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Regulation of GSK-3: A Cellular Multiprocessor

Minireview

Adrian J. Harwood[1]
MRC Laboratory for Molecular Cell Biology
and Department of Biology
University College London
Gower Street
London, WC1E 6BT
United Kingdom

The structures of protein kinases, as resolved by X-ray crystallography, have provided a set of useful paradigms to explain kinase regulation. The control of a handful of kinases, however, has proven more difficult to understand. Among these, glycogen synthase kinase-3 (GSK-3) has been particularly perplexing, having a number of unconventional characteristics and acting within at least two very different signal pathways. Recent results, including the resolution of the GSK-3 protein structure, have now begun to illuminate a sophisticated regulatory mechanism that enables it to process different signals within the same cell.

Insulin and Wnt Both Regulate GSK-3β

GSK-3 was discovered and named as one of many protein kinases that phosphorylate and inactivate glycogen synthase (Embi et al., 1980). Despite the specificity inferred by its name, GSK-3 is an important regulatory kinase with a plethora of significant cellular targets, including cytoskeletal proteins and transcription factors. GSK-3 is of medical importance, being involved in metabolic control, animal development, and oncogenesis. GSK-3 is a downstream target of insulin stimulation and regulates glycogen synthase, the protein translation initiation factor eIF2B, and the transcription factor C/EBPα (Cohen, 1999). Its regulation by insulin is important for the understanding of non-insulin-dependent diabetes mellitus, the most common metabolic disorder. GSK-3 associates with presenilin and phosphorylates the microtubule-associated protein tau, implicating GSK-3 in the progression of Alzheimer's disease. GSK-3 is also a central element of the Wnt signaling pathway and is required for pattern formation during embryonic development and regulation of cell proliferation (Dale, 1998). The GSK-3 protein family is highly conserved and has been found in animals, yeasts, Dictyostelids, and plants. Two GSK-3 genes (α and β) have been cloned from vertebrates (Plyte et al., 1992).

GSK-3 has strikingly different behavior to other protein kinases. GSK-3 has a high basal activity within the cell and both insulin and Wnt stimulation lead to a decrease in kinase activity. In the case of insulin this is by activation of protein kinase B (PKB), which phosphorylates a serine residue in the N terminus, residue Ser-9 in GSK3β and Ser-21 in GSK3α, and inhibits GSK-3 activity. Wnt stimulation, on the other hand, acts on GSK-3β in a multiprotein complex that also includes axin, adenomatous polyposis coli-associated protein (APC), and β-catenin. GSK-3β phosphorylates all three of these proteins; however, phosphorylation of β-catenin leads to its degradation. Wnt stimulation inhibits the activity of GSK-3β within this complex, though mechanisms that may involve axin binding to the proteins Dishevelled (Li et al., 2001) and LRP-5 (Mao et al., 2001). This allows unphosphorylated β-catenin to accumulate in the cytoplasm and nucleus. By binding to TCF family transcription factors, nuclear β-catenin regulates transcription of target genes such as c-*myc* and cyclin D1. Mutations that perturb the function of the Axin-APC complex, such as truncation of APC or deletion of the GSK-3 sites of β-catenin, are present in 90% of colon cancers (Polakis, 2000).

Sequestration of GSK-3 within the axin complex does not appear to be sufficient to prevent cross-talk with components of the insulin pathway. PKB phosphorylation of GSK-3β in the axin complex has been observed after chronic and prolonged Wnt stimulation (Fukumoto et al., 2001), and alanine substitution of Ser-9 increases activity of GSK-3β within the Wnt pathway (Ding et al., 2000). PKB phosphorylation, however, does not elicit the Wnt response (Ding et al., 2000). This makes sense, as the effects of insulin and Wnt are different, even in cells responsive to both signals. So how is GSK-3 regulated and how is cross-talk between the Wnt and insulin pathways prevented?

Structural Mechanisms for Kinase Regulation

The structure of GSK-3 suggests a mechanism for PKB regulation of kinase activity; however, before considering this structure, it is useful to think about the structural basis of the regulation of other kinases. The MAP kinase family is regulated by kinase activation through the specific realignment of the residues that bind substrate and catalyze phosphate transfer. This "activation segment" is formed by phosphorylation at Thr-183 and Tyr-185, and the switch between inactive and active conformations results in greater than a 1000-fold increase in kinase activity (Canagarajah et al., 1997).

In contrast, the cAMP-dependent protein kinase (PKA) is constitutively phosphorylated with its activation segment held in an active conformation. PKA uses a pseudo-substrate mechanism to regulate kinase activity (Zheng et al., 1993). A pseudo-substrate is a short protein sequence that conforms to the structure of the kinase substrate, but as it lacks an appropriately placed serine, threonine or tyrosine residue, it cannot be phosphorylated. As a consequence, a pseudo-substrate is a competitive inhibitor of kinase activity. The pseudo-substrate of PKA is present on a separate protein, the regulatory subunit (R subunit), which binds the kinase catalytic domain (C subunit). Kinase inhibition is removed by dissociation of the R subunit due to binding of cAMP. Pseudo-substrate mechanisms are also employed by other kinases, for example protein kinase C, where the pseudo-substrate is present in the same protein as the kinase domain.

Structure of GSK-3β

Two groups, Dajani et al. (2001) and ter Haar et al. (2001), report the resolution of the GSK-3β crystal structure.

[1] Correspondence: a.harwood@ucl.ac.uk

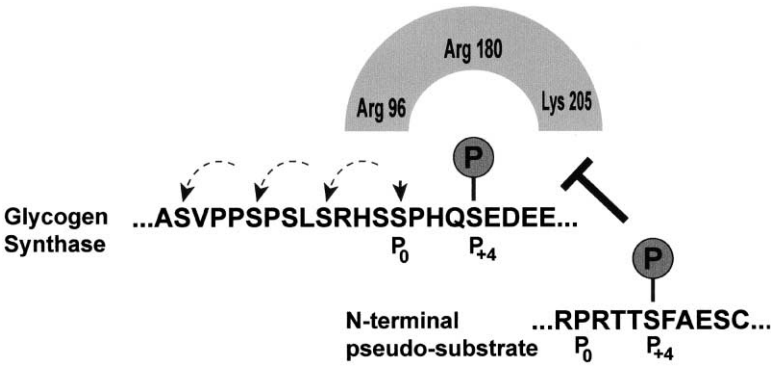

Figure 1. A Pseudo-Substrate Mechanism

The "priming" phosphate (P_{+4}) of substrates such as glycogen synthase interact with a binding pocket in GSK-3 formed by Arg-96, Arg-180, and Lys-205. Glycogen synthase is phosphorylated on a serine (P_0), which then forms the priming phosphate for sequential phosphorylations (dotted lines). A pseudo-substrate within the N terminus can displace the substrate from the binding pocket.

Based on protein sequence, GSK-3 is closely related to CDK and MAP kinase families. Consistent with its primary sequence, GSK-3 conforms to the general structure of an activation segment protein kinase and much of its structure resembles MAP kinase. It differs from MAP kinase in the N-terminal portion of the kinase domain, which more closely resembles a tyrosine kinase, such as Src-kinase. This is interesting as GSK-3 has been reported to have both serine/threonine and tyrosine phosphorylation activity (Wang et al., 1994). The most surprising feature of the structure, however, is that it forms an activation-segment in the absence of any phosphorylation.

The majority of GSK-3 substrates are formed by prior phosphorylation from another kinase, an event known as "priming," to give the structure S/T-X-X-X-S/T-$P0_4$; where S/T corresponds to serine or threonine and X to any other residue. Priming phosphorylation occurs at position P_{+4} and GSK-3 transfers phosphate to position P_0 (Figure 1). In the case of glycogen synthase GSK-3 phosphorylation site priming is due to casein kinase II. Once this site is phosphorylated by GSK-3, it then acts as the priming phosphate to create an adjacent GSK-3 site. This then creates a third site, and subsequently generates a fourth GSK-3 site. This unusual substrate preference not only sets up the conditions for GSK-3 to promote the incorporation of a large number of phosphate moieties; it also forms the basis of its regulation by PKB. Both structures are resolved without substrate, but are bound to either a HEPES molecule (Dajani et al., 2001) or a phosphate ion (ter Haar et al., 2001). In both cases, these species interact with the positively charged side chain head groups of Arg-96, Arg-180, and Lys-205. In MAP kinase the equivalent three residues interact with phosphorylated Thr-183 residue, but in GSK-3 they are positioned within the substrate binding cleft such that they could bind the primed residue. In support of this, mutagenesis of residue Arg-96 greatly reduces phosphorylation of primed GSK-3 substrates (Frame et al., 2001). This structural feature may explain the preference of GSK-3 for primed substrates and furthermore substrate binding may act to stabilize the active conformation of the enzyme.

The presence of a specific recognition site for primed substrates suggests a pseudo-substrate mechanism for GSK-3 regulation. Here PKB phosphorylation of Ser-9 could mimic the priming phosphorylation at P_{+4}, but as there is no corresponding serine or threonine at P_0 GSK-3 phosphorylation would not occur (Figure 1). Consistent with this idea, peptides based on the sequence around Ser-9 compete for substrate phosphorylation in a dose-dependent manner, but only when Ser-9 is phosphorylated (Dajani et al., 2001, Frame et al., 2001). In the converse experiment, the Arg-96 mutant maintains activity after Ser-9 phosphorylation and hence is resistant to inactivation by PKB (Frame et al., 2001). Finally the residues between Ser-9 and the kinase domain form a loop long enough to reach the putative phospho-substrate interaction site (ter Haar et al., 2001). These results strongly suggest that insulin-induced phosphorylation can inhibit GSK-3 activity by generation of a pseudo-substrate.

Specificity of Signaling Pathways

Based on their observations, Frame, Cohen, and Biondi (2001) further suggest a mechanism for maintaining signal pathway specificity. They observe that although introduction of Arg-96 mutation causes a dramatic reduction in the ability of GSK-3 to utilize primed substrates, it has little effect on its phosphorylation of unprimed substrates. Furthermore, short peptides of less than eight residues derived from the pseudo-substrate block activity of wild-type protein against primed substrates, but leave unprimed ones unaffected. This suggests that GSK-3 may interact differently with the two forms of substrate and complements results obtained using a peptide known as FRATtide (Thomas et al., 1999). This peptide is derived from the FRAT/GBP protein found in vertebrates and is an inhibitor of GSK-3 activity in the Wnt signaling pathway. In contrast to pseudo-substrate peptides, FRATtide has little effect on GSK-3 phosphorylation of primed substrates, but inhibits unprimed substrates (Figure 2). This is best illustrated with tau, a protein substrate whose phosphorylation by GSK-3 is virtually unaffected by FRATtide in its primed form but is strongly inhibited when not subject to prior phosphorylation. These results again suggest that primed and unprimed may have different interactions with GSK-3.

Both axin and β-catenin proteins can be phosphorylated in vitro by GSK-3 without first carrying out a priming phosphorylation, and both are sensitive to FRATtide inhibition (Thomas et al., 1999). This raises the possibility that by binding to GSK-3, FRAT/GBP could selectively prevent phosphorylation of axin and β-catenin. This has also been seen by Farr et al. (2000) who again found that FRAT/GBP does not block phosphorylation of primed substrates, but does block GSK-3-mediated β-catenin

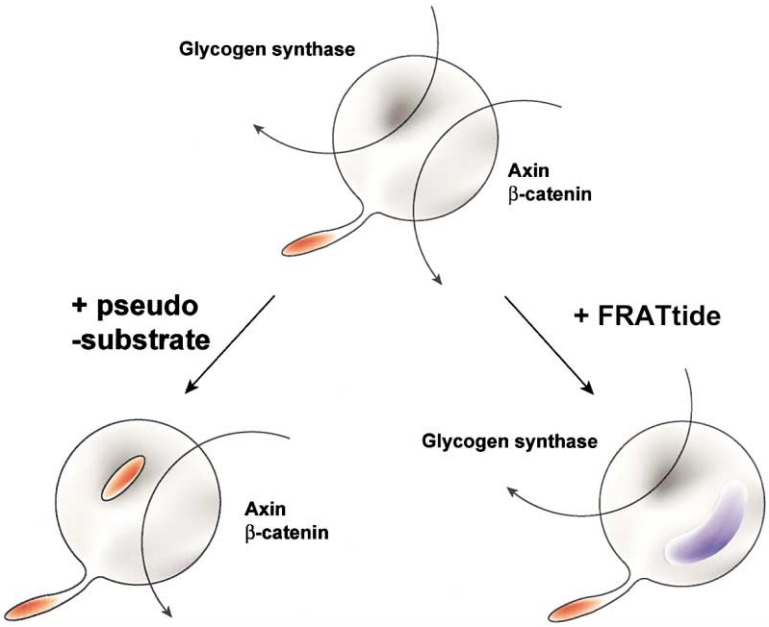

Figure 2. Differential Activity toward Primed and Unprimed Substrates

In vitro phosphorylation of glycogen synthase (a primed substrate) is inhibited by binding of a pseudo-substrate (red), whereas interaction with axin and β-catenin (unprimed substrates) is inhibited by binding FRATtide (blue).

degradation. Furthermore, Wnt stimulation via the protein Dishevelled has been suggested to promote entry of FRAT/GBP into the axin-GSK-3β complex (Li et al., 2001). FRAT/GBP may however have an alternative effect by interfering with GSK-3 binding to axin (Farr et al., 2000). These two putative roles could be combined if FRAT/GBP remains bound to GSK-3 after dissociation from the axin and perhaps prevents phosphorylation of axin and β-catenin, while at the same time allowing GSK-3 activity toward glycogen synthase and regulation by insulin. In this way, FRAT/GBP could biochemically restrict the effect of Wnt stimulation but allow GSK-3 to proceed with its other cellular functions.

The critical test for this restriction mechanism is to demonstrate that it can occur within the cell, and it must be pointed out that it has not been demonstrated that either axin or β-catenin is unprimed in vivo. Furthermore, GSK-3 has 400- to 1000-fold less activity against unprimed peptide substrates than primed (Frame et al., 2001), a situation that is suggested to pertain for protein substrates in vitro (Thomas et al., 1999). It is therefore unclear whether unprimed proteins substrates are true cellular GSK-3 targets, although bringing GSK-3 and its substrates together in a complex could promote phosphorylation of what may be a biochemically unfavorable reaction. Examination of the in vivo behavior of the Arg-96 mutant should establish the physiological relevance of GSK-3 activity against unprimed substrates. A final problem is that FRAT/GBP has not been found outside the vertebrates, this means that either FRAT/GBP inhibition is not the only mechanism to regulate GSK-3 activity in the axin complex, or other FRAT-like proteins await discovery. In principle, however, any mechanism that displaces GSK-3 or an unprimed β-catenin protein from the axin complex could prevent its phosphorylation.

Tyrosine Phosphorylation and Dimerization

The structure of GSK-3 also sheds light on other mechanisms of GSK-3 regulation. In addition to being under negative regulation, GSK-3 can be activated and a tyrosine kinase, ZAK1, has been identified which activates GSK-3 activity during *Dictyostelium* development. In addition, phosphorylation at position Tyr-216, the equivalent of Tyr-185 in MAP kinase, has been observed in GSK3β (Harwood, 2000). However, if phosphorylation of this tyrosine is not required for generation of the activation segment, what role could it play? In the conformation seen in the GSK3β structure, the unphosphorylated Tyr-216 side chain is directed downward toward the bottom of the substrate binding cleft, an orientation which could impede, but not totally block, entry of a phosphorylated residue at position P_{+4} of the substrate (Dajani et al., 2001; ter Haar et al., 2001). By comparison to MAP kinase, phosphorylation could move Tyr-216 away from the phosphate binding pocket. This suggests that while not necessary for GSK-3 activation, phosphorylation could facilitate substrate binding and hence kinase activity.

The crystal structure of GSK3β shows it to be a tightly opposed dimer. Dimers have been observed in crystals of other protein kinases, including the MAP kinase ERK2. Here, dimerization leads to accumulation of ERK2 in the nucleus (Cobb and Goldsmith, 2000). The GSK-3β dimer is in a head-to-tail orientation with the surface of one monomer interacting with the predicted substrate binding site of the other. This could block entry of substrate into the dimer. Interestingly, the side chain of Tyr-216 participates in the dimer interface and phosphorylation of Tyr-216 would be predicted to interfere with dimerization by causing rotation to the "up" conformation (Dajani et al., 2001). This offers the potential for a novel mechanism of GSK-3 regulation and a possible additional role for tyrosine phosphorylation. None of these predictions have been verified either in vitro or in vivo.

Concluding Remarks

The structure, biochemistry and effect of mutagenesis suggest a more complex regulation of GSK-3 than previously imagined. This protein kinase is able to process at least two different stimuli and deliver distinct out-

comes. This in part may be due to compartmentalization of GSK-3 action within the cell, but what these recent results suggest is that specificity could also be achieved by intrinsic properties of GSK-3. These could allow a common pool of GSK-3 to participate in multiple pathways, but limit inappropriate cross-talk. These results may also lead to the design of function-specific drugs that disrupt some actions of GSK-3, but leave others intact. To date much of our knowledge of GSK-3 activity is based on in vitro studies. With the GSK-3 structure, we are now in the position to design mutant proteins that lack some but not all of the kinase properties and test our knowledge gained in vitro in the context of the cell.

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