Overexpression of the mouse dishevelled-1 protein inhibits GSK-3β-mediated phosphorylation of tau in transfected mammalian cells

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Received 20 May 1997

Abstract Tau is a neuronal microtubule-associated protein whose function is modulated by phosphorylation. GSK-3β is a tau kinase. GSK-3β is part of the wingless signalling pathway and stimulation by wingless is predicted to down-regulate GSK-3β activity. In *Drosophila* imaginal disc cells, overexpression of dishevelled, a component of the wingless pathway, mimics the wingless signal. We have therefore studied the effect that overexpression of the murine dishevelled-1 protein has on GSK-3β-mediated phosphorylation of tau in transfected CHO cells. We find that co-transfection with dishevelled-1 is inhibitory to GSK-3β-mediated tau phosphorylation. Tau is hyperphosphorylated in Alzheimer's disease and the possible relevance of these findings to Alzheimer's disease pathogenesis are discussed.

Key words: Tau; GSK-3; Disheveled; Wingless; Alzheimer's disease

1. Introduction

Tau is a neuronal microtubule-associated protein that is localised in axons (see [1] for review). Tau promotes microtubule assembly in vitro [2] and is probably involved in the formation and maintenance of axons [3,4].

The function of tau is influenced by its phosphorylation status; phosphorylated tau has a reduced affinity for microtubules and is less potent at promoting microtubule assembly in vitro [5–8]. Likewise, phosphorylation of tau in tau transfected cells can also reduce its ability to bind to microtubules and this can influence cellular microtubule organisation [9–12].

Glycogen synthase kinase-3 (GSK-3α and GSK-3β) are serine/threonine kinases that phosphorylate tau both in vitro and in cultured cells [11–21]. GSK-3β is the mammalian homologue of the *Drosophila zeste white 3* gene (*zw3* or *shaggy*) product, a component of the wingless signal transduction pathway ([22] and see [23–25]). Wingless, a member of the Wnt growth factor family, plays an important role in early patterning of the Drosophila embryo (see [24,25]). Recently, a member of the *Drosophila frizzled* gene family (Dfz2) has been shown to function as a wingless receptor [26]. Genetic studies have shown that dishevelled, a cytoplasmic protein downstream of Dfz-2 is essential for reception of the wingless signal (see [27]). Wingless signalling via dishevelled results in the inactivation of Zw3 and this leads to increased stability of armadillo (arm) (see [23–25,28] for reviews). Components of the wingless signalling pathway are conserved in mammals with β-catenin being the mammalian homologue of armdillo.

The molecular mechanism(s) by which stimulation of the Wnt pathway leads to inactivation of GSK-3β are not fully understood although recent studies suggest that it may involve protein kinase C isoforms [29]. However, genetic studies place *dishevelled* upstream of, and closest to, *GSK-3*β. Interestingly, elevation of dishevelled protein levels by transfection into *Drosophila* imaginal disc cell lines mimics the wingless signal by increasing the levels of arm [30]. Overexpression of dishevelled therefore appears to be an experimental route that enables downstream activation of the wingless pathway.Since stimulation of the wingless pathway appears to down-regulate GSK-3β activity and since overexpression of dishevelled by transfection can mimic such stimulation, we have examined whether overexpression of the mouse dishevelled-1 (Dvl-1) protein can influence GSK-3β-mediated phosphorylation of tau in transfected mammalian cells.

2. Materials and methods

2.1. Expression plasmids

For expression of tau, a tau cDNA comprising the smallest human tau isoform containing no amino-terminal inserts and three carboxy-terminal repeat regions cloned into pSG5 (Stratagene) as previously described was used [11]. GSK-3β was expressed using a GSK-3β cDNA cloned into pMT2 as described [16]. To express mouse dishevelled-1 (Dvl-1), a cDNA encoding mouse Dvl-1 [31] was cloned as an *Eco*RI to *Xho*I fragment into the *Eco*RI and *Sal*I sites of pCIneo (Promega). As a control, the chloramphenicol acetyltransferase (CAT) gene was also cloned into pCIneo by removing the CAT gene from pMSG-CAT (Pharmacia) as a *Sal*I fragment and cloning into the *Sal*I site of pCIneo.

2.2. Cell culture, transfection and Western blotting

Chinese hamster ovary (CHO) cells were grown in α-MEM containing 10% v/v foetal bovine serum plus supplements and transfected using a Promega Profection kit. For Western blotting, transfected cells were washed with PBS and harvested by scraping into sodium-dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) sample buffer and heating in a boiling water bath. Proteins were separated on either 8% gels or on 4–15% gradient gels, transferred to nitrocellulose and probed with primary antibodies. Tau antibodies were TP70, a phosphorylation-independent rabbit polyclonal antibody [16] and the phosphorylation-dependent mouse monoclonal antibodies AT8, 8D8 and AT180. AT8 detects tau phosphorylated on ser^{202} and thr^{205}, 8D8 detects tau phosphorylated on ser^{396} and AT180 detects tau phosphorylated on thr^{231} [13,32–34]. AT antibodies were obtained from Innogenetics Ghent, Belgium. To study Dvl-1 expression, a rabbit polyclonal antibody raised against a GST-mouse Dvl-1 fusion protein was used. β-catenin was detected with a rabbit polyclonal β-catenin antibody. Blots were developed using the Enhanced Chemiluminescence (ECL) detection system (Amersham).

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PII S0014-5793(97)00733-3

3. Results

To investigate how experimental manipulation of Dvl-1 expression might influence phosphorylation of tau, we studied tau from CHO cells transiently transfected with tau, tau+Dvl-1, tau+GSK-3β and tau+Dvl-1+GSK-3β. Since one aim of these experiments was to compare tau phosphorylation in tau+GSK-3β co-transfected cells with tau+GSK-3β+Dvl-1 co-transfected cells, we controlled for the different numbers of plasmids transfected by including in those transfections involving only one or two plasmids, either empty vector or vector expressing CAT. All transfections therefore received the same numbers and amounts of plasmids; tau only transfected cells contained tau+2 CAT (or 2 empty vector); tau+Dvl-1 transfected cells contained tau+Dvl-1+1 CAT (or 1 empty vector); tau+GSK-3β transfected cells contained tau+GSK-3β+1 CAT (or one empty vector). All transfections were performed at least three times and produced identical results. No differences were observed between transfections involving the empty vector or transfections in which the CAT plasmid was used as a control.

To study Dvl-1 expression in CHO cells, we utilised a Dvl-1 antibody to probe Western blots of Dvl-1 transfected and non- or mock/CAT-transfected cells. This antibody reacted strongly with several species that migrated as a broad band between approximately 78 and 90 kDa in Dvl-1 transfected cells (Fig. 1); (extended exposure of the blots revealed some weaker reactivity in non-transfected cells with species in the 70–90 kDa range (not shown)). The predicted molecular weight of Dvl-1 is 75 kDa [31]. *Drosophila* dishevelled is phosphorylated in response to the wingless signal and overexpression of dishevelled also induces its phosphorylation [30]. Thus, the broad smear of reactivity that we observe in the Dvl-1 transfected CHO cells might possibly be due to differential phosphorylation. Whatever the reason, the intense labelling that we observe with the Dvl-1 antibody in Dvl-1 transfected but not non-transfected cells, clearly demonstrates that Dvl-1 is being over-expressed after transfection of the plasmid.

We next studied tau phosphorylation in the transfected CHO cells. Western blot analyses of tau only transfected cells with the phosphorylation-independent antibody TP70 revealed that the tau migrated as a doublet (Fig. 2 track 2). However, co-transfection of tau with GSK-3β induced a marked reduction in electrophoretic mobility of a proportion of the tau so as to generate new slower migrating species (Fig. 2 tracks 4 and 5). These results are consistent with previous observations of tau and tau+GSK-3β transfected cells [11,12,16–18]. Tau from cells co-transfected with tau+Dvl-1 migrated in a similar fashion to cells transfected with tau alone (Fig. 2 track 3). However, tau from cells transfected with tau+GSK-3β+Dvl-1 migrated in a different pattern to cells transfected with tau+GSK-3β; instead of the marked reduction in electrophoretic mobility of the tau induced by GSK-3β, only a partial reduction in electrophoretic mobility was detected (Fig. 1 tracks 6 and 7). These results were seen in cells in which either empty vector or CAT vector was used to control for co-transfection of the additional Dvl-1 plasmid.

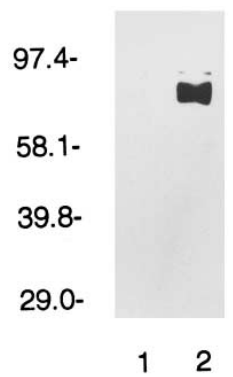

Fig. 1. Western blot with a polyclonal Dvl-1 antibody of mock transfected cells (track 1) or cells transfected with the Dvl-1 plasmid (track 2). Molecular masses of markers are shown on the left.

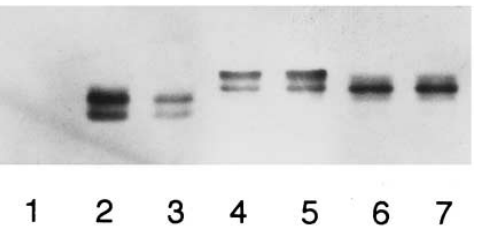

Fig. 2. Western blot with phosphorylation-independent tau antibody TP70 of cells transfected with tau, Dvl-1 and GSK-3β. Track 1, mock-transfected cells; track 2, cells transfected with tau+2CAT; track 3, cells transfected with tau+Dvl-1+1CAT; track 4, cells transfected with tau+GSK-3β+1CAT; track 5, repeat of track 4 but different transfection; track 6, cells transfected with tau+GSK-3β+Dvl-1; track 7, repeat of track 6 but different transfection.

Since the decrease in electrophoretic mobility of the tau induced by GSK-3β is due to phosphorylation of the tau [11,12,16–18], the above results suggest that co-transfection of Dvl-1 somehow inhibits phosphorylation of tau. To explore this possibility further, we utilised three phosphorylation-dependent tau antibodies (whose epitopes are all generated, at least in part, by GSK-3β) to study the phosphorylation status of tau in the various transfections. Consistent with previous observations of tau and tau+GSK-3β transfected CHO cells

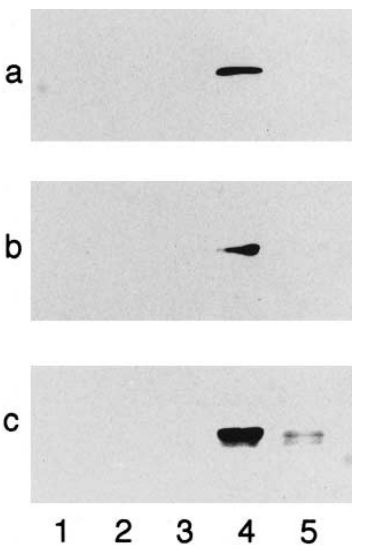

Fig. 3. Western blot of cells transfected with tau, GSK-3β and Dvl-1 with phosphorylation-dependent tau antibodies AT8 (a), 8D8 (b) and AT180 (c). Track 1, mock-transfected cells; track 2, cells transfected with tau+2 CAT; track 3, cells transfected with tau+Dvl-1+1CAT; track 4, cells transfected with tau+GSK-3β+1CAT; track 5, cells transfected with tau+GSK-3β+Dvl-1.

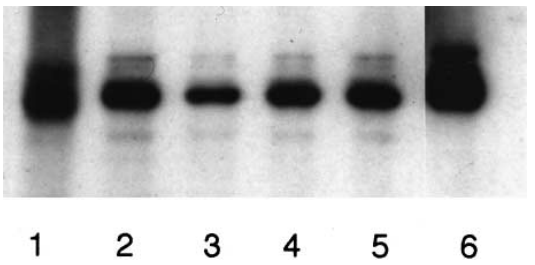

Fig. 4. Western blot of transfected cells probed with the β-catenin antibody. Track 1, mock transfected cells; track 2, cells transfected with tau+2 CAT; track 3 cells transfected with tau+GSK-3β+1 CAT; track 4, cells transfected with tau+GSK-3β+Dvl-1; track 5, as track 4, different transfection; track 6, sample of mouse brain. The slightly faster rate of migration of β-catenin seen in track 1 is due to anomalous running of the sample on this particular gel.

[11,17], tau transfected alone into cells failed to react, or reacted weakly with antibodies AT8, 8D8 and AT180 (Fig. 3a–c tracks 2) but co-transfection of tau with GSK-3β induced strong reactivity with a tau species that corresponded to the slower migrating species detected by antibody TP70 (Fig. 2 track 4). This was shown by cutting blots in half and probing one half with TP70 and the other with the phosphorylation-dependent antibodies. Co-transfection of tau with Dvl-1 produced labelling patterns that were highly similar to those seen following transfection of tau alone (Fig. 3 tracks 2 and 3). However, co-transfection of Dvl-1 with tau and GSK-3β either abolished, or markedly reduced the GSK-3β-induced reactivity with AT8, 8D8 and AT180 (Fig. 3 track 5). Thus co-transfection of Dvl-1 appears to inhibit GSK-3β-mediated phosphorylation of tau.

To determine whether this inhibitory influence on GSK-3β-mediated phosphorylation of tau was restricted solely to the transfected tau and to determine whether Dvl-1 overexpression could also inhibit the effect that GSK-3β has on an endogenous cellular protein, we studied the levels of β-catenin in the various transfections. CHO cells are highly transfectable and our immunofluorescence studies revealed that a high proportion of the cells in the transient transfections (more than 50%) express transfected proteins. Since most cells express the transfected proteins, we therefore reasoned that it was feasible to search for effects of GSK-3β/Dvl-1 on β-catenin in these transient transfected cells. GSK-3β activity down-regulates the levels of cytoplasmic β-catenin/armadillo and stimulation of the wingless pathway is predicted to inhibit this process [30,35–37]. Overexpression of GSK-3β led to a decrease in the levels of β-catenin in the CHO cells but this effect was inhibited by co-expression of Dvl-1 with GSK-3β (Fig. 4). Thus Dvl-1 also appears to inhibit the effect that GSK-3β has on the levels of endogenous β-catenin.

4. Discussion

In this report, we have investigated the effect that overexpression of the mouse Dvl-1 protein, a component of the Wnt signalling pathway, has on GSK-3β-mediated phosphorylation of tau in transfected CHO cells. Stimulation of the wingless pathway is predicted to result in down-regulation of GSK-3β activity (see [23–25,28] for reviews) and indeed, recent studies have shown that stimulation of 10T1/2 cells with wingless does indeed inhibit GSK-3 activity [29]. Overexpression of dishevelled in *Drosophila* imaginal disc cells mimics stimulation of the wingless pathway as assayed by accumulation of arm and hyperphosphorylation of dishevelled [30] although whether such overexpression alters GSK-3β activity has not been shown. Our finding that overexpression of Dvl-1 inhibits GSK-3β-mediated phosphorylation of tau is therefore consistent with these earlier reports which show that overexpression of dishevelled results in increased arm stability, probably by inhibiting Zw3 (GSK-3β).

Dvl-1 is widely expressed in adult tissues of the mouse including the brain, the major site of tau expression [31]. The ability of tau to influence cellular microtubule organisation appears to be modulated by phosphorylation and in particular by phosphorylation via GSK-3β [11,12]. Recent results generated by some of us have demonstrated that Wnt increases neurite spreading and branching in primary neuronal cultures [38] although whether such cytoskeletal rearrangements are due to changes in tau phosphorylation via Dvl-1 and GSK-3β are not established.

Tau is hyperphosphorylated in the paired helical filaments (PHF) found in the brains of patients with Alzheimer's disease and many of the sites that are phosphorylated in PHF-tau are targets for GSK-3β [11–18]. Mutations in the *presenilin* genes are the cause of most of the early onset, familial forms of Alzheimer's disease [39,40] and genetic studies in *C. elegans* have shown that a worm presenilin homologue is genetically linked to *lin-12*, a *C. elegans Notch* gene [41]. Recently, dishevelled has been shown to interact with Notch so as to connect the wingless and Notch signalling pathways [42]. Although only tentative at present, a genetic link therefore appears to exist between the presenilin family of proteins and dishevelled. In this context, our observations that dishevelled can influence tau phosphorylation suggests a possible route by which alterations in presenilin function might lead to Alzheimer-type changes in tau phosphorylation.

Acknowledgements: We thank Dr. Ken Kosik (Harvard University, USA) for kind gift of the human tau cDNA, Jim Woodgett (Ontario Cancer Institute) for GSK-3β expression plasmid, Daniel Sussman for Dvl-1 cDNA, Roel Nusse for the Dvl-1 antibody and Jackie Papkoff for the β-catenin antibody. This work was supported by grants from The Wellcome Trust, MRC, Nuffield Foundation and a donation from BAT.

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