescence signal of Akt, normalized to arbitrary units. The error bars represent means $\pm$ S.E.M. ($n = 5$). (B) Immunofluorescence of endogenous LSF in B103 cells treated with 5 μ M STS for 5 h. In hAPPwt cells, LSF is predominantly nuclear (filled arrows), whereas in mock or hAPPwt and Δ p85, LSF is mainly cytoplasmic, with no detectable or only robust nuclear LSF fluorescence (open arrows). Scale bar, 100 μ M. (C) Frequency of plots of LSF-positive nuclei (%) per total number of cells (> 300 cells) were counted in each experiment, as determined using the Image Pro-Plus software. The error bars represent means $\pm$ S.E.M. ($n = 4$). (D) Relative reduction of MTT. The error bars represent means $\pm$ S.E.M. ($n = 6$).

cells, expression of hAPPwt led to a significantly ($P < 0.003$, $n = 5$) higher phospho-Akt relative level of 7.5 ± 0.6 , compared with 0.7 ± 0.02 in FAD-mutant APP or 0.4 ± 0.05 in mock cells (Figures 5A and 5B). This result was consistent with the relatively higher endogenous Akt kinase activity of $48 \pm 3\%$ in hAPPwt, compared with 10% in mock and $18 \pm 3\%$ in hAPPmut cells (Figures 5C and 5D).

Wild-type APP-mediated LSF nuclear translocation is PI 3-kinase/Akt-dependent

We examined the possibility of LSF being a target of the PI 3-kinase/Akt pathway. To this end, we generated cells expressing

dominant negative PI 3-kinase (Δ p85), constitutively active PI 3-kinase (p110ca) and hAPPwt co-transfected with Δ p85 or p110ca. Expression of p110ca caused a significant increase ($P < 0.03$; $n = 5$) in Akt kinase activity of $68 \pm 1\%$, compared with Akt kinase activity of 10% in mock cells. Co-expression of p110ca and hAPPwt led to a marked increase in Akt kinase activity of $105 \pm 3\%$ compared with expression of hAPPwt alone with Akt kinase activity of $43 \pm 2\%$. This activity was also significantly ($P < 0.05$, $n = 5$) less than the $68 \pm 1\%$ of Akt kinase activity for p110ca expression. In contrast, expression of Δ p85 inhibited Akt kinase activity and attenuated hAPPwt-mediated increase in Akt kinase activity (Figure 6A). Expression of Δ p85 also abolished nuclear translocation of LSF in hAPPwt-transfected

Figure 7 Wild-type-APP-enhanced nuclear translocation of LSF is dependent on the Akt pathway after STS treatment

(A) Western-blot analyses, after STS treatment, of LSF in nuclear extracts from cells transfected with hAPPwt (lane 1), hAPPwt and Akt-1ca (lane 2), hAPPwt and mock (lane 3), Akt-1ca (lane 4), Akt-1dn (lane 5) and hAPPwt and Akt-1dn (lane 6). Samples (30 μ g) were separated by SDS/PAGE, transferred to nitrocellulose membranes and probed for LSF and CTCF. (B) The relative levels of nuclear LSF. The error bars represent means $\pm$ S.E.M. ($n = 5$). (C) Nuclear extracts (20 μ g) from transfected cells (upper panel) were analysed in an EMSA with DIG-labelled LSF-binding site probe. Control, no nuclear extract. Four separate experiments gave similar results. (D) The LSFwt-binding site-responsive TS-CAT reporter genes were transiently transfected with pSV β -gal genes into transfected cells (upper panel). Cells were treated, 48 h after transfections, with 5 μ M STS for 5 h. A representative TLC-CAT assay autoradiograph is shown. Five separate experiments gave similar results. (E) Frequency of the relative CAT activity. Mock-transfected cells were arbitrarily assigned the relative CAT activity of 100%. The error bars represent means $\pm$ S.E.M. ($n = 5$). (F) Determination of relative MTT reduction. The error bars represent means $\pm$ S.E.M. ($n = 5$).

cells (Figure 6B). Thus LSF was found in $3 \pm 0.03\%$ of nuclei of $\Delta p85$ cells, in $73 \pm 0.02\%$ of nuclei of hAPPwt and in $2.5 \pm 0.02\%$ of nuclei of mock cells [$\chi^2 = 28.9\%$; 1 d.f.; $P < 0.001$ ($n = 4$) for $\Delta p85$ compared with hAPPwt, and no significant difference compared with mock] (Figure 6C). The inhibition of Akt kinase activity or nuclear translocation of LSF is not a reflection of cytotoxic effects due to $\Delta p85$ or p110ca (Figure 6D). These results are consistent with the role of PI 3-kinase in APP-mediated cell survival and the fact that PI 3-kinase is upstream of Akt.

Next, we investigated the role played by Akt in hAPPwt signalling. hAPPwt was co-transfected with Akt-1ca or with Akt-1dn [29]. After STS treatment, there was a significant ($P < 0.03$, $n = 5$) increase in the relative levels of LSF of 7 ± 0.2 in hAPPwt and Akt-1ca cells, relative to hAPPwt and mock expression with relative nuclear LSF levels of 3 ± 0.2 and 2.8 ± 0.3 respectively. Expression of Akt-1dn abolished the increase in relative levels of nuclear LSF mediated by wild-type APP (Figures 7A and 7B). The relative levels of nuclear LSF in Akt-1dn of 0.3 ± 0.001 or 0.19 ± 0.003 in hAPPwt and Akt-1dn were significantly ($P < 0.001$, $n = 4$) less compared with hAPPwt and Akt-1ca, hAPPwt, and Akt-1ca, with relative nuclear LSF levels of 6.8 ± 0.01 , 3.0 ± 0.02 and 2.8 ± 0.03 respectively. These results suggest that expression of Akt-1dn alone had no effect on LSF nuclear translocation and that wild-type APP-mediated LSF nuclear translocation in STS-induced cells is dependent on Akt signalling.

Wild-type APP-mediated LSF-dependent gene transcription is via Akt signalling

We tested the functional relevance of the APP-mediated LSF nuclear translocation and the role of Akt in this process. We examined the influence of Akt-1 expression on hAPPwt-mediated LSF–DNA-binding activity and LSF-dependent gene transcription. Co-expression of Akt-1ca and hAPPwt significantly ($P < 0.001$, $n = 4$) enhanced the LSF–DNA-binding activity (by 5-fold) compared with hAPPwt, Akt-1ca or Akt-1dn expression alone (Figure 7C). Thus the value of the relative band intensity of the LSF–DNA complex in hAPPwt and Akt-1ca cells was 5 ± 0.5 compared with 1.5 ± 0.6 for hAPPwt and 0.5 ± 0.1 and 0.47 ± 0.3 for Akt-1ca- and Akt-1dn-expressing cells respectively. Although Akt-1dn prevented the LSF–DNA-binding activity mediated by hAPPwt (Figure 7C, lane 4), expression of Akt-1dn on its own had no effect on LSF–DNA-binding activity (Figure 7C, lane 6). The LSF–DNA-binding activity of hAPPwt expression was substantially higher (by 2-fold; $P < 0.05$, $n = 4$) compared with expression of Akt-1 on its own in B103 cells. Consistent with the LSF–DNA-binding activities (Figure 7C), the relative CAT activity of the LSF-responsive TS-CAT reporter gene in hAPPwt and Akt-1ca was $300 \pm 20\%$ compared with that of hAPPwt and mock transfections. In contrast, hAPPwt and Akt-1dn expression, with a relative CAT activity of $30 \pm 5\%$, was significantly ($P < 0.03$, $n = 4$) decreased, by 3-fold, compared with hAPPwt and mock transfections, assigned with the relative CAT activity of 100% (Figures 7D and 7E). Expression of Akt-1ca or Akt-1dn showed a significant decrease ($P < 0.05$, $n = 5$) with a relative CAT activity of 50 ± 3 or $49 \pm 2\%$ respectively, compared with the relative CAT activity of 100% for expression of hAPPwt and mock. These results indicate that Akt-1ca/Akt-1dn alone had no effect on LSF activation, but that there is an absolute requirement of APP for Akt to mediate LSF nuclear translocation and dependent gene transcription. It is noteworthy that Akt-1dn or Akt-1ca expression had no signifi-

cant ($P > 0.05$, $n = 4$) effect on the relative MTT reduction, compared with hAPPwt and mock transfections.

DISCUSSION

Although the C-terminus of APP interacts with proteins that may target transcription factors to promote cell survival or may affect neuronal apoptosis [30,31], few studies have examined how FAD mutations impair or counteract specific APP functions [32,33]. Largely unexplored are the molecular mechanisms involved in the regulation of apoptosis by APP and its mutant forms, and how alterations in APP contribute to neurodegeneration in AD brain [34,35]. Therefore the elucidation of APP pathways involved in inhibiting or promoting apoptosis, mediated by transcription factors [36,37], is important in understanding the pathogenesis of AD and in approaches to its treatment or prevention. We report that wild-type but not FAD-mutant APP rescued B103 cells from STS-induced apoptosis, and that the expression of FAD-mutant APP did not induce apoptosis. Furthermore, we examined the possible role of the AD-modifying nuclear factor LSF [18,19] in this anti-apoptotic APP effect. Although expression of LSFdn alone did not induce apoptosis, because another neurogenic-element-binding transcription factor family of proteins may compensate for the loss of LSF–DNA-binding activity [38], LSFdn enhanced the STS-induced apoptosis. This apoptosis was partially blocked by wild-type APP (Figures 2A and 2B), suggesting that LSF is critical in anti-apoptotic APP signalling. The expression of wild-type APP did not effect cytoplasmic accumulation or nuclear export of LSF, because in basal conditions LSF was predominantly cytoplasmic and no apparent difference was observed in the endogenous relative levels of nuclear LSF (Figures 3A and 3B). In the presence of STS, wild-type but not FAD-mutant APP effectively induced LSF nuclear translocation (Figures 3C–3H). Although the precise mechanisms that lead to activation of LSF are yet to be defined, it is possible that under stress, APP mediates rapid phosphorylation of LSF, leading to nuclear translocation. Alternatively, APP may promote a signal-inducible degradation of an LSF-binding inhibitor (which sequesters LSF in the cytoplasm), a situation that may be analogous to the nuclear factor κ B activation by Akt through phosphorylation and degradation of inhibitory κ B [39]. The increased LSF–DNA-binding activity and LSF-dependent transcription that was observed in wild-type APP-expressing cells (Figure 4) may reflect functional significance of the LSF nuclear translocation. The results so far indicate that APP may inhibit apoptosis via activation of LSF.

As the Ras/PI 3-kinase/Akt pathway has been shown to inhibit apoptosis and promote cell survival mediated by neurotrophins and growth factors [26,27], we examined the possible role of this pathway in APP signalling. This assumption is reinforced by the observations that APP may be a neurotrophic factor [40,41] and that APP activates PI 3-kinase in the insulin signalling pathway for neuronal activity [42]. In support of this idea, expression of APP caused an increase in Akt phosphorylation and kinase activity in cells under apoptotic stress (Figure 5), and this activity was abolished and LSF activation was enhanced by dominant-negative and constitutively active PI 3-kinase (Figure 6). Akt is a proximal downstream effector of the anti-apoptotic Ras/PI 3-kinase pathway [43] and is known to target proapoptotic proteins by phosphorylation [44]. This raises the possibility that APP expression leads to Akt kinase-dependent phosphorylation of LSF. This is most probable, because LSF seems to have an ideal Akt phosphorylation motif [45] located in a RXRXXT-HYD consensus peptide and, also, phosphorylation

Figure 8 PI 3-kinase/Akt links APP with LSF in anti-apoptotic signalling

STS treatment induced hAPPwt to activate LSF via the PI 3-kinase/Akt pathway. Activated Akt may transiently associate with and phosphorylate LSF to interact with nuclear chaperone proteins (CP), or Akt may phosphorylate an inhibitory protein (IP) to release LSF.

of LSF leads to increased DNA-binding activity [12]. Consistent with this suggestion, APP-mediated LSF nuclear translocation was markedly enhanced by expression of Akt-1ca. In contrast, Akt-1dn abrogated this effect (Figures 7A and 7B). These results suggest that Akt is a component of a previously unrecognized signalling pathway activated by wild-type but not FAD-mutant APP, leading to LSF activation. In support of this idea, APP-mediated LSF–DNA-binding activity and LSF-dependent transcription after STS treatment were significantly enhanced by Akt-1ca and were prevented by Akt-1dn expression (Figures 7C–7E). We also found that although Akt is necessary for APP-mediated activation of LSF, it is not sufficient on its own to induce activation of LSF in the presence of an apoptosis-inducing stimulus. This implies that other APP cellular targets independent of Akt may be involved in mediating the activation of LSF, e.g. through protein kinase C (PKC), because PKC can be stimulated by APP [46] and can also associate with Akt [47].

Because Akt is an activated survival factor [48], our study demonstrates that a signalling pathway mediated by APP culminating in phosphorylation of Akt is necessary for the activation of LSF. Once activated, LSF regulates neuronal survival by stimulating LSF-dependent gene transcription of survival proteins or suppressing transcription of proapoptotic genes as a cellular response to stress. Based on these results, we present a model that illustrates the relationship between APP and the Ras/PI 3-kinase/Akt pathway that converges on LSF (Figure 8). Since Akt can activate LSF only in the presence of APP, it is possible that APP can activate LSF independent of Akt. Such pathways may involve phosphorylation of nuclear chaperone proteins or LSF-inhibitory proteins mediated by PKC. How does this model apply to the pathogenesis of AD? Disruption of this function by mutations in APP and aberrations in LSF could enhance neuronal apoptosis due to secondary insults and possibly contribute to neuronal loss in AD brain.

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