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Reduced amyloidogenic processing of the amyloid β -protein precursor by the small-molecule Differentiation Inducing Factor-1

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Abstract

The detection of cell cycle proteins in Alzheimer's disease (AD) brains may represent an early event leading to neurodegeneration. To identify cell cycle modifiers with anti-A β properties, we assessed the effect of Differentiation-Inducing Factor-1 (DIF-1), a unique, small-molecule from *Dictyostelium discoideum*, on the proteolysis of the amyloid β -protein precursor (APP) in a variety of different cell types. We show that DIF-1 slows cell cycle progression through G0/G1 that correlates with a reduction in cyclin D1 protein levels. Western blot analysis of DIF-treated cells and conditioned medium revealed decreases in the levels of secreted APP, mature APP, and C-terminal fragments. Assessment of conditioned media by sandwich ELISA showed reduced levels of A β 40 and A β 42, also demonstrating that treatment with DIF-1 effectively decreases the ratio of A β 42 to A β 40. In addition, DIF-1 significantly diminished APP phosphorylation at residue T668. Interestingly, site-directed mutagenesis of APP residue Thr668 to alanine or glutamic acid abolished the effect of DIF-1 on APP proteolysis and restored secreted levels of A β . Finally, DIF-1 prevented the accumulation of APP C-terminal fragments induced by the proteasome inhibitor lactacystin, and calpain inhibitor N-acetyl-leucyl-leucyl-norleucinal (ALLN). Our findings suggest that DIF-1 affects G0/G1-associated amyloidogenic processing of APP by a γ -secretase-, proteasome- and calpain-insensitive pathway, and that this effect requires the presence of residue Thr668.

Keywords

Amyloid β -protein precursor; Alzheimer's disease; Phosphorylation; Differentiation inducing factor; Cell cycle; Proteolysis

1. Introduction

Alzheimer's disease (AD) is a form of dementia that affects the elderly and is broadly characterized by memory loss and cognitive deterioration. It is a slow, progressive neurodegenerative disorder defined pathologically by increased extracellular deposits of β -amyloid (plaques) and the formation of neurofibrillary tangles in specific neuronal regions of the brain often accompanied by atypical protein phosphorylation [1–3]. The amyloid β -

protein precursor (APP) is a highly conserved posttranslationally modified type I transmembrane glyco-protein expressed in most cells, but enriched in neurons [4]. APP is a member of a gene family that includes two highly similar homologues, the amyloid precursor-like proteins 1 (APLP1) and 2 (APLP2) [5,6]. However, the amyloidogenic A β domain, the main component of β -amyloid plaques, is only generated by APP catabolism [7]. APP is processed in at least two different pathways that produce different carboxyl-terminal cleavage fragments (CTFs) [4,7]. The cleavage of APP by the disintegrin and metalloproteinase (α -secretase) or β -site APP-cleaving enzyme 1 (BACE1), produces soluble N-terminal fragments s α APP or s β APP, and C83 (α CTF) and C99 (β CTF) membrane-bound CTFs, respectively [4,7]. The CTFs are further cleaved by γ -secretase leading to the release of either the p3 peptide or the amyloidogenic peptides A β 40 and A β 42 [4,7]. Processing by α -secretase(s) dominates APP metabolism in most cell types, whereas BACE1 production of β -CTFs is less frequent [4,7,8]. A complete understanding of the cellular events that regulate APP processing may lead to methods of early detection, drug intervention, and ultimately the prevention of AD.

Many theories regarding the pathogenesis of AD have been proposed including abnormal A β accumulation, tau phosphorylation, inflammation, oxidative stress, metal ion dysregulation and Ca²⁺-dyshomeostasis, but the mechanisms leading to A β accumulation remain unclear [9–11]. However, mounting evidence suggests that irregular cell cycle re-entry in selective neuronal populations may have an important role in the initiation of AD pathogenesis [12–14]. A variety of cell cycle regulatory proteins, including proliferating cell nuclear antigen, cyclin D1, Cdk4, and cyclin B1, have been detected in animal models of AD well before the presence of plaques, and in human brain regions that display AD pathology [15–17]. Interestingly, APP processing is also elevated in tumors, pancreatic cancer, oral squamous cell carcinomas and colon carcinomas, which collectively imply that APP catabolism is linked to mechanisms involved in cellular proliferation [18–20]. Though the degree of APP processing is clearly altered in both neuronal and oncogenic signaling pathways, the relevance of neuronal cell cycle re-entry to AD pathogenesis remains unclear.

The social amoeba *Dictyostelium discoideum* is an excellent model organism to study many eukaryotic cellular processes. The formation of multicellular fruiting bodies requires the secretion of small chemical morphogens that coordinate the differentiation of amoeba into prestalk or prespore cells [21–23]. One particular morphogen is a unique chlorinated alkyl phenone, (1-[3, 5-dichloro-2, 6-dihydroxy-4-methoxyphenyl]-1-hexanone) which has been termed Differentiation-Inducing Factor-1 (DIF-1) [23]. The intracellular DIF-1 targeted molecules are still unknown even in *Dictyostelium*, and no structural analogs of DIF-1 have yet to be reported in mammalian systems. However, DIF-1 has been suggested to inhibit proliferation of mammalian tumors *via* transient activation of glycogen synthase kinase-3 β (GSK3 β), resulting in decreased levels of cyclin D1 and β -catenin [24,25]. Cyclin D1 expression is critical for entry into S-phase, such that it serves as a marker of cell cycle progression [26]. The levels of cyclin D1 are further linked to the stabilization of cytosolic β -catenin/human T-cell factor-mouse lymphoid enhancer factor complexes that activate transcription of cell cycle genes [27]. Of interest, mutant presenilin variants that bind β -catenin modify cell growth through errant β -catenin signaling *in vivo* [28–30]. Based on these lines of evidence, we posit that DIF-1 might modulate APP catabolism. Here, we show that the amyloidogenic processing of APP is in fact reduced by DIF-1. Our data suggest that DIF-1 can significantly modulate amyloidogenic processing of APP in a proteasome- and calpain-insensitive pathway and that this mechanism requires the C-terminal Thr668 residue.

2. Materials and methods

2.1. Reagents and antibodies

DIF-1 (1-(3, 5-dichloro-2, 6-dihydroxy-4-methoxyphenyl)-1-hexanone) was purchased from Affiniti Research Products (BioMol). Pre-cast 14% and 8% Tris-glycine gels, SeeBlue plus pre-stained MW markers, G418, zeocin and hygromycin were from Invitrogen. The biochemical enzyme inhibitors γ -secretase inhibitor IX (DAPT), kenpaullone, 1-azakenpaullone, roscovitine and LiCl were all purchased from Calbiochem. BCA protein assay reagent and SuperSignal ECL were purchased from Pierce. Proteasome inhibitors ALLN and lactacystin were purchased from Sigma. Antibodies and their suppliers were anti-APPT668; anti-APP C-terminal (8717) (Sigma); anti-GAPDH (MAB374), and anti-N terminus APP (22C11) (Chemicon); anti-cyclin D1 (H-295); Anti-APP residues 1–17 of A β (6E10). Anti-N-terminus amino acids 595–611 of APP (r1736) (was kindly provided by Dr Dennis Selkoe, Harvard Medical School, Boston, MA, USA). Polyclonal anti-APLP1-W1Ct and anti-APLP2-W2Ct were raised to human APLP1 and APLP2, are specific for each protein and do not cross-react with APP.

2.2. Cell culture, proliferation assays and flow cytometry

Naïve CHO, N2a, MEF and SH-SY5Y cells were maintained in DMEM containing 4.5 mg/mL D-glucose supplemented with 10% fetal bovine serum and 100 units/mL penicillin, 100 μ g/mL streptomycin, 2 mM L-glutamine (Sigma) at 37 °C in a 5% CO₂ atmosphere. CHO cells stably expressing human wild type APP⁷⁵¹ (CHO-7W), wild type human APLP2 (CHO-A2) or wild type human APLP1 (CHO-A1) were grown in media supplemented with hygromycin. CHO cells stably expressing human APP⁷⁵¹ and human BACE1 (CHO-CAB) were maintained in G418 and zeocin. H4 neuroglioma cells stably expressing human wild type APP⁷⁵¹ or the Swedish mutant form APP^{sw} were maintained in G418.

For proliferation assays, 1×10^4 cells in 24-well plates were treated with vehicle (ethanol or DMSO) or various amounts of DIF-1 for a period of 4 days. Cells were harvested by trypsin/EDTA treatment, stained with Trypan blue and counted in triplicate twice using a hemocytometer. Data represents the mean cell number $\pm$ the standard deviation for 3 independent experiments.

In order to analyze the cell cycle distribution, we collected cells by trypsin/EDTA treatment and counted them using a hemocytometer. Cells (1×10^5) were deposited into 10 cm tissue culture dishes containing fresh media and grown for 24 h prior to DIF-1 treatment (25 μ M). Cells were grown in the presence of DIF-1 for approximately 18–20 h. Cells were collected by trypsin/EDTA treatment and were suspended in a hypotonic fluorochrome solution containing 50 μ g/mL of propidium iodide, 0.1% sodium citrate, and 0.1% Triton X-100. Cells ($\sim 5 \times 10^4$) were analyzed for fluorescence using the Massachusetts General Hospital cytology core. Cell cycle distribution was compared between vehicle and DIF-1 treated naïve CHO cells, and CHO-7W cells. The percentage of cells in G0/G1, S-phase and G2/M represents the mean cell number $\pm$ the standard deviation from 3 independent experiments.

2.3. Construction of APPT668A, APPT668E mutations and stable cell lines

Constructs were generated using APP⁷⁵¹ in which residue Thr724 was mutated to an alanine residue such that phosphorylation could not occur, or a glutamic acid residue that should mimic constitutive phosphorylation of APP. To create the desired mutations, the APP⁷⁵¹ codon for threonine 724 (ACC) was mutated to code for alanine (A) (GCC) or glutamic Acid (E) (GAA) using Stratagene's QuickChange Site-Directed Mutagenesis Kit as recommended by the manufacturer. Primers were designed for the T724A mutation: the forward primer sequence is 5'-GACGCCGCTGTCCGCCAGAGGAGCGC-3', and the

reverse primer sequence is 5'-GCGCTCCTCTGGGGCGACAGCGGCGTC-3'. Primers designed for the T724E mutation: the forward primer sequence is 5'-GTTGACGCCGCTGTGGAACCAGAGGAGCGCCAC, and the reverse primer sequence is 5'-GTGGCGCTCCTCTGGTTCGACAGCGGCGTCAAC-3'. Mutated codons in each primer are underlined. The following PCR conditions were used with 50 ng of template DNA: one denaturing cycle for 30 s at 95 °C followed by 12 cycles consisting of 30 s at 95 °C, 1 min at 57.4 °C or 55.3 °C, and 12 min at 68 °C. PCR products were transformed into *E. coli* using Invitrogen's MAX Efficiency DH5- α Competent Cells and plated on LB agar containing ampicillin. Colonies were selected and grown in LB media supplemented with ampicillin. Plasmids were purified using Qiagen's QIAprep Spin Miniprep Kit and sequenced at the Massachusetts General Hospital's Sequencing Core. All primers were purchased from Operon.

Naïve CHO cells were transfected with 2 μ g of plasmid DNA using the AMAXA nucleofactor kit optimized for CHO cells. Cells were diluted and plated in multiple 10 cm tissue culture dishes. Stable colonies were selected in G418 over 3 weeks according to standard protocols. Protein extracts from stable cells were assessed for APPT724A and APPT724E expression levels compared to naïve cells by western blot using the anti-APP C-terminal antibody (C66). However, for clarity purposes these mutations are referred to as APPT668A and APPT668E (APP⁶⁹⁵ numbering) throughout the manuscript.

2.4. Western blot detection of cellular APP, APLP1 and APLP2

Cells were lysed in ice cold RIPA buffer (20 mM Tris-HCl (pH 7.4) containing 150 mM NaCl, 2 mM EDTA, 1% Nonidet P-40 (NP-40), 50 mM NaF, 1 mM Na₃VO₄, 1 mM Na₂MoO₄, 10 mg/mL aprotinin, and 10 mg/mL leupeptin), and supplemented with protease inhibitor tablets (Roche). Protein extracts were fractionated by SDS-PAGE and transferred to PVDF membranes. The membranes were blocked for 1 h with 5% fat-free powdered milk or 5% BSA, incubated overnight at 4 °C with the appropriate primary antibodies, anti-APP C-terminal (C66) (1:5000), anti-APLP1-W1Ct and anti-APLP2-W2Ct (1:1000), anti-pAPPT668 (1:1000), anti-N terminus APP (22C11) (1:1000) anti-APP amino acids 595–611 of APP (r1736), (1:1000) anti-cyclin D1 (H-295) (1:500), anti-GAPDH (MAB374) (1:1000), washed with TTBS and then incubated at room temperature for 1 h with the appropriate peroxidase-conjugated secondary antibodies (1:5000). Target proteins were detected using the enhanced chemiluminescence (ECL) system (Amersham Pharmacia Biotech) and XOMAT Kodak film.

2.5. Detection and quantification of secreted levels of A β 40 and A β 42

The levels of secreted A β was quantified by sandwich ELISA and viewed by western blot using anti-APP antibody (6E10) (1:500) that recognizes residues 1–17 of A β . Cells (5 $\times$ 10⁵) were deposited into 6-well tissue culture dishes in triplicate and allowed to grow for 24 h. The media was aspirated and the cells were washed 2 $\times$ in warm PBS. Fresh media (2 mL) and media supplemented with vehicle or DIF-1 was added to each well. After 18–20 h, conditioned media was collected from vehicle and DIF-1 (30 μ M) treated cells, and centrifuged at 14,000 rpm for 10 min to remove any cells or debris. Since DIF-1 exerts a cytostatic effect, the cell number was counted for all treatments following removal of conditioned media and used to normalize the levels of A β . It should be noted that after 18–20 h, the cell number for vehicle treated cells is ~10% higher than DIF-1 treated cells. The concentration of A β 40 and A β 42 was subsequently detected in triplicate using the β -Amyloid 1–40 or 1–42 Colorimetric ELISA kit (Biosource International, Inc.) according to the manufacturer's instructions. Since DIF-1 is cytostatic, our A β sandwich ELISA data was normalized against vehicle total protein levels and cell number.

2.6. Treatment with cell cycle/kinase and proteasome inhibitors

For the kinase assays, cells were pre-treated (~3 h) with inhibitors of GSK or Cdk5 (5–10 mM LiCl, 10 μ M kenpaullone, 10 μ M 1-azakenpaullone and 10 μ M roscovitine, respectively), and then DIF-1 (30 μ M) was added to each treatment and incubated overnight for 18–20 h. Cells were pre-treated (~3 h) with the proteasomal inhibitors, lactacystin (10 μ M) and ALLN (10 μ M). DIF-1 (30 μ M) was then added to each treatment. Cells were collected in RIPA buffer as described above.

2.7. Densitometry and statistical analysis

Densitometry analysis of western blot data was performed using QuantityOne software. Loading variations between western blot lanes were normalized according to the GAPDH signal prior to quantification. Data points represent the mean value $\pm$ the standard deviation. Significant differences between the mean values of vehicle-treated and DIF-1 treated cells were revealed using two tailed paired *t* tests. The number of samples (*n*) in each experimental condition is indicated in each figure legend.

3. Results

3.1. DIF-1 reduces proliferation of CHO cells stably expressing APP

DIF-1 is a small hydrophobic chlorinated alkyl phenone (Fig. 1). To examine whether DIF-1 had an anti-proliferative effect on naïve CHO cells, the growth of cells treated with DIF-1 (25 μ M) was compared to that of cells treated with vehicle alone. As can be seen in Fig. 2, DIF-1 significantly inhibited the proliferation of CHO cells compared to vehicle alone (Fig. 2a, $P < 0.001$). Washing the cells with PBS restored their rate of proliferation (Fig. 2a). To gain insight as to how affects proliferation, cell cycle distribution was measured by flow cytometry. After 20 h of exposure, $37.8 \pm 1.22\%$ of vehicle treated cells were in G0/G1, whereas the percentage of DIF-1 treated cells in G0/G1 was increased to $52.6\% \pm 0.89$ (Fig. 2b).

APP has been suggested to play a role in the proliferation of neural stem cells and keratinocytes [31,32]. To assess whether increased levels of APP could alter the cytostatic effect of DIF-1, CHO cells that stably overexpress human wild type APP⁷⁵¹ (CHO-7W) were treated with DIF-1 and cell proliferation was examined. DIF-1 decreased the proliferation of CHO-7W cells in a dose-dependent manner (Fig. 2c). Analysis of the cell cycle distribution revealed an increase in the percentage of cells in G0/G1 similar to what was observed in naïve CHO cells (Fig. 2d). A similar cytostatic response was seen in mouse N2a and human SH-SY5Y cells suggesting that the effect of DIF-1 extends across a variety of mammalian cell types (Fig. 2e, f). Analysis of cell cycle distribution after 72 h revealed DIF-1 treatment resulted in a marginal increase in the percentage of cells in G0/G1 compared to 24 h exposures, and that treatment did not result in all cells being blocked in G0/G1 (data not shown).

3.2. DIF-1 reduces the expression of cyclin D1 in CHO cells

The cytostatic effect of DIF-1 coupled with the reported detection of cyclin D1 in AD-diseased brain regions [13,14,17]; prompted us to use western blot analysis to assess DIF-1 effects on cyclin D1 expression. As illustrated in Fig. 3a, DIF-1 induced a marked decrease in cyclin D1 levels. Densitometry analysis showed an average decrease of $82.41 \pm 0.74\%$ in cyclin D1 levels (Fig. 3b). When cells were treated with the γ -secretase inhibitor DAPT the level of cyclin D1 remained unchanged, however, when co-treated with DAPT and DIF-1 the decrease in cyclin D1 expression was similar to that seen for DIF-1 alone (Fig. 3b). The level of cyclin D1 remained unchanged when cells were treated with different cell cycle inhibitors including kenpaullone, 1-azakenpaullone and roscovitine (data not shown). This

suggests that the cytostatic effect of DIF-1 is correlated with a decrease in the level of cyclin D1.

3.3. DIF-1 reduces processing of APP, APLP2 but not APLP1

Since reports have shown increased levels of cyclin D1 in human brain regions that display AD pathology we wanted to see if cyclin D1 levels correlated with APP processing. We used western blotting to assess whether DIF-1 affected APP processing in CHO-7W cells. DIF-1 (30 μ M) reduced APP maturation and decreases α CTF levels, and appears to preferentially decrease β CTF levels (Fig. 4a) suggesting that the level of APP processing in CHO cells is linked to the G0/G1 phase of the cell cycle (Fig. 4a). Alternatively, the observed decrease in APP CTFs implies that DIF-1 may potentiate γ -secretase activity. To explore this possibility, we examined the combined effect of DAPT, a potent inhibitor of γ -secretase and DIF-1 (Fig. 4b). As expected, DAPT alone increased the levels of both APP CTFs, whereas DIF-1 alone decreased α CTF and β CTF levels (Fig. 4c). Co-treatment with DAPT and DIF-1 did not increase the total level of CTFs compared to DAPT alone (Fig. 4c). DAPT treatment led to a comparable increase in APP α CTF levels in the presence of DIF-1, but this was not the case for the β CTF. The level of presenilin N-terminal and C-terminal fragments was not affected by DIF-1 (data not shown). We then assessed whether DIF-1 alters BACE1 cleavage of APP using cells that stably overexpress both APP⁷⁵¹ and BACE1 (CHO-CAB). Increased BACE1 expression did not attenuate the effect of DIF-1 on mAPP, or total CTF levels and suggest that BACE1 activity is most likely not inhibited by DIF-1 (Fig. 4d, e, f). The above data suggest that DIF-1 might not affect γ -secretase function but potentially limits the available CTF substrate.

To determine whether the effect of DIF-1 on APP processing occurs as a result of a general disruption in metabolic processing of type I transmembrane proteins, we investigated its effect on two highly related proteins, APLP1 and APLP2 [33]. DIF-1 treated CHO cells that stably overexpress APLP2 showed a decrease in APLP2 CTF levels, without affecting APLP2 maturation (Fig. 4g). Comparatively, DIF-1 did not affect APLP1 maturation or CTF production in CHO cells that stably overexpress APLP1 (Fig. 4h). We then analyzed endogenous APP⁷⁵¹ processing in mouse embryonic fibroblasts (MEFs) to ensure that our results were not due to overexpression of APP. MEFs treated with DIF-1 reduced both mAPP and CTF levels (Fig. 4i). Collectively these findings suggest that the effect of DIF-1 on APP and APLP2 processing might involve a selective regulatory mechanism that does not affect APLP1 all type 1 transmembrane proteins.

3.4. DIF-1 reduces secretion of A β

To examine the effects of DIF-1 on the secretion of APP and A β , DIF-1 conditioned medium was analyzed by western blotting using three different anti-APP antibodies. DIF-1 reduced the level of secreted APP compared to controls (Fig. 5a). Western blotting and sandwich ELISA showed that DIF-1 decreased the level of A β secreted by CHO-7W cells (Fig. 5b). Treatment of CHO-CAB cells with increasing concentrations of DIF-1 revealed a dose-dependent reduction in mAPP, CTFs and A β levels (Fig. 5c, d). Fig. 5e illustrates the DIF-1 associated differential reduction in the levels of A β 40 (44.2 $\pm$ 3.4%) and A β 42 (66.9 $\pm$ 2.6%), demonstrating that treatment with DIF-1 effectively decreases A β secretion and the ratio of A β 42 to A β 40. Notably, DIF-1 reduced mAPP levels without affecting levels of immature APP. The above data show that DIF-1 decreases the secretion of both APP and A β .

3.5. APP phosphorylation at residue Thr668 is inhibited by DIF-1

DIF-1 dose-dependently decreased APP metabolism and A β generation with a concomitant increase in the percentage of cells in G0/G1. Interestingly, APP that is phosphorylated at

residue Thr668 appears to be a preferred substrate for amyloidogenic cleavage [34,35]. This observation prompted us to assess whether DIF-1 affects the level of APPT668 phosphorylation (Fig. 6). The basal level of pAPPT668 in each cell line was below the limits of detection using anti-pAPPT668 antibodies for western blotting (Fig. 6a, b). To address this issue, we used DAPT treatment of CHO-7W, CHO-CAB and H4APP^{Sw} cells to increase the total amount of APP CTFs. As expected, DAPT increased the level of detectable pAPPT668 in each of these cell types, (Fig. 6b–e). However, under these conditions we detect a large reduction ($88.7 \pm 3.16\%$) in pAPPT668 CTFs when cells are co-treated with DIF-1 (Fig. 6a, b, and f). This finding suggests the DIF-1-associated decrease in APP CTF levels is correlated with a decrease in Thr668 phosphorylation.

We have shown that while DIF-1 affects APP and APLP2 metabolism, it appears to have no effect on APLP1. To gain insight as to how DIF-1 might differentially affect the processing of these very similar protein, we aligned and examined the last 99 amino acids of APP, APLP2 and APLP1 and as can be seen in Fig. 6g, APP and APLP2 each contain a potential proline-directed phosphorylation site (Ser/Thr-Pro) [36,37] that includes residue Thr668, while APLP1 does not. This observation has led us to hypothesize that modulation of APP and APLP2 processing by DIF-1 is associated with the Thr668 residue, which precedes the proline residue in this motif (Thr668-Pro).

3.6. Mutation of APP Thr668 abolishes the effect of DIF-1 on APP processing

In order to determine the importance of the Thr668 residue with respect to the effect of DIF-1 on APP metabolism, we generated constructs that result in changes of the Thr668 residue to either Thr668A or Thr668E which would result in the production of APP molecules that cannot be phosphorylated at Thr668. These constructs were used to generate CHO cells that stably overexpress APP Thr668A and Thr668E. Clones that overexpress mutated APP at a level similar to that seen in CHO-7W cells were chosen for analysis. Surprisingly, DIF-1 had no effect on the metabolic processing of either APPT668A or APPT668E as compared to wild type APP⁷⁵¹. The APPT668E and APPT668A mutations abolished the effect of DIF-1 on APP maturation, CTF levels and total A β production as assessed by western blotting and sandwich ELISA (Fig. 7a–c). This result was surprising since the T668E mutation was expected to mimic a permanently phosphorylated form of APP, and suggests that a glutamic acid residue cannot compensate for the actual phosphorylation of Thr668 with respect to the mechanism of DIF-1 action on APP processing. Bioinformatic analysis of APP and APLP2 predicts that mutating residue Thr668 to any other amino acid (with the exception of serine) in this region no longer meets the criteria as a group IV WW-binding motif (Ser/Thr-Pro) [36,37] as is the case for APLP1.

3.7. The effects of DIF-1 on APP CTF levels are insensitive to GSK and cdk5 inhibitors

The proline-directed kinase GSK3 β and cell cycle-dependent kinase cdk5 have both been shown to phosphorylate APP at Thr668 [38,39]. Since DIF-1 reduces phosphorylation of Thr668 and reports suggest that DIF-1 might activate GSK3 β [25], we examined the effects of three structurally distinct GSK inhibitors (LiCl, kenpaullone and 1-azakenpaullone) on DIF-1 modulation of APP processing (Fig. 8a). LiCl (10 mM) treatments alone increased both α CTF and β CTF levels as compared to control cells, whereas LiCl and DIF-1 together resulted in only a slight increase in α CTF levels (Fig. 8a). In contrast, neither of the more potent and specific cell cycle/GSK inhibitors kenpaullone (10 μ M) or 1-azakenpaullone (10 μ M) [40] had an effect on APP processing (Fig. 8a). Furthermore, kenpaullone or 1-azakenpaullone did not attenuate or potentiate the effect of DIF-1 on APP processing (Fig. 8a). Thus; it appears that the effect of DIF-1 on APP processing is not likely due to increased GSK3 α/β activity. The effects of DIF-1 on APP processing compared with those of kenpaullone, 1-azakenpaullone and roscovitine which also affect cell cycle progression (G0/

G1, G2 and M phase) [40], suggests the effect of DIF-1 may be linked to G0/G1-associated metabolic pathways, but is not simply a result of the cell cycle phase.

3.8. DIF-1 prevents proteasome- and calpain-mediated accumulation of APP CTF levels

The group IV WW-protein interaction motif (Ser/Thr-Pro) has been shown to serve as a potential signaling module in the regulation of protein degradation *via* the proteasome [36,37] and the finding that T668A and T668E mutations abolish the effect of DIF-1, suggests that this domain is critical for the effect of DIF-1 on APP processing. To determine if the proteasome is involved in this process, CHO-CAB cells were pre-treated with two structurally different proteasome inhibitors: lactacystin (10 μ M) and Acetyl-L-Leucyl-L-Leucyl-L-Nor-leucinal (ALLN) (10 μ M). Western blotting was used to assess the effect of both inhibitors on APP processing, alone or combined with DIF-1 (Fig. 8b, c). As a positive control for proteasomal degradation, we also monitored protein levels of cyclin D1, which has been shown to be degraded by the proteasome [26] (Fig. 8b, c). As expected, cyclin D1 levels were increased when cells were treated with either lactacystin or ALLN. Proteasomal inhibition by lactacystin increased APP CTF levels compared to control cells (Fig. 8b). Interestingly, co-treatment with DIF-1 diminished the effect of lactacystin on APP CTF accumulation (Fig. 8b). However, even when cells were pre-treated with lactacystin, the addition of DIF-1 resulted in the reduction of APP CTF levels (Fig. 8b). We next assessed the effect of ALLN on the accumulation of APP CTFs, and as can be seen in Fig. 8c, low concentrations of ALLN (10 μ M) led to an accumulation of APP CTF levels. When cells were pre-treated with ALLN, the addition of DIF-1 resulted in a reduction of APP CTFs (Fig. 8c), consistent with our observation for effects of lactacystin. Taken together, these findings suggest that the effect of DIF-1 on APP processing is insensitive to proteasome- or calpain-inhibition.

4. Discussion

The normal function of APP has remained elusive since its' initial discovery in 1987 [2,3] and consequently, knowledge of the biology behind AD pathogenesis remains limited. The use of chemical biological tools provides another resource that will compliment existing models and may allow for the discovery of alternative pathways that regulate A β production. In this paper, we show that the small-molecule DIF-1 affectively modulates processing of intracellular APP through a proteasome- and calpain-insensitive pathway and that this process requires the C-terminal Thr668 residue. APP is phosphorylated at Thr668 under physiological and pathological conditions, but the mechanism and significance of this phosphorylation remain unclear.

Anti-proliferative effects of DIF-1 were observed in a variety of mammalian cell types. Extended treatment did not result in the arrest of all cells in G0/G1 suggesting that DIF-1 significantly slows progression through G0/G1 rather than acting as a cell cycle block. In addition to the effect on the cell cycle, DIF-1 reduces the levels of mature APP and of APP C-terminal fragments, but does not lead to an accumulation of or changes in the levels of immature APP. It would appear that treatment with DIF-1 may decrease the availability of mature APP or the resulting CTFs as a substrate for the α -, β - and γ -secretases. A reduced availability could be a result of DIF-1 effects on APP maturation through the secretory system or alternatively, DIF-1 could conceivably affect substrate recognition via Thr668 posttranslational modification. The striking reduction in APP phosphorylation at Thr668 supports this hypothesis; however, we cannot yet differentiate whether this mechanism is a direct or indirect effect of DIF-1 on APP processing. Future experiments are aimed at identifying the molecular targets that are bound by DIF-1. Still, this possibility is consistent with the observed reduction in secreted APP and substantial decrease in production of A β 40 and A β 42 that was observed following DIF-1 treatment. Of particular importance is the

effect that DIF-1 has on decreasing the ratio of A β 42 to A β 40, as A β 42 has been demonstrated to be more toxic than A β 40 (e.g., [9]).

DIF-1 treatment degrades cyclin D1 with a concomitant decrease in APP processing. Neurons in the developed brain are typically maintained in a differentiated state (G0/G1) that prevents their ability to migrate or proliferate. However, controlled mitogenic signaling may allow neuronal cells to re-enter the G1 phase of the cell cycle in order to maintain synaptic remodeling and neuronal plasticity [41]. Altered cyclin D1 levels in AD brain could conceivably be a consequence of aberrant cycling associated with age-dependent, environmentally acquired proteasomal defects in late-onset AD. Of particular importance is the accumulating evidence that suggests both early and late-onset AD pathology display an increase in the expression of cell cycle proteins [12,14]. Furthermore, transgenic mouse models also show evidence of cell cycle re-entry prior to the development of senile plaques [15]. The reason for increased, localized levels of a number of specific cell cycle proteins in AD brains remains unclear. We believe that our findings set the stage for the use of DIF-1 as a molecular probe to further elucidate cell cycle-dependent signaling mechanisms that regulate amyloidogenic processing of APP in mammalian systems. Of particular importance is the consistent DIF-1 mediated reduction in APP catabolism seen in a variety of cell types. It is tempting to speculate that cells contain a conserved target (or targets) of DIF-1 that modulate amyloidogenic processing of APP in a manner that utilizes G0/G1-associated signaling pathways.

Cellular proteasomal machinery is tightly regulated. Even minor alterations can result in abnormal protein expression that lead to malignancies, inflammation and neurodegenerative disease [42]. The downregulation of ubiquitin 1 increases APP trafficking into the secretory system and elevates A β [43]. Conversely, the modifier of cell adhesion protein (MOCA) reduces APP metabolism by directing APP molecules away from the secretory system [44]. Our findings that inhibition of the proteasome led to increases in both cyclin D1 and APP CTF levels are consistent with previous reports [26,42,44]. Interestingly, we have found that treatment of cells with DIF-1 in combination with lactacystin or ALLN attenuates the proteasome inhibitor associated accumulation of APP CTFs. Our data suggests that the effect of DIF-1 in modulating processing of APP occurs through a proteasome- and calpain-insensitive pathway. Alternatively, the effects of DIF-1 could precede these modes of protein degradation which would in effect prevent the accumulation of CTFs. This scenario would suggest alternative, non-amyloidogenic degradation pathways for APP, and as such we are currently exploring the likelihood of this possibility. Preliminary western blot analysis has shown that DIF-1 effects on APP processing are insensitive to lysosomal inhibition using NH₄Cl (unpublished data).

Considering that healthy neurons are post-mitotically blocked, the consequence of APP phosphorylation at residue Thr668 in AD pathology currently remains a matter of debate. The DIF-1 induced decrease in pAPPT668 is interesting since the cell cycle-dependent nature of this phosphorylation alludes to a specific role in APP catabolism. Notably, the alteration of the APPT668 residue (APPT668A and APPT668E) abolishes the effect of DIF-1 on APP processing. One possibility is that DIF-1 differently affects the processing of unphosphorylated and phosphorylated APP C-terminal fragments. However, this seems unlikely since the low level of detectable Thr668 phosphorylation would impede detection of the DIF-induced decrease of CTF levels by western blot. Recent *in vivo* results suggest that the APP T668A mutation in knock-in mice does not modify APP processing [45]. Although the limitations of this prior study do not allow it to address the role of pThr668 in AD pathogenesis our findings are in agreement with these results. The cells used in our study do not represent a pathological state equivalent to AD. It would appear that the modulating effect of DIF-1 on APP processing could be a result of the Thr668 residue itself

where it may serve as a critical signal within the context of the surrounding residues in APP. If so, transient posttranslational modifications that occur during this window of the cell cycle could have significant physiological effects on APP catabolism and be difficult to identify in cycling cells. Flux through signaling pathways is controlled, in large part, by regulated protein degradation and reversible serine or threonine phosphorylation. In some cases, negatively charged amino acids such as aspartate or glutamate do not substitute for phosphorylated residues to positively regulate receptor ubiquitination and internalization [46].

In summary, we have presented evidence that treatment of cultured cells with the small-molecule DIF-1 results in reduced levels of APP C-terminal fragments, and that this effect requires the presence of residue Thr668 which has previously been shown to undergo phosphorylation. Our data is consistent with previous reports that suggest a role for the Ser/Thr-Pro motif in maintaining an intricate balance of protein expression within specific phases of the cell cycle. The mechanism of DIF-1 in modulating APP processing involves G0/G1-associated signaling pathways that are proteasome- and calpain-insensitive. As such, aberrant expression of cell cycle proteins such as cyclin D1 may serve as markers for dysregulated G0/G1 signaling pathways and the production of A β . Our findings further suggest that *D. discoideum* is a potential resource for unique molecules with biomedical relevance.

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Abbreviations

Aβ	amyloid beta peptide
AD	Alzheimer's disease
ALLN	Acetyl-L-Leucyl-L-Leucyl-L-Norleucinal
APP	amyloid- β protein precursor
APLP1	amyloid precursor-like protein 1
APLP2	amyloid precursor-like protein 2
αCTF (C83)	C-terminal fragment of APP produced by α -cleavage
βCTF (C99)	C-terminal fragment of APP produced by β -cleavage
DAPT	(N-[N-(3,5-difluorophenacetyl-L-alanyl)]-S-phe-nylglycine tert-butyl ester)
DIF-1	(Differentiation-Inducing Factor-1) (1-[3, 5-dichloro-2, 6-dihydroxy-4-methoxyphenyl]-1 hexanone)
MEF	mouse embryonic fibroblast

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Fig. 1. Chemical structure of DIF-1. Differentiation-Inducing Factor-1 (DIF-1) (1-[3, 5-dichloro-2, 6-dihydroxy-4-methoxyphenyl]-1-hexanone) is a chlorinated alkyl phenone, isolated from the social amoeba *Dictyostelium discoideum*.

Fig. 2.

Cytostatic effect of DIF-1 on naïve and CHO APP⁷⁵¹ cells. (a) Naïve CHO cells were incubated with 25 μ M DIF-1 for the time period indicated and then counted with a hemocytometer. Results shown represent the average increase of cells/mL $\pm$ S.D. over a four day period. *Black*—untreated, *dotted*—vehicle (ethanol), *Green*—DIF-1, *Red*—washed cells. (b) The mean cell cycle distribution ($\pm$ S.D.) for vehicle and DIF-1 treated cells was measured using flow cytometry. The average percentage of cells in G0/G1, S-phase and G2/M from 3 independent experiments is shown below each histogram. (c) CHO-7W cells stably expressing human APP⁷⁵¹ were incubated with 1, 10 or 25 μ M DIF-1 for the period indicated and then counted with a hemocytometer. *Black*—untreated, *Purple*—vehicle (ethanol), *Green*—1 μ M DIF-1, *Grey*—10 μ M DIF-1, *Red*—25 μ M DIF-1, *Blue*—washed cells. Results shown represent the average increase of cells/mL $\pm$ S.D. over a four day period. (d) The mean cell cycle distribution ($\pm$ S.D.) for vehicle treated cells expressing human APP⁷⁵¹ was measured using flow cytometry. The average percentage of cells in G0/G1, S-phase and G2/M from 3 independent experiments is shown below each histogram. In each case, a two-tailed paired *t* test was performed from three independent experiments ($n=3$ for each experiment) and compared against vehicle treated cells. One asterisk represents a *P* value < 0.008 , two asterisks represent $P < 0.005$ and three asterisks represent $P < 0.001$. (e) Naïve mouse N2a cells were deposited in 6-well plates and incubated with 25 μ M DIF-1 or vehicle for the time period indicated and then counted with a hemocytometer. (f) Naïve human SH-SY5Y cells were deposited as described above and incubated with 25 μ M DIF-1 or vehicle. *Black*—untreated, *Hatched line*—vehicle, *Red*—25 μ M DIF-1. The results shown in (e) and (f) represent the mean $\pm$ S.D. of 3 separate experiments. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Fig. 3.

Reduced expression of cyclin D1 in CHO-7W cells treated with DIF-1. (a) CHO-7W cells were treated for 18–20 h with vehicles (DMSO or ethanol), DIF-1 (30 μ M), DAPT (250 nM) or DIF-1 and DAPT together. Fifteen micrograms of total protein was loaded per lane. Cyclin D1 and GAPDH levels were detected by western blot. This blot is representative of at least 3 separate experiments. (b) The level of cyclin D1 expression was normalized to GAPDH levels. DIF-1 reduces expression of cyclin D1 in all cells tested on average $82.41 \pm 0.74\%$. *Black bar*—Non-treated cells; *Grey bar*—Vehicle-treated cells; *White bar*—DIF-1 treated cells. In each case, a two-tailed paired *t* test was performed from three independent experiments ($n=3$) for all cell types tested and compared to vehicle treated cells. One asterisk represents a *P* value <0.0003 .

Fig. 4.

DIF-1 reduces APP⁷⁵¹ maturation and the production of α CTF and β CTF levels in a variety of cell types. (a) CHO-7W cells were treated with 30 μ M DIF-1 and the level of mature APP was detected. Lane 1 represents endogenous APP and CTFs levels. Lane 2 shows the level of APP α CTF, and β CTF expression in CHO-7W cells. Lane 3 represents the vehicle control. DIF-1 significantly decreases the levels of mature APP, α CTF and β CTF levels. (b) CHO-7W cells were treated with 250 nM DAPT. Lane 1—non-treated cells, lane 2—DMSO, lane 3 equals 250 nM DAPT. (c) Cells treated with DIF-1 (lane 2). Lane 4—DIF-1 in the presence of DAPT. (d) CHO-CAB cells were treated as previously described. Lane 4—DIF-1 treated cells. (e) CHO-CAB cells treated with DAPT. (f) Co-treatment with DAPT and DIF-1. (g) Analysis of DIF-1 on CHO cells stably expressing APLP2. No obvious trend is observed regarding APLP2 maturation. However, co-treatment with DAPT and DIF-1 greatly reduced the low molecular weight APLP2 CTF. (h) Analysis of DIF-1 on CHO cells stably expressing human APLP1. DIF-1 had no effect on maturation or production of APLP1 CTFs. (i) Effects of DIF-1 on endogenous APP in mouse embryonic fibroblasts (MEF). Non- treated (lanes 1 and 2), vehicle treated (lanes 3 and 4) and DIF-1 treated cells (lanes 5 and 6) are shown. DIF-1 substantially reduces maturation of endogenous APP and CTF levels. Molecular weight markers are shown *on the left*. GAPDH loading controls are shown for each panel. These results are representative of five independent experiments to ensure the reproducibility of DIF-1 on APP processing.

Fig. 5.

DIF-1 reduces secretion of APP and inhibits A β production. (a) CHO-7W cells were treated with DIF-1 as previously described. Conditioned media was analyzed for secreted APP using three separate anti-APP N-terminus antibodies as indicated on the right of the panel. Molecular weight markers are shown *on the left*. DIF-1 (30 μ M) reduces the level of total secreted APP. (b) Western blot analysis of total secreted A β in CHO-7W conditioned media. A β was detected using the monoclonal anti-APP antibody 6E10. Vehicle-treated cells secrete levels of A β similar to non-treated cells. DIF-1 (30 μ M) significantly reduces the levels of A β in conditioned media. Loading controls for A β are shown (1000, 500, 250 and 125 pg/mL). (c) Levels of A β 40 and A β 42 were measured by sandwich ELISA. The graph represents the percent average reduction of A β ($\pm$ S.D.) ($n=3$) compared to controls. *Black bars* = non-treated cells, *grey bars* = vehicle and *white bars* = DIF-1 treated CHO-7W cells. A two-tailed paired t test was used to assess significance. One asterisk represents a P value <0.003 and two asterisks represent a P value <0.001 . (d) Extracts from CHO-CAB cells treated with vehicle (1st lane) or increasing concentrations of DIF-1 (1, 5, 10, 20 and 30 μ M) were assessed by western blot using antibodies to APP (e) The levels of secreted A β 40 and A β 42 in conditioned media from CHO-CAB cells were measured by sandwich ELISA. A two-tailed paired t test was used to assess significance. One asterisk represents a P value <0.003 , two asterisks represent a P value <0.001 , and three asterisks represent a P value <0.0005 . Results represent 3 separate experiments ($n=3$) measured in triplicate.

Fig. 6. DIF-1 reduces phosphorylation of APP⁷⁵¹ at threonine 668. Panels a through e represent different cell lines with equal amounts of total protein (15 μ g) loaded as follows: lane 1=untreated, lane 2=DMSO vehicle control (DAPT), lane 3=250 nM DAPT, lane 4=ethanol vehicle control (DIF-1), lane 5=30 μ M DIF-1, and lane 6=250 nM DAPT and 30 μ M DIF-1. Cell lines treated are indicated above the panel. Western blots were carried out with an anti-APP antibody (C66) (1:5000) (a) or an anti-pAPPT668 (1:1000) (b–e). All panels are representative of three independent experiments. (f) The average percent reduction of pCTFs in all four cell lines was calculated from all treatments ($n=12$). Statistical analysis was performed using a two-tailed paired t test. One asterisk represents a P value < 0.001 (g). Multiple sequence alignment was used to compare the last 47 shared residues between APP, APLP2 and APLP1. The predicted proline-directed group IV WW phosphorylation-dependent interaction motif (S/TP) is highlighted in grey. This motif is not found in APLP1, highlighted in dark grey. Molecular weight markers are shown on the left. GAPDH loading control levels are shown below each blot.

Fig. 7.

Site-directed mutation of APP threonine 668 to either alanine or glutamic acid abolishes the effects of DIF-1 on APP metabolism. CHO cells stably expressing APP⁷⁵¹ where the threonine 668 has been mutated to (a) glutamic acid (APPT668E) or (b) to alanine (APPT668A) were treated with appropriate vehicle (DMSO or ethanol), or with DAPT (250 nM), DIF-1 (30 μ M) or DAPT and DIF-1 combined. Protein extracts were assessed for expression of full length APP and APP CTFs by western blot analysis. GAPDH was used as a loading control and molecular weight markers are shown *on the left*. Extracts from naïve cells, expressing endogenous levels of APP are shown in the first lane as indicated, and levels of stably produced APPT668E or APPT668A are shown in the control lanes. DAPT induced the accumulation of APPT668E and APPT668A CTF levels, however, DIF-1 no longer reduced maturation of this form of APP or reduced the levels of APPT668E-derived or APPT668A-derived α CTF and β CTF. These blots are representative of three individual experiments. (c) The levels of secreted A β ₄₀ and A β ₄₂ (pg/mL) were measured in triplicate in conditioned media from APPT668A and APPT668E cell lines by sandwich ELISA. No detectable differences in total A β production were detected compared to CHO-7W cells ($n=3$). The levels of A β for all three cell lines were normalized to the level detected in naïve CHO cells. *Black bars* represent CHO-7W cells, *grey bars* represent T668A cells and *white bars* represent T668E cells.

Fig. 8.

The effect of cell cycle/GSK kinase inhibitors and proteasome inhibition on the DIF-1 dependent reduction in APP processing. (a) Three specific GSK kinase inhibitors (LiCl, kenpaullone and 1-azakenpaullone) were assessed for their ability to inhibit APP processing in the presence or absence of DIF-1, as indicated above the panel. CHO-CAB cells treated with lithium chloride (10 mM) caused an increase in APP CTFs, whereas kenpaullone and 1-azakenpaullone (10 μ M) had no effect on APP processing. Neither inhibitor could restore APP CTF levels in the presence of DIF-1. The Cdk5 inhibitor roscovitine (10 μ M) was used as a control to ensure APP processing by LiCl, kenpaullone or 1-azakenpaullone was not a result of non-specific inhibition of Cdk5. (b) The effect of proteasome inhibitors on DIF-1 processing of APP. Treatment with lactacystin (10 μ M) induced an increase in α CTF and β CTF levels. DIF-1 (30 μ M) greatly reduced the effect of lactacystin. (c) The effect of a second proteasome inhibitor, ALLN, on APP processing. ALLN (10 μ M) also induced an increase in CTF levels compared to vehicle treated cells. Co-treatment with DIF-1 reduced the effect of ALLN on APP processing. To ensure that the results observed were due to proteasome inhibition, we assessed the effects of lactacystin and ALLN on cyclin D1 levels. Cyclin D1 is targeted for degradation via ubiquitin-mediated 26S proteasome activity. Molecular Weight markers (kDa) are shown *on the left*. GAPDH levels are shown *below* each blot. The data represents that of three separate experiments.