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GSK-3 inhibition by adenoviral FRAT1 overexpression is neuroprotective and induces Tau dephosphorylation and β-catenin stabilisation without elevation of glycogen synthase activity

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Abstract Glycogen synthase kinase 3 (GSK-3) has previously been shown to play an important role in the regulation of apoptosis. However, the nature of GSK-3 effector pathways that are relevant to neuroprotection remains poorly defined. Here, we have compared neuroprotection resulting from modulation of GSK-3 activity in PC12 cells using either selective small molecule ATP-competitive GSK-3 inhibitors (SB-216763 and SB-415286), or adenovirus overexpressing frequently rearranged in advanced T-cell lymphomas 1 (FRAT1), a protein proposed as a negative regulator of GSK-3 activity towards Axin and β-catenin. Our data demonstrate that cellular overexpression of FRAT1 is sufficient to confer neuroprotection and correlates with inhibition of GSK-3 activity towards Tau and β-catenin, but not modulation of glycogen synthase (GS) activity. By comparison, treatment with SB-216763 and SB-415286 proved more potent in terms of neuroprotection, and correlated with inhibition of GSK-3 activity towards GS in addition to Tau and β-catenin.

Key words: Glycogen synthase kinase-3; FRAT1; Neuroprotection; Tau; β-Catenin; PC12 cell

1. Introduction

Glycogen synthase kinase-3 (GSK-3) is a ubiquitously expressed serine/threonine protein kinase which was discovered by its ability to phosphorylate and inactivate glycogen synthase (GS) [1–5]. It is clear, however, that GSK-3 is important not only in the regulation of glycogen synthesis, but also modulates several other cellular processes including apoptosis (reviewed in [6]). Pharmacological analysis of GSK-3 activity has been possible through the use of lithium, a relatively specific but weak inhibitor of GSK-3. Recently we have demonstrated that ATP-competitive and selective small molecule GSK-3 inhibitors, SB-216763 and SB-415286 [7], protect primary neurones from cell death [8]. This has strengthened our understanding of the role of GSK-3 in metabolism and neuronal death, but as general inhibitors of all GSK-3 activities, functions of specific effector pathways in such cellular processes are not addressed by such routes.

GSK-3 is active in resting cells, but its activity is physiologically inhibited by two distinct signalling pathways. Insulin and/or growth factors stimulate a relatively well characterised phosphatidylinositide 3-kinase/protein kinase B-dependent pathway that results in inhibition of GSK-3 activity by phosphorylation of specific N-terminal serine residues (Ser-9 in GSK-3β and Ser-21 in GSK-3α [9]). However, GSK-3 can also be inhibited in response to Wnt signalling, in which a role for frequently rearranged in advanced T-cell lymphomas 1 (FRAT1) has been implicated. Wnt molecules control numerous developmental processes by altering specific gene expression patterns [10], and deregulation of Wnt signalling can contribute to tumorigenesis [11,12]. Wnt signalling during embryogenesis inhibits GSK-3-catalysed phosphorylation of β-catenin. Since phosphorylated β-catenin is targeted for ubiquitin-dependent proteolysis, GSK-3 inhibition results in accumulation of β-catenin in the nucleus where it regulates gene expression via interactions with the TCF/LEF family of transcription factors (reviewed in [13]).

It is presently unclear how Wnt signalling results in an inhibition of GSK-3 towards β-catenin, but it appears to be independent of protein kinase B, and instead involves the regulation of a signalling complex which includes Dishevelled (Dvl), FRAT1, GSK-3, Axin and β-catenin. Axin, itself a substrate of GSK-3 [14], acts as a scaffold to facilitate the phosphorylation of β-catenin by GSK-3. Axin also interacts with Dvl to form a quaternary complex. [15–19]. Wnt-induced changes to the signalling complex cause dissociation of the GSK-3/Axin/β-catenin complex, and hence prevent GSK-3-mediated phosphorylation of β-catenin, resulting in β-catenin stabilisation. Precisely how this occurs is still unclear. However, GSK-3 binding protein (GBP)/FRAT1 has been implicated.

GBP was first identified in *Xenopus* as a protein that inhibits GSK-3 in vivo, appearing to act as a positive regulator of the Wnt signalling pathway by stabilising β-catenin [20]. Further studies performed in *Xenopus* showed that GBP inhibited GSK-3 activity towards β-catenin, at least in part, by preventing Axin binding to GSK-3 [21]. GBP is homologous to the mammalian FRAT1 protein, which may co-operate with oncogenes to promote tumorigenesis in T-cells [22]. Transfection

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studies in mammalian cells have confirmed the role of FRAT1 in the stabilisation of β-catenin and have shown the presence of FRAT1 in complexes with Dvl, GSK-3 and Axin [23]. Taken together, these findings suggest that Wnt signalling causes a recruitment of FRAT1 into such complexes, leading to FRAT1-mediated dissociation of GSK-3 from Axin.

A 39-residue peptide from the C-terminus of FRAT1, termed FRATtide, has been shown to be sufficient to bind GSK-3 and prevent binding of Axin in vitro [24]. In addition, FRATtide was also shown to selectively inhibit phosphorylation of Axin and β-catenin in vitro, but did not inhibit GSK-3 activity towards peptides derived from eIF2B or GS. The selective nature of FRAT/GBP-mediated inhibition of GSK-3 activity is also supported by the observation that GBP complexed with GSK-3 isolated from *Xenopus* oocytes does not inhibit phosphorylation of a CREB-derived peptide substrate by GSK-3 [21]. Hence FRAT1 provides a potentially useful tool to discriminate between substrates of GSK-3 that may be important in the regulation of apoptosis. By contrast, ATP-competitive compounds are likely to act against all substrates of GSK-3, and so do not facilitate the dissection of neuroprotective GSK-3 effector pathways.

Here, we have used binding assays performed in vitro to define the affinity of FRATtide for GSK-3 and have used such assays to investigate the possibility of overlap between binding of FRATtide and ATP-competitive small molecule selective GSK-3 inhibitors. To investigate the cellular effects of both methods of GSK-3 inhibition, we compared the neuroprotective properties of treatment with ATP-competitive GSK-3 inhibitors and adenoviral FRAT1 overexpression in PC12 cells. Recombinant adenoviral vectors were used rather than microinjection or conventional cell transfection, since the efficiency of adenoviral infection facilitates biochemical analysis of the selective nature of GSK-3 inhibition by FRAT1. Consequently, the effects on substrates of GSK-3 that may play a role in the regulation of apoptosis (namely β-catenin, Tau and GS) were examined, and correlated to neuroprotection.

2. Materials and methods

2.1. Fluorescence binding assay for FRATtide/GSK-3 interactions in vitro

A binding assay using FRATtide labelled by TAMRA-X-SE (Molecular Probes) on the N-terminal amine group (TAMRA-FRATtide) was employed to investigate FRATtide binding to GSK-3. TAMRA-FRATtide (10 nM) was incubated with varying concentrations of GSK-3β to determine binding affinity. To determine if ATP-competitive small molecule GSK-3 inhibitors or GSK-3 interacting domain (GID, a 25-amino acid sequence in Axin that binds GSK-3) peptide were antagonists of the GSK-3/FRATtide interaction, GSK-3β (120 nM) and TAMRA-FRATtide (10 nM) were incubated with a series of concentrations of SB-415286 or SB-216763 or increasing concentrations of GID peptide. All experiments were performed in a buffer composed of 50 mM HEPES, pH 7.5, 1 mM CHAPS, 10 mM $MgCl_2$, and in a final volume of 10 μl. The fluorescence anisotropy of the TAMRA-FRATtide was measured using a commercial plate reader (LJL Acquest).

2.2. Generation of recombinant adenovirus

A full-length human FRAT1 cDNA tagged at the N-terminus with the myc epitope was generated by RT-PCR and verified by sequence analysis. This cDNA was cloned into the shuttle vector pAdTrack-CMV to generate recombinant adenoviruses using the AdEasy system as described previously [25]. Adenoviruses made in this way overexpress green fluorescent protein in addition to the gene of interest. Hence, two adenoviruses were made: AdFRAT, which overexpresses both myc-tagged FRAT1 and green fluorescent protein (GFP), and as a control for adenoviral infection, AdGFP, which overexpresses GFP alone. Adenoviral titres from high-titre CsCl gradient-purified preparations were determined by plaque assay on HEK293 cells.

2.3. Culture and infection of PC12 cells

PC12 cells were cultured in DMEM (Gibco, high glucose) containing 10% heat-inactivated foetal bovine serum, 5% heat-inactivated horse serum and 2 mM glutamine. Cells were routinely infected at a multiplicity of infection (MOI) of 50 which resulted in the infection of the vast majority of cells ($>90\%$, as assessed by GFP expression 32 h after infection). In agreement with other reports of PC12 infection with adenovirus [26], this MOI did not result in toxicity as measured by cell death assays below (data not shown). For all studies, cells were infected with either AdFRAT or AdGFP in a minimal volume of medium for 1 h at 37°C. Growth medium was then added to increase the volume, and cells were left for approximately 32 h before further treatments.

For cell death assays, cells were placed into medium containing LY-294002 (50 μM) in the presence or absence of SB-415286 (30 μM) and SB-216763 (3 μM) [12]. Cell death was then measured 24 h after treatment with compounds.

For GS assays and immunoblotting studies, cells were placed into serum-free medium for 2 h before treatment with SB-415286 (30 μM) or SB-216763 (3 μM). Lysates were prepared after 1.5 h and 4 h of compound treatment for GS assays and immunoblotting studies respectively. Control cells were treated with dimethylsulfoxide (DMSO). DMSO concentrations did not exceed 0.1%.

2.4. Cell death assays

PC12 cells were seeded into wells of collagen I-coated 48-well plates at a density of 1.5×10^5 cells per well. Cells were infected as described in Section 2.3. Apoptosis was measured using an ELISA that detects the enrichment of mono- and oligonucleosomes that occurs in apoptotic cells (Roche). Data are expressed relative to the apoptosis observed on LY-294002 treatment of cells infected with AdGFP.

2.5. Immunoblotting

PC12 cells were seeded into wells of collagen I-coated 6-well plates at a density of 1×10^6 cells per well. Cells were infected and treated as described in Section 2.3, and harvested by washing in ice-cold phosphate-buffered saline prior to scraping and lysis (for Tau immunoblots, lysis buffer contained: 25 mM Tris–HCl, 3 mM EDTA, 3 mM EGTA, 50 mM NaF, 2 mM sodium orthovanadate, 0.27 M sucrose, 10 mM sodium β-glycerophosphate, 5 mM sodium pyrophosphate, 0.5% (v/v) Triton X-100, 0.1% (v/v) β-mercaptoethanol, Boehringer 'Complete' protease inhibitors, pH 7.5; Boehringer, Lewes, UK; for immunoblots of cytoplasmic β-catenin, hypotonic lysis buffer contained; 50 mM Tris–HCl, 3 mM EDTA, 3 mM EGTA, 0.5 mM NaF, 1 mM sodium orthovanadate, 10 mM β-glycerophosphate, 5 mM sodium pyrophosphate, 0.1% (v/v) β-mercaptoethanol, Boehringer 'Complete' protease inhibitors, pH 7.5). Cell lysates were centrifuged at $15\,000\times g$ for 15 min at 4°C. Equal amounts of protein lysate were subjected to sodium dodecylsulphate polyacrylamide gel electrophoresis, electrophoretically transferred to nitrocellulose membranes and immunoblotted for: myc-tagged FRAT1 (clone 9E10; Autogen Bioclear, Calne, UK), β-tubulin (Autogen Bioclear), Tau phosphorylated on Ser-396 and Ser-404 (clone AD2 [27]), Tau dephosphorylated on the epitope Ser-189–206 (clone Tau-1; Boehringer Mannheim) and β-catenin (Transduction Laboratories, Oxford, UK). Blots were developed using an Enhanced Chemiluminescence kit (Amersham-Pharmacia, Little Chalfont, UK).

2.6. GS assays

PC12 cells were seeded into 10-cm collagen coated dishes at a density of 5×10^6 cells per dish. Cells were infected and treated as described in Section 2.3, and harvested by lysis as detailed above for preparation of lysates for immunoblotting to detect Tau phosphorylation. Lysates were assayed for GS activity in buffer (67 mM Tris–HCl pH 7.5, 5 mM dithiothreitol, 6.7 mM EDTA, 13 mg/ml glycogen, 8.9 mM [^{14}C]UDP-glucose) in the presence or absence of 20 mM glucose 6-phosphate as described previously [28]. Data are expressed as GS activity ratios.

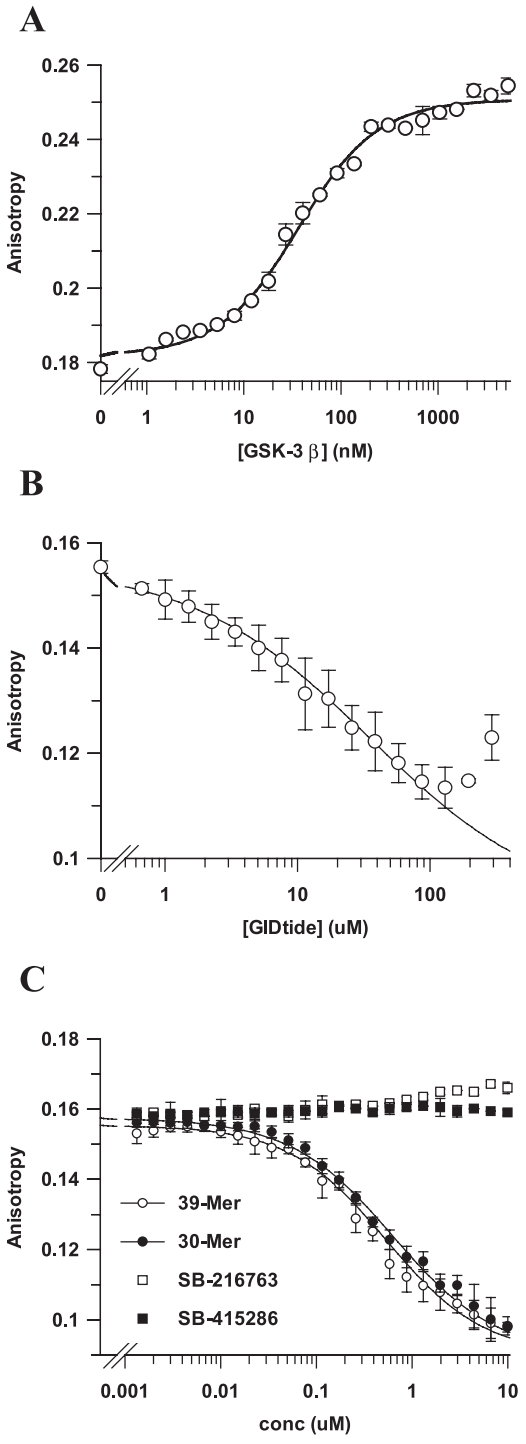

Fig. 1. Fluorescence anisotropy measurements of FRATtide/GSK-3 interactions. A: Anisotropy of TAMRA-labelled FRATtide (39-mer) was measured in the presence of increasing concentrations of GSK-3. The anisotropy of peptide increases on association with larger protein due to the slower rotation rate of the complex. B: Anisotropy of TAMRA-labelled FRATtide (39-mer) bound to GSK-3 was measured in the presence of increasing concentrations of unlabelled GID to determine whether GID could displace TAMRA-labelled FRATtide binding. The decrease in anisotropy observed with increasing concentrations of GID indicates displacement of TAMRA-labelled FRATtide. Increases in anisotropy at high concentrations of GID peptide are due to scattering caused by GID insolubility. C: Anisotropy of TAMRA-labelled FRATtide (39-mer) bound to GSK-3 was measured in the presence of increasing concentrations of unlabelled 39-mer FRATtide, unlabelled 30-mer FRATtide (lacking nine amino acids from the N-terminus) and ATP-competitive small molecule inhibitors. The decrease in anisotropy observed with increasing concentrations of unlabelled 39-mer and 30-mer indicate displacement of TAMRA-labelled FRATtide. Increasing concentrations of ATP-competitive small molecule inhibitors did not have any effect on anisotropy, indicating a lack of displacement of FRATtide.
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3. Results

3.1. FRATtide binding to GSK-3 is displaced by GID but not by ATP-competitive small molecule inhibitors SB-415286 and SB-216763

Studies performed in vitro using FRATtide have shown that this peptide binds GSK-3 and is sufficient to prevent binding of Axin [24]. However, the affinity of such interactions is not known. To this end, we developed a fluorescence-based ligand displacement assay of peptide binding to GSK-3 (Fig. 1). FRATtide was labelled with TAMRA on the N-terminal amine and the increase in fluorescence anisotropy upon binding to GSK-3 was monitored. These measurements revealed that FRATtide bound GSK-3 with a K_d of 26 ± 12 nM (Fig. 1A). Displacement of the labelled 39-mer FRATtide by unlabelled 39-mer (K_d 39 ± 7 nM) or a 30-mer that lacks nine N-terminal residues (K_d 52 ± 6 nM) indicated that fluorophore had not greatly affected binding and the essential interactions were preserved in the shorter peptide (Fig. 1C). Moreover, it was demonstrated that GID peptide [29] (K_d 3.2 ± 1.1 μM) could displace labelled 39-mer FRATtide, indicating mutually exclusive binding sites (Fig. 1B). In addition, FRATtide binding to GSK-3 was not displaced by ATP-competitive compounds, providing evidence that the mechanisms by which ATP-competitive compounds and FRAT1 inhibit GSK-3 activity differ (Fig. 1C).

3.2. Adenoviral FRAT1 overexpression or treatment with selective GSK-3 inhibitors (SB-216763 and SB-415286) is neuroprotective

PC12 cells infected with AdFRAT or AdGFP were induced to undergo apoptosis by treatment with LY-294002 (50 μM). Cells were also treated with or without SB-415286 (30 μM) and SB-216763 (3 μM). These concentrations have previously been found to give maximal inhibition of GSK-3 activity as assessed by GSK-3 activity assays, and assays of neuroprotection and β-catenin-dependent gene transcription [7,8]. Apoptosis was measured using an ELISA that detects the enrichment of mono- and oligonucleosomes occurring in apoptotic cells (Fig. 2). ELISA readings obtained from cells that had been induced to undergo apoptosis by LY-294002 treatment were denoted as maximal levels of apoptosis, and hence readings were fixed at 100%. Basal levels of apoptosis were typically found to be ~25% of this maximum. In agreement with previous findings [8], treatment of cells with small molecule ATP-competitive GSK-3 inhibitors protected cells from apoptosis induced by LY-294002. SB-415286 (30 μM) was observed to inhibit apoptosis to approximately basal or sub-basal levels of apoptosis, and treatment of cells with SB-216763 (3 μM) was observed to inhibit apoptosis to approximately 30–40% of maximum. Overexpression of recombinant adenoviral FRAT1 in cells in the absence of ATP-competitive inhibitors resulted in an inhibition of LY-294002-induced apoptosis to ~60% of maximum. These experiments were per-

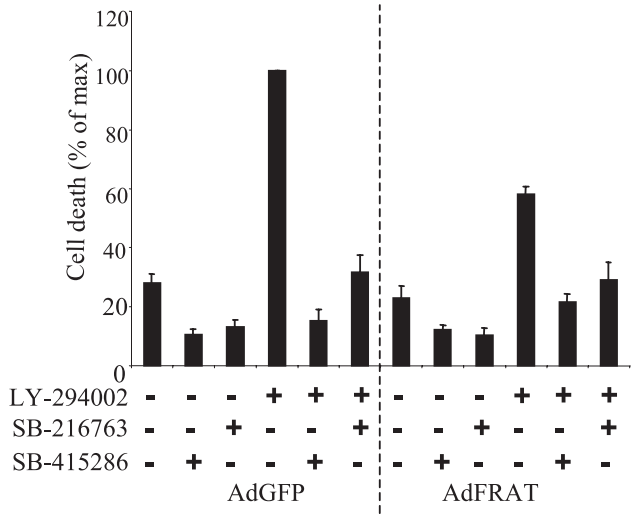

Fig. 2. SB-415286, SB-216763 or adenoviral FRAT1 overexpression protect PC12 cells from apoptosis induced by treatment with LY-294002. Cells were infected with AdGFP or AdFRAT (MOI 50). Cell death was induced by treatment with LY-294002 (50 μM) in the presence or absence of SB-415286 (30 μM) or SB-216763 (3 μM). Cell death was quantified 24 h after compound treatment using an ELISA that detects the enrichment of histone-associated DNA fragments in apoptotic cells. Data are expressed relative to the maximum levels of apoptosis observed in GFP-expressing cells treated with LY-294002 in the absence of GSK-3 inhibitors, which was defined as 100% (±S.E.M., $n=4$).

formed using an MOI of 50, which resulted in the vast majority of cells (>90%) overexpressing FRAT1, and increasing the MOI did not result in a greater inhibition of apoptosis (data not shown). Hence, while FRAT1 overexpression was observed to be neuroprotective, both small molecule inhibitors proved more potent in terms of neuroprotection.

3.3. *Adenoviral FRAT1 overexpression or treatment with selective GSK-3 inhibitors (SB-216763 and SB-415286) results in cytoplasmic β-catenin stabilisation and dephosphorylation of Tau*

As substrates of GSK-3, β-catenin and Tau have been speculated to play a role in the regulation of apoptosis. We decided therefore to examine whether treatment with selective small molecule ATP-competitive inhibitors or adenoviral FRAT1 overexpression modulated cytoplasmic β-catenin stabilisation and Tau phosphorylation with a view to correlating such effects to neuroprotection.

FRAT1 overexpression in mammalian cells has previously been shown to lead to LEF-1-dependent transcription through stabilisation of β-catenin levels [23]. In agreement with these findings, adenoviral-mediated FRAT1 overexpression in PC12 cells resulted in elevated endogenous cytoplasmic β-catenin levels compared to cells that had been transfected with adenovirus expressing GFP only (Fig. 3A). Treatment of cells with SB-415286 or SB-216763 also stabilised cytoplasmic β-catenin levels, consistent with GSK-3 inhibition [7,8].

We next determined whether adenoviral FRAT1 overexpression would result in Tau dephosphorylation. Two epitopes in endogenous Tau present in PC12 cells were examined by Western blotting with phosphospecific antibodies. AD2 antibody recognises phosphoserine 396 and phosphoserine 404 [27]. Tau-1 antibody recognises specifically dephosphory-

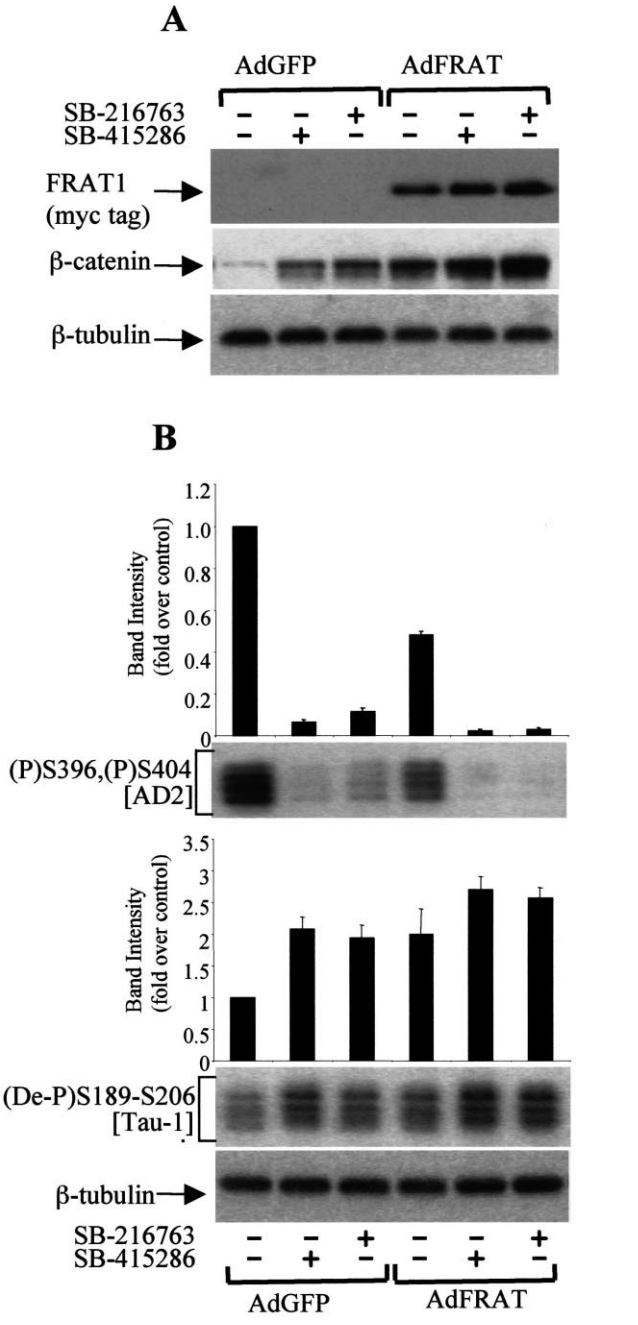

Fig. 3. SB-415286, SB-216763 or adenoviral FRAT1 overexpression stabilises endogenous cytoplasmic β-catenin and results in Tau dephosphorylation. A: Cells infected with AdGFP or AdFRAT were serum-starved prior to treatment with SB-415286 (30 μM) or SB-216763 (3 μM) for 4 h. Lysates were prepared using a hypotonic lysis buffer. Immunoblotting of lysates confirmed the expression of myc-tagged FRAT1, and an anti-tubulin antibody was used to correct for protein loading. Stabilisation of cytoplasmic β-catenin was detected by immunoblotting. A representative blot from three separate experiments is shown. B: Lysates from cells treated as described in panel A were prepared for immunoblotting to determine levels of Tau phosphorylated on Ser-396 and Ser-404 (AD2) or Tau dephosphorylated on Ser-189–206 (Tau-1). Blots shown are from a representative experiment, with the immunoreactivity from each experiment quantified by densitometric analysis and plotted (±S.D., $n=3$).

lated serine residues in the amino acid stretch 189–206 [30], and consequently inhibition of phosphorylation of residues within this region increases detection by this antibody. Residues detected by both these antibodies have been reported to be phosphorylated by GSK-3 both in vitro and in cultured cells [8,31–34]. Treatment of cells with SB-415286 and SB-216763 confirmed phosphorylation at these epitopes to be regulated by GSK-3 activity since a decrease in AD2 detection, consistent with a dephosphorylation of Ser-396/404, and an increase in Tau-1 detection, consistent with a dephosphorylation of serine residues between 189 and 206, was noted (Fig. 3B). FRAT1 overexpression was also observed to decrease AD2 detection and increase Tau-1 detection, consistent with an inhibition of GSK-3 activity to these sites.

3.4. GS activity is elevated by treatment with selective GSK-3 inhibitors SB-216763 or SB-415286, but not by adenoviral FRAT1 overexpression

Having observed that FRAT1 overexpression can inhibit GSK-3 activity towards β-catenin and Tau, we addressed the selective nature of FRAT1 inhibition of GSK-3 by examining the effect of FRAT1 overexpression on GS activity. GS activity was assayed in PC12 cells infected with adenovirus-overexpressing FRAT1 or infected with adenovirus-expressing GFP only as a control (Fig. 4). Cells were also treated with SB-415286 (30 μM) and SB-216763 (3 μM). Use of adenovirus ensures that the vast majority of cells express FRAT1. Hence, in contrast to conventional transfection techniques, results can be interpreted without the concern that a background of non-transfected cells may obscure a FRAT-mediated effect on GS. Consistent with an inhibition of GSK-3 activity towards GS, treatment with ATP-competitive inhibitors elevated GS activity by two- to four-fold. However, FRAT1-overexpressing cells did not show elevated levels of GS activity compared to cells expressing GFP only, suggesting that phosphorylation of GS by GSK-3 was not inhibited by FRAT1.

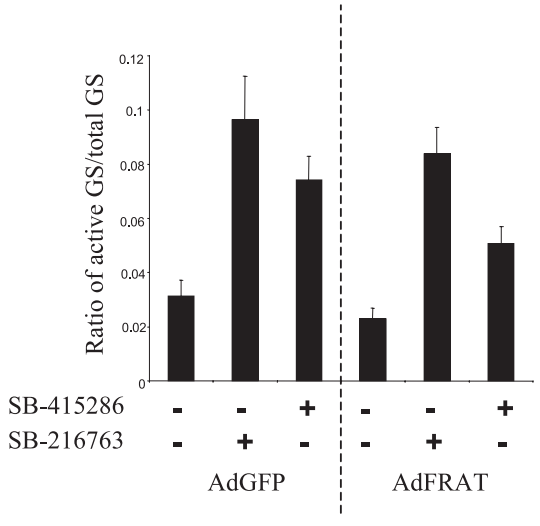

Fig. 4. GS activity is elevated by SB-415286 and SB-216763, but not by adenoviral FRAT1 overexpression in PC12 cells. Cells infected with AdGFP or AdFRAT were serum-starved prior to treatment with SB-415286 (30 μM) or SB-216763 (3 μM) for 1.5 h. Lysates were assayed for GS activity using incorporation of [^{14}C]UDP glucose into glycogen. Results are expressed as a ratio of active GS:total GS present in lysates (±S.E.M., $n=6$ from three independent experiments).

4. Discussion

Here, we have observed that adenoviral FRAT1 overexpression in mammalian cells is neuroprotective, and that neuroprotection correlates with inhibition of GSK-3 activity towards Tau and β-catenin, but not GS. Our results demonstrate for the first time that FRAT1 overexpression results in selective inhibition of GSK-3 towards specific cellular substrates in mammalian cells. By comparison, treatment with selective small molecule ATP-competitive GSK-3 inhibitors was found to be more potent in terms of neuroprotection than FRAT1 overexpression, and resulted in inhibition of GSK-3 activity towards Tau and β-catenin and elevated GS activity.

Using in vitro ligand displacement assays, we extended previous findings to define the affinity of FRATtide and GID for GSK-3, and also illustrated that whereas the binding sites on GSK-3 for FRATtide and GID overlap, binding of FRATtide does not interfere with binding of ATP-competitive inhibitors. These results are in good agreement with information gained recently from the crystal structure of GSK-3β complexed with FRATtide [35], which reveals that the FRATtide binding site is on the C-terminal lobe of the kinase domain, and does not obstruct the ATP binding site. Together, these results demonstrate the biophysical differences in the mechanisms of inhibition of GSK-3 by FRAT1 and by the selective small molecule ATP-competitive inhibitors SB-216763 and SB-415286. Moreover, we have shown that they also differ in modulation of GSK3 substrates, so facilitating molecular dissection of pathways of neuroprotection mediated by GSK-3 inhibition.

Microinjection studies have also suggested that FRAT overexpression protects neurones from apoptosis, but biochemical analysis of cellular substrates of GSK-3 that may contribute to neuroprotection has not been possible by this route [36,37]. The biochemical analyses of cellular substrates of GSK-3 performed here have shown that GS activity is not modulated by FRAT1-mediated inhibition of GSK-3 under conditions where phosphorylation of Tau and β-catenin is inhibited. This suggests that adenoviral FRAT1 overexpression is not simply acting by non-specific sequestration of GSK-3 into complexes in which GSK-3 can no longer access cellular substrates. The selective effect of FRAT1 overexpression on GSK-3 activity is therefore distinct to GSK-3 inhibition by small molecule ATP-competitive inhibitors, which according to their mechanism of action would inhibit GSK-3 activity towards all available substrates.

Several mechanisms have been suggested to explain such selective inhibition (reviewed in [38]). Since FRAT1 overexpression did not appear to inhibit GSK-3 activity towards GS (a primed substrate), our data are consistent with a hypothesis that FRAT1 may be unable to inhibit GSK-3 activity towards substrates that contain a 'priming' phosphoserine located at n+4 (where n is the site of phosphorylation). The crystal structure of GSK-3β complexed with FRATtide also suggests FRAT1 is unable to inhibit GSK-3 activity towards primed substrates, since FRATtide does not obstruct the binding site for the priming phosphoserine residue [35]. Here, we have shown that FRAT1 overexpression does, however, result in inhibition of GSK-3 activity towards Tau and β-catenin. It should be noted that Ser-396 and Ser-404 (detected by AD2 antibody) and Ser-199 and Ser-202 (detected by Tau-1 antibody) have been shown to be phosphorylated in bacterial

recombinant Tau incubated with GSK-3 in vitro [31,32,39], suggesting that in vitro at least, these sites are unprimed. However, it is presently unclear whether β-catenin is unprimed in a cellular context, and also whether the Tau phosphorylation sites assayed are primed or unprimed in cells.

Alternatively (or in addition), the selective nature of GSK-3 inhibition by FRAT1 may well be explained by the disruption of protein complexes that are necessary for GSK-3 activity towards specific cellular substrates. FRAT1 and Axin have overlapping binding sites on GSK-3 [19,22] (Fig. 1A) and disruption of Axin binding to GSK-3 prevents β-catenin phosphorylation and degradation. The data presented here demonstrate that the binding affinity of FRATtide for GSK-3 is two orders of magnitude higher than GID. Such a large difference in affinity raises questions regarding the mechanism by which FRAT recruitment may displace Axin binding; a more closely aligned binding affinity between the two would be more consistent with the hypothesis that an increased local concentration of FRAT (as a result of FRAT1 recruitment) could compete for GSK-3 binding. However, it is possible that the binding affinity of Axin for GSK-3 is higher, since GID might not optimally represent the GSK-3 binding region of Axin. With respect to FRAT1-mediated inhibition of Tau phosphorylation, it is also possible that FRAT1 displaces a cellular interacting protein that facilitates Tau phosphorylation by GSK-3.

The inhibition of β-catenin and Tau phosphorylation observed may well contribute to FRAT1-mediated neuroprotection, since both of these substrates have been implicated in neurodegeneration, and have been observed to be modulated in GSK-3-overexpressing transgenic mice that demonstrate neurodegenerative loss [40]. β-Catenin as a transcriptional regulator would seem a likely candidate for a substrate of GSK-3 that may play a role in the regulation of apoptosis; previous studies have shown that dominant negative β-catenin and Lef-1 sensitise neurones to β-amyloid-induced toxicity [41]. However, other reports argue against a neuroprotective role [36]. GSK-3 has been observed to phosphorylate Tau both in vitro and in cultured cells [31–34,42,43]. Tau promotes microtubule assembly in vitro [44,45], and Tau function is influenced by its phosphorylation [43,46–48]. Whether Tau hyperphosphorylation by GSK-3 contributes to neuronal death is presently unclear [49].

The observation that adenoviral FRAT1 overexpression proved a less potent method of neuroprotection than treatment with structurally distinct small molecule ATP-competitive inhibitors is intriguing. Although increasing viral load did not improve neuroprotection, it is possible that the differing extent to which individual cells express FRAT1 may be critical to reaching a 'threshold' of GSK-3 inhibition required for survival. Alternatively, the difference in potency of neuroprotection could reflect the possibility that maximal survival promoted by GSK-3 inhibition may require multiple effector pathways. For example, the additional ability of small molecule ATP-competitive compounds to elevate GS activity may contribute to neuroprotection, since cellular energy homeostasis is fundamental to the regulation of cell survival, and glycogen accumulation has been proposed as being neuroprotective against insults such as ischaemia [50]. It is probable that ATP-competitive GSK-3 inhibitor compounds modulate kinase activity not only towards GS, but also towards additional substrates unaffected by FRAT1 overexpression that are important to survival. Further dissection of neuroprotective effector pathways resulting from GSK-3 inhibition could be of therapeutic value in the treatment of neurodegenerative disorders such as Alzheimer's disease or stroke.

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