GSK-3: tricks of the trade for a multi-tasking kinase

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Journal of Cell Science 116, 1175-1186 © 2003 The Company of Biologists Ltd
doi:10.1242/jcs.00384

Summary

Glycogen synthase kinase 3 (GSK-3) is a multifunctional serine/threonine kinase found in all eukaryotes. The enzyme is a key regulator of numerous signalling pathways, including cellular responses to Wnt, receptor tyrosine kinases and G-protein-coupled receptors and is involved in a wide range of cellular processes, ranging from glycogen metabolism to cell cycle regulation and proliferation. GSK-3 is unusual in that it is normally active in cells and is primarily regulated through inhibition of its activity. Another peculiarity compared with other protein kinases is its preference for primed substrates, that is, substrates previously phosphorylated by another kinase. Several recent advances have improved our understanding of GSK-3 regulation in multiple pathways. These include the solution of the crystal structure of GSK-3, which has provided insight into GSK-3's penchant for primed substrates and the regulation of GSK-3 by serine phosphorylation, and findings related to the involvement of GSK-3 in the Wnt/β-catenin and Hedgehog pathways. Finally, since increased GSK-3 activity may be linked to pathology in diseases such as Alzheimer's disease and non-insulin-dependent diabetes mellitus, several new GSK-3 inhibitors, such as the aloisines, the paullones and the maleimides, have been developed. Although they are just starting to be characterized in cell culture experiments, these new inhibitors hold promise as therapeutic agents.

Key words: Signal transduction, Phosphorylation, Wnt signalling, Insulin signalling, Protein structure-function

Introduction

Glycogen synthase kinase 3 (GSK-3) is a serine/threonine kinase that was first isolated and purified as an enzyme capable of phosphorylating and inactivating the enzyme glycogen synthase (Embi et al., 1980; Woodgett and Cohen, 1984). We now know that, beyond its role in glycogen metabolism, GSK-3 acts as a downstream regulatory switch that determines the output of numerous signalling pathways initiated by diverse stimuli (reviewed in Frame and Cohen, 2001; Grimes and Jope, 2001; Woodgett, 2001). The pathways in which GSK-3 acts as a key regulator, when dysregulated, have been implicated in the development of human diseases such as diabetes, Alzheimer's disease, bipolar disorder and cancer.

Given its involvement in many pathophysiological processes and diseases, GSK-3 is a tempting therapeutic target. However, its involvement in multiple pathways also raises the issue of selectivity – how might we impact one process while leaving others untouched? We must also assess the full spectrum of GSK-3 functions to avoid later surprises. For example, although inhibition of GSK-3 may be desirable in one context (e.g. in preventing neuronal apoptosis), it could have serious implications for another – for example, it might accelerate hyperplasia by deregulating β-catenin. That said, the recent development of small molecule inhibitors of GSK-3 has provided new tools for visualizing the cell from the perspective of GSK-3. Since it is highly active in most cell types, regulation of substrate phosphorylation occurs either by inactivation of GSK-3 or by changing substrate accessibility or recognition. Here, we present an overview of the various processes in which GSK-3 plays a key role and describe the current status of knowledge of GSK-3 regulation.

GSK-3 variants

There are two mammalian GSK-3 isoforms encoded by distinct genes: *GSK-3α* and GSK-3β (Woodgett, 1990) (Fig. 1). GSK-3α has a mass of 51 kDa, whereas GSK-3β is a protein of 47 kDa. The difference in size is due to a glycine-rich extension at the N-terminus of GSK-3α. Although highly homologous within their kinase domains (98% identity), the two gene products share only 36% identity in the last 76 C-terminal residues (Woodgett, 1990). Homologues of GSK-3 exist in all eukaryotes examined to date and display a high degree of homology; isoforms from species as distant as flies and humans display >90% sequence similarity within the kinase domain (reviewed in Ali et al., 2001).

GSK-3α and GSK-3β, although structurally similar, are not functionally identical. This became obvious upon ablation of the GSK-3β isoform in mice, which results in an embryonic lethal phenotype (Hoeflich et al., 2000). Embryos carrying homozygous deletions of exon 2 of GSK-3β die around embryonic day 16 owing to massive liver degeneration caused by extensive hepatocyte apoptosis. The inability of GSK-3α to rescue the GSK-3β-null mice indicates that the degenerative liver phenotype arises specifically from the loss of the beta isoform. This phenotype is remarkably similar to that of animals lacking RelA or I-κB kinase 2 (Beg et al., 1995; Li et al., 1999), components of the NF-κB signalling pathway (Ghosh and Karin, 2002; Rothwarf and Karin, 1999). Regulation of NF-κB nuclear import is not disrupted in embryonic fibroblasts isolated from the GSK-3β-null mice, which indicates that GSK-3 affects some other level of NF-κB regulation. The specific target(s) of GSK-3β in the regulation of NF-κB signalling has not been identified. Other protein

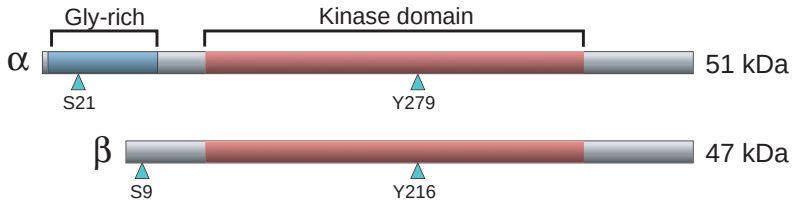

Fig. 1. Schematic representation of mammalian GSK-3α and GSK-3β. Sites of serine and tyrosine phosphorylation are indicated with blue arrowheads. The glycine-rich N-terminal domain unique to GSK-3α and the conserved kinase domain shared by both isoforms are highlighted.

kinases can phosphorylate the p65 subunit of NF-κB, increasing transactivation (Jang et al., 2001; Wang et al., 2000). GSK-3β can phosphorylate the C-terminal domain (residues 354-551) of p65 in vitro (Schwabe and Brenner, 2002), but further studies are required to verify if p65 is a physiological GSK-3β substrate and to assess the effect of this modification. The phenotype of mice lacking GSK-3α has not yet been reported.

A minor (~15% of total) splice variant of GSK-3β, GSK-3β2, has recently been identified and contains a 13-residue insert within the kinase domain (Mukai et al., 2002). Analysis of the in vitro kinase activity of GSK-3β2 revealed reduced activity towards the microtubule-associated protein tau, compared with 'unspliced' GSK-3β. An antibody selective for the novel splice-insertion polypeptide revealed that GSK-3β2 is localized primarily to neuronal cell bodies, unlike unspliced GSK-3β, which is also found in neuronal processes. It is unclear whether these substrate and subcellular localization differences can be generalized to other proteins or cells. Given the location of the insert within a highly conserved sequence, it probably forms a loop/hook, which might allow differential binding of the splice variant to scaffolding proteins that then expose the isoform to a distinct subset of target proteins (Mukai et al., 2002).

GSK-3 phosphorylation: insights from crystal structures

1) T-loop phosphorylation

Three groups recently determined the crystal structure of GSK-3 (Bax et al., 2001; Dajani et al., 2001; ter Haar et al., 2001). The structure has provided insight into both its regulation and its preference for primed, pre-phosphorylated substrates. Protein kinases related to GSK-3, such as CDK2, p38γ and ERK2, require phosphorylation of residues in their activation loops (T-loops) as a prerequisite for activity (Bellon et al., 1999; Brown et al., 1999; Canagarajah et al., 1997). A phosphothreonine is used by all three related kinases to align key β-strand and α-helical domains. The phosphorylation of a T-loop tyrosine is also required by p38γ and ERK2 to open up the catalytic site for substrate access. The T-loop of GSK-3 is tyrosine phosphorylated at Y216 and Y279 in GSK-3β and GSK-3α, respectively, but not threonine phosphorylated. Y216/Y279 phosphorylation could play a role in forcing open the substrate-binding site, but there appear to be no constraints preventing the open conformation in the unphosphorylated state (Dajani et al., 2001). Thus, T-loop tyrosine phosphorylation of GSK-3 might facilitate substrate phosphorylation but is not strictly required for kinase activity (Dajani et al., 2001).

GSK-3 has an unusual preference for target proteins that are pre-phosphorylated at a 'priming' residue located C-terminal to the site of GSK-3 phosphorylation (Fiol et al., 1987). The consensus sequence for GSK-3 substrates is Ser/Thr–X–X–X-Ser/Thr-P, where the first Ser or Thr is the target residue, X is any amino acid (but often Pro), and the last Ser-P/Thr-P is the site of priming phosphorylation. Although not strictly required, priming phosphorylation greatly increases the efficiency of substrate phosphorylation of most GSK-3 substrates by 100-1000-fold (Thomas et al., 1999). For example, glycogen synthase, the prototypical primed substrate, requires priming phosphorylation by casein kinase II (CK2) and then undergoes sequential multisite phosphorylation by GSK-3 (Fiol et al., 1988; Fiol et al., 1990).

The function expected for the 'missing' phosphothreonine in the GSK-3′s T-loop is believed to be replaced by the phosphorylated residue of a primed substrate, which binds to a positively charged pocket comprising R96, R180 and K205 (residue numbers for GSK-3β). This not only optimizes the orientation of the kinase domains but also places the substrate at the correct position within the catalytic groove for phosphorylation to occur. There are some substrates that lack a priming site. These proteins often display negatively charged residues at or near the priming position that may mimic a phospho-residue.

2) Inhibitory serine phosphorylation

Stimulation of cells with insulin causes inactivation of GSK-3 through a phosphoinositide 3-kinase (PI 3-kinase)-dependent mechanism. PI-kinase-induced activation of PKB (also termed Akt) results in PKB phosphorylation of both GSK-3 isoforms (S9 of GSK-3β; S21 of GSK-3α) (Cross et al., 1995), which inhibits GSK-3 activity. This leads to the dephosphorylation of substrates including glycogen synthase and eukaryotic protein synthesis initiation factor-2B (eIF-2B), resulting in their functional activation and consequent increased glycogen and protein synthesis (reviewed in Cohen et al., 1997).

Numerous other stimuli also lead to inactivation of GSK-3 through S9/S21 phosphorylation, including growth factors such as EGF and PDGF that stimulate the GSK-3-inactivating kinase $p90^{RSK}$ (also known as MAPKAP-K1) through MAP kinases (Brady et al., 1998; Saito et al., 1994), activators of p70 ribosomal S6 kinase (p70S6K) such as amino acids, (Armstrong et al., 2001; Krause et al., 2002; Terruzzi et al., 2002), activators of cAMP-activated protein kinase (PKA) (Fang et al., 2000; Li et al., 2000; Tanji et al., 2002) and PKC activators (Ballou et al., 2001; Fang et al., 2002).

The crystal structure of GSK-3 also helps to explain the inhibitory role of this serine phosphorylation (see Fig. 2A). Phosphorylation of S9/S21 creates a primed pseudosubstrate that binds intramolecularly to the positively charged pocket mentioned above. This folding precludes phosphorylation of substrates because the catalytic groove is occupied. Note that

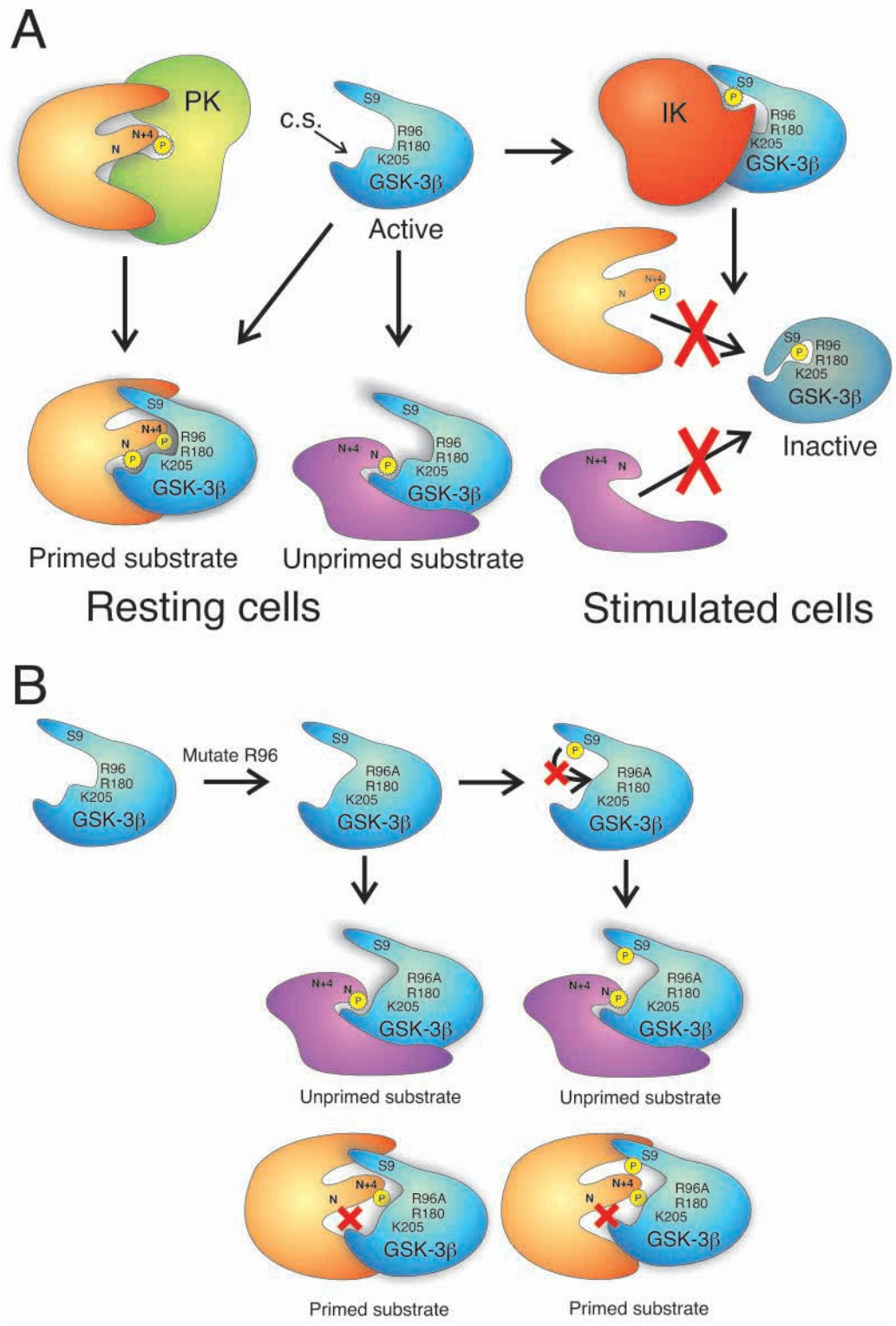

Fig. 2. (A) Regulation of GSK-3β activity by serine phosphorylation. In the resting cell, GSK-3β is constitutively active. Both unprimed substrates and substrates phosphorylated by a priming kinase (PK) are capable of being phosphorylated by the active GSK-3β. The priming phospho-residue at position N + 4, binds a pocket of positive charge arising from the arginine (R) and lysine (K) residues indicated. This directs a serine or threonine at position N to the active catalytic site (C.S.). When an inactivating kinase (IK) such as PKB/Akt phosphorylates GSK-3β on serine 9 (S9), the phosphorylated N-terminus becomes a primed pseudo-substrate that occupies the positive binding pocket and active site of the enzyme, acting as a competitive inhibitor for true substrates. This prevents phosphorylation of any substrates. (B) Effect of mutating arginine 96 to alanine (R96A) on GSK-3β activity. Since arginine 96 is a crucial component of the positive pocket that binds primed substrates, its mutation to an uncharged alanine residue disrupts the pocket so that primed substrates can no longer bind. The enzyme retains activity. Also, the S9-phosphorylated pseudosubstrate is no longer capable of inactivating the enzyme. As a consequence, GSK-3β, whether S9-phosphorylated or not, can phosphorylate unprimed substrates, but not primed substrates. Note that unprimed and primed substrates interact with GSK-3 through different interfaces.

the mechanism of inhibition is competitive. A consequence of this is that primed substrates, in high enough concentrations, out-compete the pseudosubstrate and thus become phosphorylated (Frame et al., 2001). Thus, although less efficient, unprimed substrates may provide a more accurate measure of GSK-3 activity in kinase assays performed in vitro. Mutation of Arg96 to alanine disrupts the pocket of positive charge (Fig. 2B) (Frame et al., 2001). Interestingly, although a peptide of 11 residues that matches the phosphorylated N-terminus of GSK-3β peptide-11 competitively inhibits both primed and unprimed substrates, a truncated version of this peptide (NTptide-8) inhibits phosphorylation only of primed substrates (Frame et al., 2001). Thus, small molecule inhibitors modeled to fit in the positively charged pocket of the GSK-3 kinase domain could potentially be very effective for selective inhibition of primed substrates.

Physiological roles for tyrosine phosphorylation

The physiological significance of phosphorylation of Y216 of GSK-3β in mammalian cells is unclear, since this phosphorylation is constitutive in resting cells (Hughes et al., 1993). The kinase(s) responsible has not been identified, although the tyrosine kinase (Zak1) that phosphorylates the Y216 equivalent is known in the slime mould (Kim et al., 1999). Mammalian GSK-3β expressed in bacteria shows evidence of autophosphorylation on tyrosine, serine and threonine residues, which raises the possibility that tyrosine phosphorylation of GSK-3 in mammalian cells is an autocatalytic event (Wang et al., 1994). GSK-3 immunoprecipitated from mammalian cells does not show this behaviour, however (Hughes et al., 1993).

Although GSK-3 is tyrosine phosphorylated in resting cells, the level may not be stoichiometric. Apoptotic stimuli such as staurosporine treatment or neurotrophic factor withdrawal increase GSK-3 activity and tyrosine phosphorylation in certain neuronal cell lines (Bhat et al., 2000; Bijur and Jope, 2001). Moreover, LPA, through a pathway involving the G proteins $G_{\alpha 12}$ and $G_{\alpha 13}$, also increases GSK-3 activity in neuronal cells at least in part through enhanced tyrosine phosphorylation of Y216/Y279 (Sayas et al., 2002). Since treatment of primary neurons with LPA results in neurite retraction, treatment of a neuroblastoma cell line, Neuro2A, results in cell rounding (Sayas et al., 2002), and LPA also causes apoptosis of adult neurons (Steiner et al., 2000), this provides further circumstantial evidence for a role for tyrosine phosphorylation in apoptosis.

Recent work has revealed two possible candidates for kinases that might tyrosine phosphorylate GSK-3 under these circumstances. Transient increases in intracellular calcium increase GSK-3-mediated phosphorylation of the microtubule-associated protein tau, and the increased GSK-3 activity is attributed to elevated GSK-3 tyrosine phosphorylation (Hartigan and Johnson, 1999). The tyrosine kinase that may be responsible for the phosphorylation of GSK-3 is proline-rich tyrosine kinase 2 (PYK2)†, a calcium-sensitive enzyme. Active, but not kinase-dead, PYK2 increases tyrosine phosphorylation of GSK-3 both in PYK2-transfected cells and in vitro, and PYK2 and GSK-3β co-immunoprecipitate in cells transfected with PYK2 (Hartigan et al., 2001). The Fyn tyrosine kinase is another potential player: insulin treatment of SH-SY5Y cells for 1 minute causes an increase in association of Fyn with GSK-3β, a transient increase in GSK-3β activity with a concomitant transient increase in GSK-3β phosphorylation on Y216 and a transient increase in tau phosphorylation. The short duration of insulin treatment is crucial for the observed increase in GSK-3 activity (Lesort et al., 1999). Prolonged treatment with insulin, besides the well characterized inhibition of GSK-3 through serine phosphorylation (discussed above), has also been reported to inhibit GSK-3 through tyrosine dephosphorylation in Chinese hamster ovary cells (Murai et al., 1996). Further characterization of the proposed GSK-3-phosphorylating tyrosine kinases is required to substantiate a role for them in physiological processes.

The role of tyrosine phosphorylation of GSK-3 in *Dictyostelium* is better understood. Zak1 is essential for activation of GSK-3 during developmental patterning and is regulated downstream of certain cyclic AMP receptors involved in chemotaxis (Kim et al., 1999; Plyte et al., 1999). Unfortunately, identification of a mammalian orthologue of Zak1 has so far proven elusive. Kim et al. have recently described an opposing role for a protein tyrosine phosphatase, which dephosphorylates GSK-3 on tyrosine residues and results in a prestalk fate (Kim et al., 2002). Interestingly, phosphorylation of two tyrosine residues, both residing in the activation loop of GSK-3, appears to be modulated by cAMP in *Dictyostelium* (Kim et al., 2002). Both of these tyrosine residues, Y214 and Y220, are conserved in mammals (Y216 and Y219, respectively). Thus, Y219 – in addition to Y216 – might therefore play a role in GSK-3 regulation in mammals.

The role of GSK-3 in the Wnt/β-catenin pathway

The Wnts are a family of secreted, cysteine-rich, glycosylated, protein ligands that influence cell growth, differentiation, migration and fate (Miller, 2002; Polakis, 2000; Smalley and Dale, 1999). Members of the Wnt family have been identified in organisms ranging in complexity from the cnidarian Hydra to humans; at least 19 different Wnts exist in mammals (Miller, 2002). Severe developmental defects usually occur in mice that have defective Wnt signalling. One of the pathways regulated by Wnt molecules is termed the canonical Wnt pathway or the Wnt/β-catenin pathway (reviewed in Huelsken and Behrens, 2002; Polakis, 2000; Seidensticker and Behrens, 2000; Sharpe et al., 2001) (Fig. 3). Here, we limit discussion of Wnt signalling to this signalling pathway. Wnt signal transduction ultimately results in the activation of genes regulated by the T-cell factor (TCF)/lymphoid enhancer factor (LEF) family of architectural transcription factors (reviewed in Barker et al., 2000; Brantjes et al., 2002; Novak and Dedhar, 1999). The effector molecule responsible for activating TCF/LEF-responsive genes is β-catenin, which serves as a transactivator that binds to DNA-bound TCF/LEFs. As in insulin signalling, GSK-3 plays a key inhibitory role in the Wnt pathway. In unstimulated cells, GSK-3 phosphorylates the N-terminal domain of β-catenin, thereby targeting it for ubiquitylation and proteasomal degradation. Exposure of cells to Wnts leads to inactivation of GSK-3 through an as yet unclear mechanism. The phosphoprotein Dishevelled is required, after receptor-ligand interaction, to transduce the signal that results in the inactivation of GSK-3. As a result, β-catenin is dephosphorylated (van Noort et al., 2002) and escapes the ubiquitylation-dependent destruction machinery. Unphosphorylated β-catenin accumulates in the cytoplasm and translocates to the nucleus, where it can associate with the TCF/LEFs and become a transcriptional transactivator. Mutations in β-catenin that prevent its phosphorylation by GSK-3 (gain-of-function mutations) have been found in cancers of the skin, colon, prostate, liver, endometrium and ovary (reviewed in Polakis, 2000). Many of the components of the Wnt pathway, and their functional roles, were first identified in studies of the Wnt pathway in *Drosophila melanogaster*, which is driven by the Wnt homologue, Wingless (Dierick and Bejsovec, 1999; Manoukian and Woodgett, 2002; Siegfried and Perrimon, 1994). In *Drosophila*, the GSK-3 homologue is termed Shaggy (Bourouis et al., 1990) or Zeste-white3 (Siegfried et al., 1990).

Phosphorylation of β-catenin by GSK-3 occurs in a complex

†Also known as cell adhesion kinase beta; focal adhesion kinase 2; protein tyrosine kinase 2 beta, cell adhsion kinase beta, calcium-dependent kinase, realted adhesion focal tyrosine kinase.

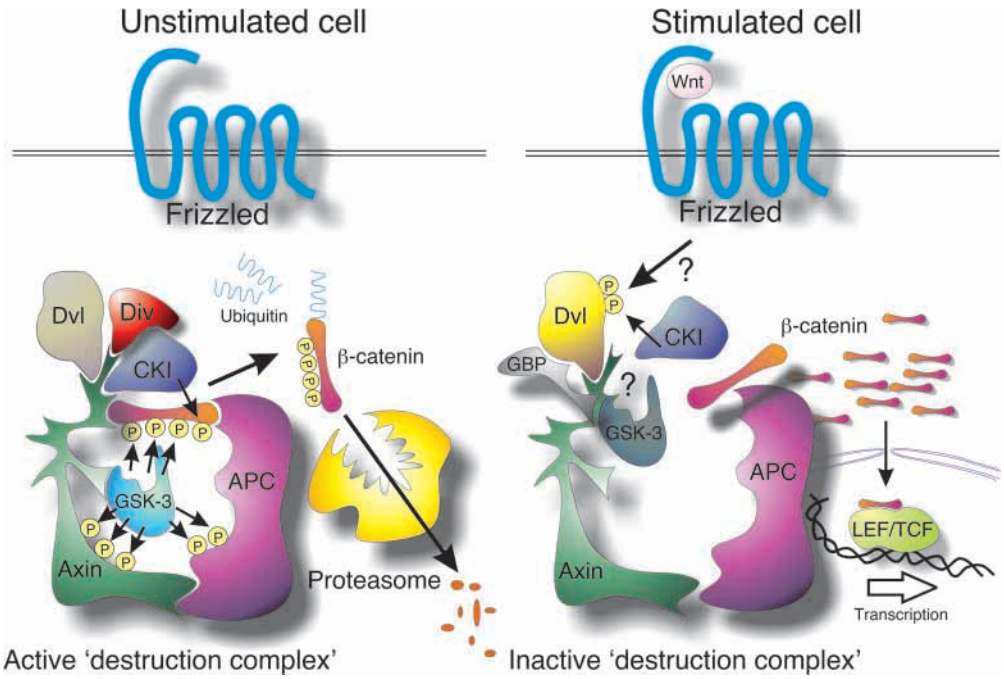

Fig. 3. Central role of GSK-3 in the Wnt/β-catenin pathway. In unstimulated cells, CKI phosphorylates β-catenin on S45, priming it for subsequent phosphorylation by GSK-3 (S41, S37, S33), which targets β-catenin for ubiquitylation and proteasomal degradation. The ankyrin repeat protein, Diversin (Div), may help recruit CKI to the destruction complex. Wnt stimulation activates the receptor Frizzled, which then signals through Dishevelled (Dvl), using an unclear mechanism, to inactivate β-catenin phosphorylation. Unphosphorylated β-catenin accumulates and then translocates to the nucleus where it transactivates genes regulated by TCF/LEF transcription factors. The GSK-3-binding protein (GBP/FRAT) may be involved in transmission of a Wnt signal by regulating binding of GSK-3 to the scaffold protein, axin.

sometimes referred to as the destruction complex, which consists minimally of the proteins GSK-3, β-catenin, axin/conductin and adenomatous polyposis coli (APC) (Hinoi et al., 2000). APC is a tumour suppressor protein commonly deleted in familial adenomatous polyposis and sporadic colorectal cancer (Polakis, 1997). Axin (and a related protein known as conductin or axil) harbors several protein-protein interaction domains and serves as a scaffolding protein that holds together the elements of the β-catenin destruction complex. Both Axin and APC are phosphorylated by GSK-3. Phosphorylation of axin by GSK-3 increases its stability and binding to β-catenin (Ikeda et al., 1998; Jho et al., 1999; Yamamoto et al., 1999). Phosphorylation of APC increases its binding to β-catenin (Rubinfeld et al., 1996).

Five groups have recently determined that β-catenin is also a primed substrate for GSK-3, with casein kinase I (CKI) acting as the priming kinase (Amit et al., 2002; Hagen et al., 2002; Hagen and Vidal-Puig, 2002; Liu et al., 2002; Sakanaka, 2002; Yanagawa et al., 2002). CKI phosphorylates S45, which lies four residues C-terminal to three GSK-3 targets at serines S33, S37 and S41. As a priming kinase, CKI functions as a negative regulator of Wnt signalling since it promotes GSK-3 function. Such a role contrasts with that proposed in several previous reports, which identified CKI as a positive transducer of Wnt signalling (Gao et al., 2002; Kishida et al., 2001; Lee et al., 2001; McKay et al., 2001). CKI binds to axin and Dishevelled and phosphorylates not only β-catenin but also axin, Dishevelled and APC. Polakis has proposed a model in which CKI can act as both a positive and a negative regulator of Wnt signalling (Polakis, 2002). In this model, CKI plays a role in the destruction complex as the priming kinase for GSK-3 and is required for transmission of the Wnt signal by assisting in the activation of Dishevelled, perhaps by increasing its affinity for signalling intermediates (see below). A novel ankyrin-repeat-containing protein, Diversin, has been reported to recruit CKI to the destruction complex (Schwarz-Romond et al., 2002).

These data raise the possibility that GSK-3 plays only a latent role in regulation of β-catenin. For example, if CKI activity is directly regulated by Wnt signalling, then phosphorylation of S45 would act as the trigger for subsequent phosphorylation by GSK-3. In this scenario, the activity of GSK-3 could be totally independent of Wnt regulation. However, phosphorylation of S45 appears to be constitutive, at least in some cell types. Although S45 phosphorylation has been reported to decrease upon Wnt stimulation, the phospho-specific antibodies used in those studies also detect S41, one of the GSK-3 targets (Amit et al., 2002). Antibodies selective for phosphoserine 45 do not reveal changes in stoichiometry in response to Wnt (Liu et al., 2002). Clearly, cells have evolved complex mechanisms to titrate β-catenin levels, presumably to allow multiple layers of control.

Another interesting player in the regulation of the Wnt pathway, at least in vertebrates, is a GSK-3-binding protein termed GBP (also known as FRAT‡) (Farr et al., 2000; Ferkey and Kimelman, 2002; Fraser et al., 2002; Sumoy et al., 1999; Yost et al., 1998). Binding of GBP to GSK-3 precludes GSK-3 from binding to axin and thus interferes with β-catenin phosphorylation. A small peptide derived from FRAT called FRAT-tide is sufficient to prevent axin-GSK-3 interaction and prevents phosphorylation of both axin and β-catenin (Thomas et al., 1999). Comparison of mutations that affect binding of GSK-3 to axin and GBP, as well as analysis of the crystal structure of a GSK-3-FRAT-tide complex, indicates that the binding sites on GSK-3 for GBP/FRAT and axin overlap (Bax et al., 2001; Ferkey and Kimelman, 2002; Fraser et al., 2002). Treatment of *Xenopus* embryo extracts with CKIε increases binding of GBP to Dishevelled (Lee et al., 2001).

GBP also plays a role in the nuclear export of GSK-3 (Franca-Koh et al., 2002). A mutant of GSK-3 that cannot bind to GBP accumulates in the nucleus. Moreover, a peptide that

‡Frequently rearranged in advanced T-cell lymphocytes

interferes with GBP binding to GSK-3 causes endogenous GSK-3 to accumulate in the nucleus. These findings suggest that GBP regulates access of GSK-3 to substrates partitioned between nuclear and cytoplasmic compartments. Since there are two known mammalian GBP homologues (Freemantle et al., 2002), each with dynamically regulated expression patterns during development, GBP could play an important role in modulating GSK-3 function, especially during development. Rather surprisingly, GBP homologues have not been identified in *Drosophila* or *C. elegans*, which indicates that the protein is not a core component of canonical Wnt signalling.

A critical aspect of GSK function in the Wnt pathway is that GSK-3 appears to be insulated from regulators of GSK-3 that lie outside of the Wnt pathway. For example, insulin signalling leads to inhibition of GSK-3 via serine S9/S21 phosphorylation but does not cause accumulation of β-catenin. Conversely, Wnt signalling does not affect insulin signalling (Ding et al., 2000; Yuan et al., 1999). How this insulation occurs is unclear, but it probably stems from the effective sequestration of a fraction of GSK-3 with axin in the destruction complex. Note that tissues from mice lacking GSK-3β do not show evidence of accumulated β-catenin even though total GSK-3 levels are reduced by 50% and there is zero cellular GSK-3β. Immunoprecipitation of axin from these tissues reveals that GSK-3β is simply replaced by GSK-3α (in wild-type cells, both GSK-3α and GSK-3β are found bound to axin) (E. Rubie and J.R.W., unpublished). Since cellular levels of GSK-3 exceed those of axin, the destruction machinery compensates for the loss of GSK-3β by substituting GSK-3α.

GSK-3 and the Hedgehog pathway

Groups examining Hedgehog (Hh) signalling in *Drosophila* recently discovered yet another novel function for GSK-3 (Jia et al., 2002; Price and Kalderon, 2002). The Hh pathway is somewhat similar to the Wnt pathway: both Wnt and Hh are secreted signalling proteins involved in embryonic patterning that often work in concert (reviewed in Ingham and McMahon, 2001). Whereas the Wnt pathway uses β-catenin to transduce its signals to the nucleus, the Hh pathway employs a protein called Cubitus interruptus (Ci) in flies or Gli in mammals (Ingham and McMahon, 2001). In the absence of a Hh signal, Ci is targeted for proteolysis. Unlike in Wnt signalling, proteolysis does not result in total degradation of Ci but instead processes it from a 155-residue form (Ci155) to a truncated 75-residue amino acid form (Ci75) that functions as a transcriptional repressor (Aza-Blanc et al., 1997). GSK-3, in combination with CKI and the priming kinase PKA, phosphorylates Ci155 to target it for proteolytic processing in the absence of a Hh signal (Jia et al., 2002; Price and Kalderon, 2002). Activation of Hh signalling results in translocation of full-length Ci155 to the nucleus, where it activates Hh target genes. As in the Wnt pathway, several of the molecules involved in Hh signal transduction have been implicated in cancer (reviewed in Taipale and Beachy, 2001). Notably, familial germline mutations in one allele of the Hh receptor, Patched, are associated with an almost complete penetrance of basal cell carcinoma (Johnson et al., 1996; Taipale, 2001). Although GSK-3 phosphorylation of the mammalian homologues of Ci has yet to be reported, all contain multiple GSK-3 consensus sites next to PKA sites (Ruis i Altaba, 1999; Jia et al., 2002). It seems likely, on the basis of the conservation of the Wnt pathway, that the Gli proteins will soon be confirmed as GSK-3 substrates.

Other GSK-3 substrates

Numerous putative GSK-3 substrates have roles in a wide spectrum of cellular processes, including glycogen metabolism, transcription, translation, cytoskeletal regulation, intracellular vesicular transport, cell cycle progression, circadian rhythm regulation and apoptosis. Phosphorylation of these substrates by GSK-3 is most often inhibitory, as in the cases of glycogen synthase, β-catenin and Ci. Although a detailed description of the function of all of the substrates is beyond the scope of this review, a list of many of the best-characterized substrates as well as some newly identified substrates, with a brief description of their function and the role of GSK-3 phosphorylation, is shown in Table 1.

Other GSK-3-binding proteins

Besides GBP, axin and APC, several other new GSK-3-binding partners have recently been identified. The tumour-suppressor protein p53, for example, has recently been shown to bind and activate GSK-3 in the nucleus of neuroblastoma cells treated with camptothecin, a topoisomerase I inhibitor that causes DNA damage and subsequently elicits a p53 response (Watcharasit et al., 2002). The activation of GSK-3 occurs without changes in its phosphorylation and is restricted to nuclear GSK-3 and involves an unknown mechanism. In addition, a yeast two-hybrid screen identified the A-kinase-anchoring protein AKAP220 as a GSK-3-binding partner (Tanji et al., 2002). AKAPs are scaffolding proteins, and the interaction between AKAP220 and GSK-3 occurrs in a complex that also containsPKA and type 1 protein phosphatase. The interaction between AKAP220 and GSK-3 enhances PKA-mediated inhibition of GSK-3.

Presenilin 1 (PS1) is one of the two mammalian presenilins identified through association of mutations in these proteins with early-onset familial Alzheimer's disease. Besides having a role in proteolytic processing of proteins at the cell membrane such as amyloid precursor protein, presenilin also binds to β-catenin and has been implicated in regulating its cellular levels. PS1 may also function as a scaffolding protein that binds PKA, GSK-3 and β-catenin (Palacino et al., 2001). This novel quaternary complex functions in a similar manner to the axin complex, using PS1 to couple the priming phosphorylation by PKA (on S45 of β-catenin) to subsequent phosphorylation by GSK-3.

GSK-3 and human disease

Many of the pathways that use GSK-3 as a regulator have links to human diseases. As described above, GSK-3 is a core component of two pathways involved in cell fate determination and morphology, the Wnt and Hedgehog pathways, which are both involved in several forms of human cancer. There are also numerous studies linking GSK-3 to Alzheimer's disease, on the basis of GSK-3-mediated hyperphosphorylation of tau, which is associated with neurofibrillary tangles, one of the hallmarks of Alzheimer's disease (reviewed in De Ferrari and Inestrosa, 2000;

Table 1. Putative GSK-3 substrates

Putative substrate	Function	Effect of phosphorylation by GSK-3	References
Kinesin light chain	Regulatory component of motor protein involved in vesicular transport	Inhibits anterograde vesicular movement	Morfini et al., 2002
Presenilin 1 (PS1)	Transmembrane protein linked to Alzheimer's disease; also binds β-catenin	Increases degradation of C-terminal PS1 fragment	Kirschenbaum et al., 2001
Tau	Microtubule-associated protein; stabilizes microtubules	Reduced microtubule binding; decreased microtubule stability	Hanger et al., 1992
β-catenin	Transcription transactivator	Targets for degradation	Yost et al., 1996
Axin	Scaffold protein in Wnt Pathway	Increased affinity for β-catenin and increased axin stability	Ikeda et al., 1998; Jho et al., 1999; Yamamoto et al., 1999
Adenomatous Polyposis Coli	Wnt pathway component	Increased β-catenin binding; decreased microtubule binding	Rubinfeld et al., 1996
Timeless	Transcription factor regulating *Drosophila* circadian rhythm	Increased heterodimerization with clock gene called period or increased nuclear transport suggested	Martinek et al., 2001
Nuclear factor of activated T-cells c	Transcription factor; early immune response genes	Decreased DNA binding; increased nuclear export	Beals et al., 1997
Heat shock factor-1	Transcription factor; regulates genes in response to potentially lethal stressors	Inactivates transcription factor activity	Chu et al., 1996
c-Jun	Transcription factor; component of activator protein-1 (AP-1) that regulates many diverse genes	Decreased DNA binding and transactivation	Boyle et al., 1991
c-Myc	Transcription factor; regulates genes involved in cell growth, differentiation and apoptosis	Targets for degradation	Pulverer et al., 1994; Sears et al., 2000
cAMP response element binding protein	Transcription factor; regulates cAMP-responsive genes	Increased transcription factor activity	Fiol et al., 1994
Microphthalmia-associated transcription factor	Transcription factor; regulates tyrosinase expression	Increased binding to tyrosinase promoter	Khaled et al., 2002; Takeda et al., 2000
Cylin D1	Transcription factor; cell cycle regulation	Increased nuclear export; targets for degradation	Diehl et al., 1998
eIF-2B translation factor	Critical for translation initiation	Inhibits activity	Singh et al., 1996; Welsh and Proud, 1993
Inhibitor-2	Regulatory subunit of phosphatase	Activates phosphatase	Park et al., 1994
Glycogen synthase	Glycogen metabolism	Inhibits enzyme activity	Dent et al., 1989; Dent et al., 1990; Fiol et al., 1988
Insulin receptor substrate 1	Insulin signalling	Inhibits insulin receptor signalling	Eldar-Finkelman and Krebs, 1997
Acetyl CoA carboxylase	Key lipogenic enzyme	Inactivates enzyme	Hughes et al., 1992
ATP-citrate lyase	Fatty acid synthesis	Inactivates enzyme	Benjamin et al., 1994; Hughes et al., 1992
Mucin 1/ DF3 antigen	Transmembrane glycoprotein that binds β-catenin; overexpressed in human carcinomas	Decreases interaction with β-catenin	Li et al., 1998

Substrates involved in Alzheimer's disease are shaded blue, components of the Wnt pathway are shaded yellow, transcription factors are shaded green and proteins involved in protein, insulin and lipid metabolism are shaded red.

Maccioni et al., 2001; Mattson, 2001). The GSK-3 inhibitor lithium (Stambolic et al., 1996), used for decades as a therapy for bipolar disorder, also implicates GSK-3 activity in the underlying causes of this disease (Detera-Wadleigh, 2001; Gould and Manji, 2002; Manji et al., 1999). In support of a role for GSK-3 in mood regulation is the fact that another mood-stablilizing drug, valproate, also inhibits GSK-3, although probably through an indirect mechanism (Chen et al., 1999; De Sarno et al., 2002). Note that lithium and valproate also affect neuronal inositol metabolism, and their mood-stabilizing properties might be related to this effect (Williams, 2002; Hall et al., 2002; Li et al., 2002). Apart from a role in neurological pathologies, GSK-3 may also play a role in the development of non-insulin-dependent diabetes mellitus (NIDDM), a disease often associated with chronic inhibition of muscle glycogen synthase (reviewed in Kaidanovich and Eldar-Finkelman, 2002).

Small molecule inhibitors of GSK-3

Given the association of abnormal GSK-3 activity and various human diseases, GSK-3 is emerging as a potential therapeutic target, particularly true where its over-expression may be linked to pathology – for example, in NIDDM and neurodegenerative diseases (Eldar-Finkelman, 2002) – but it may also be relevant for some of the more recently identified functions of GSK-3, such as its role in activating NF-kB, a well characterized target for anti-inflammatory agents (Hoeflich et al., 2000). The best-characterized inhibitor of GSK-3 is lithium (Klein and Melton, 1996; Stambolic et al., 1996). Although fairly specific for GSK-3, compared with other protein kinases, lithium also affects other enzymes, and a relatively high dose is required (Ki is mM) to inhibit GSK-3 activity in cell culture (Stambolic et al., 1996). The mode of inhibition is through competition for Mg^{2+} (Ryves and Harwood, 2001). Recently, the bivalent form of zinc, which mimics insulin action, has also been shown to inhibit GSK-3 when used at a concentration of 15 μM in a cell culture system (Ilouz et al., 2002). In vitro, another metal ion, beryllium, inhibits GSK-3 to half maximal activity at a concentration of 6 μM (Ryves et al., 2002).

Several new GSK-3 inhibitors have recently been developed, most of which are ATP competitive (Martinez et al., 2002a; Martinez et al., 2002b). A list of these drugs, their mode of

Table 2. GSK-3 inhibitors

Drug type	Specific drug examples	Mode of action	Clinical effects/effects in cell culture	Reference
Aloisines	Aloisine A, Aloisine B	ATP-competitive	Inhibits cell proliferation	Mettey et al., 2003
Beryllium	N/A	Competes for Mg^{2+} and ATP	No data	Ryves et al., 2002
Bivalent zinc	N/A	Undetermined; does not compete for substrate	Insulin-mimetic	Ilouz et al., 2002
Hymenialdisine	Dibromo- hymenialdisine	ATP-competitive	Suppresses inflammation; inhibits tau phosphorylation	Breton and Chabot-Fletcher, 1997; Meijer et al., 2000
Indirubins	5,5′-dibromo-indirubin	ATP-competitive	Anti-mitotic; anti-tumoural; inhibits tau phosphorylation	Damiens et al., 2001; Leclerc et al., 2001
Lithium	N/A	Competes for Mg^{2+}	Mood stabilization; prevents polyglutamine toxicity in Huntington's disease	Carmichael et al., 2002; Klein and Melton, 1996; Ryves and Harwood, 2001; Stambolic et al., 1996
Maleimides	Ro 31-8220, SB-216763, SB-415286	ATP-competitive	Insulin-mimetic; prevention of death in culture neuronal	Coghlan et al., 2000; Cross et al., 2001; Hers et al., 1999; Lochhead et al., 2001; Smith et al., 2001
Muscarinic agonists	AF102B, AF150	Unclear	Inhibits tau phosphorylation	Forlenza et al., 2000

N/A, non-applicable.

action and some of their reported effects in cell culture and patients is shown in Table 2.

There may be a skeleton in the closet, however, for anti-GSK-3 therapeutics. In addition to the problem of its broad range of functions, inhibition of the enzyme could presumably lead to enhanced accumulation of β-catenin, a known oncogene. To mitigate this complication, drugs that selectively target non-axin-associated GSK-3 would be desirable, especially for chronic diseases, such as diabetes. On the positive side, long-term lithium treatment is not associated with enhanced incidence of cancer, although the lithium-treated patient cohort is not typical of the general population.

Perspectives

An enzyme that has as many proposed substrates as GSK-3 requires numerous levels of regulation to confer signal-dependent specificity. Several different mechanisms regulating the phosphorylation of GSK-3 substrates have been described: (1) inactivation of GSK-3 through serine phosphorylation; (2) activation of GSK-3 through tyrosine phosphorylation; (3) inactivation of GSK-3 through tyrosine dephosphorylation; (4) covalent modification of substrates through priming phosphorylation; (5) inhibition or facilitation of GSK-3-mediated substrate phosphorylation through interaction of GSK-3 with binding or scaffolding proteins; (6) targeting of GSK-3 to different subcellular localizations; (7) differential usage of isoforms or splice variants to alter subcellular localization or substrate specificity; and (8) integration of parallel signals conveyed by a single stimulus.

Although our understanding of GSK-3 regulation is increasing, there are still several outstanding issues: (1) putative GSK-3 substrates need to be verified and classified as to whether they require priming phosphorylation; (2) proteins that have been identified as GSK-3-interacting proteins require characterization to identify sites of interaction and mechanisms that regulating binding; (3) the mechanism of Wnt-mediated inactivation of GSK-3 remains to be established; (4) the basis for the specific requirement for GSK-3β in regulating NFκB function remains unclear (GSK-3α does not substitute); and (5) the specificity of newly emerging GSK-3 inhibitors must be validated.

GSK-3 has reliably and regularly provided surprises ever since its discovery, and several of these have proven to be new signalling paradigms. There is little reason to doubt that there are more revelations in store, but with enhanced tools, such as selective inhibitors, knockout mice and modified binding proteins, there is hope that the true cellular roles for this multi-talented kinase will be appreciated and its utility as a therapeutic target be realized. But there are certainly easier molecules to study…

J.R.W. is a Canadian Institutes of Health Research (CIHR) Senior Scientist and International Scholar of the Howard Hughes Medical Institute and is supported by grants from the Canadian Institutes of Health Research, the Terry Fox Foundation and the National Cancer Institute of Canada. B.W.D. is supported by a CIHR post-doctoral fellowship.

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