Glycogen Synthase Kinase-3: Properties, Functions, and Regulation

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Received December 21, 2000

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I. Introduction

Glycogen synthase kinase-3 (GSK-3) is a serine−threonine kinase encoded by two isoforms in mammals, termed GSK-3α and GSK-3β.[1] Initially GSK-3 was implicated in muscle energy storage and metabolism, but since its cloning, a more generalized role in cellular regulation has emerged, highlighted by the wide array of substrates controlled by this enzyme that includes cytoplasmic proteins and nuclear transcription factors. GSK-3 targets encompass proteins implicated in Alzheimer's disease, neurological disorders, and cancer. GSK-3 genes are highly conserved and have been identified in every eukaryote investigated to date. Studies of GSK-3 homologues in various organisms have revealed physiological roles for the enzyme in differentiation, cell fate determination, and spatial patterning to establish bilateral embryonic symmetry. GSK-3 plays an important role in at least two signal transductory systems, namely, the Wnt/wingless and PI'3 kinase pathways which influence proliferation and cell survival, respectively. This review focuses on the biochemical and functional properties of GSK-3 and discusses recent advances in understanding the involvement of this unusual enzyme in human pathophysiology. Such insights have uncovered new paradigms in signaling and point to GSK-3 as a potential therapeutic target.

A. Isolation and Characterization of GSK-3

GSK-3 was originally identified as one of five protein kinases that phosphorylate the rate-limiting enzyme of glycogen synthesis, glycogen synthase (GS).[2−4] Following peptide sequencing of enzyme purified from skeletal muscle, a screen of a rat brain cDNA library revealed that GSK-3 is encoded by two independent genes, GSK-3α and GSK-3β, with molecular weights of 51 and 47 kDa, respectively.[1] Purified GSK-3α and GSK-3β exhibit similar biochemical and substrate properties.[5] The two genes display 85% overall sequence identity (see Table 1), which is even higher in the catalytic domain (93%). Chromosomal mapping identified the cytological location of human GSK-3α as 19q13.2, whereas human GSK-3β maps to 3q13.3.[6] The GSK-3β gene promoter contains several CAAT boxes as well as positive and negative transcriptional response elements.[7] Northern blot analysis has shown that GSK-3α and GSK-3β are somewhat variably expressed in different mammalian tissues, but there is poor correlation between the levels of mRNA and protein.[1,8] This may represent differential modes of transcriptional and translational regulation for the two isoforms.

II. Regulation of GSK-3 Activity

Besides glycogen synthase, a number of other GSK-3 substrates have been identified.[9,10] These include translation initiation factor eIF2Bϵ, PKA, phosphatase subunit RGI, and ATP-citrate lyase (see Table 2). GSK-3 also phosphorylates several transcription factors such as CREB, c-Jun, c-Myc, c-Myb,

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10.1021/cr000110o CCC: $36.00
Published on Web 07/04/2001

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as well as heat shock transcription factor HSF-1 in vivo[11] and in vitro.[12] Brain-associated proteins such as amyloid precursor protein, Tau, and neurofilament protein are also targets of GSK-3.[13] Phosphorylated Tau has a lower affinity for microtubules, and hyperphosphorylation appears to promote association into paired helical filaments, a pathological feature of Alzheimer's disease.[14−16]

GSK-3 is a serine/threonine-selective kinase that recognizes and phosphorylates the consensus sequence SXXXS(P) in certain proteins.[17] For some substrates, like glycogen synthase, phosphorylation by GSK-3 requires prior phosphorylation of a serine residue C-terminal to the target site. The specificity of this priming site has been probed with a synthetic peptide corresponding to a GSK-3 phosphorylation site in eIF2Bϵ. Peptides phosphorylated at serine or

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threonine in the priming position were conducive to subsequent GSK-3 phosphorylation.[18] Phosphotyrosine, however, was not a specificity determinant for GSK-3. Although prior phosphorylation of the consensus site seems to be a common mode by which GSK-3 targets its substrates, this priming mechanism is not requisite for all substrates. For example, GSK-3 phosphorylation of β-catenin does not appear to require priming (see section IV).[19] This differential requirement for substrate interaction may provide a control mechanism by which the specificity of signal transduction is influenced by the contemporaneous activation of other signaling pathways (which modulate the activity of the various priming kinases).

In studies in fruit fly and frog embryos, expression of kinase-deficient GSK-3 induces phenotypes which mimic Wnt/wingless (Wg) signaling and result in the stabilization of β-catenin/armadillo (see sections III and IV; Figure 1).[20−24] This is consistent with a model in which GSK-3 is constitutively active and negatively regulated by upstream signals. In *Drosophila*, the GSK-3 homologue is termed shaggy or zeste-white3 (herein denoted as $Zw3^{sgg}$). A series of epistasis analyses indicated that $Zw3^{sgg}$ activity is inhibited by Wg protein, an effect mimicked by expression of downstream components of Wg signaling, namely, frizzled-2 (DFz-2) and dishevelled (Dsh).[25] The degree of $Zw3^{sgg}$ kinase activity regulates the phosphorylation of armadillo, which in turn modulates its stability (see below). The molecular mechanism by which activity of $Zw3^{sgg}$ is regulated by Wg is unclear. Work in our laboratory has shown that Wg-dependent inactivation of $Zw3^{sgg}$ is accompanied by serine phosphorylation, although the identity of the relevant kinase acting on GSK-3 is currently unknown. The most proximal upstream regulatory protein in this pathway is dishevelled. This protein is also phosphorylated by a Dsh-associated kinase (DAK). The finding that casein kinase-Iϵ (CK−Iϵ) and casein kinase-II (CK−II) can bind Dsh suggested a possible role for the casein kinases (and DAK) in the transduction of

Table 1. GSK-3 Is Widely Conserved throughout Evolution[a]

		full-length protein% identity (similarity)		catalytic domain% identity (similarity)	
species	homologues	GSK-3α(human)	GSK-3β (human)	GSK-3α (human)	GSK-3β (human)
Homo sapiens	GSK-3α	100	83 (89)	100	91 (97)
Homo sapiens	GSK-3β	76 (84)	100	91 (97)	100
Rattus norvegicus	GSK-3α	93 (93)	83 (89)	99 (99)	91 (96)
Rattus norvegicus	GSK-3β	76 (84)	92 (92)	90 (96)	99 (99)
Mus musculus	GSK-3β	76 (84)	93 (93)	91 (97)	100 (100)
Danio rerio	ZGSK-3α	80 (86)	78 (84)	92 (96)	91 (97)
Danio rerio	ZGSK-3β	77 (84)	92 (93)	90 (96)	99 (99)
Xenopus laevis	XGSK-3β	75 (83)	90 (92)	90 (96)	91 (97)
Ciona intestinalis	GSK-3	80 (87)	83 (89)	90 (96)	91 (97)
Paracentrotus lividus	SUGSK-3	71 (83)	79 (88)	84 (92)	87 (94)
Drosophila melanogaster	zw3^{sgg}	71 (81)	79 (87)	82 (92)	85 (94)
Hydra vulgaris	GSK-3	66 (80)	72 (86)	78 (91)	80 (92)
Caenorhabditis elegans	gsk-3	77 (87)	77 (87)	80 (89)	80 (89)
Petunia hybrida	shaggy	69 (82)	67 (80)	80 (89)	81 (89)
Nicotiana tabacum	shaggy	62 (76)	69 (80)	71 (84)	73 (85)
Arabidopsis thaliana	ASK/ATK1	62 (76)	65 (81)	70 (85)	71 (87)
Oryza sativa	OSK	63 (77)	65 (80)	71 (84)	71 (85)
Medicago sativa	MSK1−3	62−63 (76)	64−68 (76−79)	70−71 (83−84)	70−72 (83−85)
Dictyostelium discoideum	gskA	58 (72)	61 (73)	69 (85)	69 (83)
Schizosaccharomyces pombe	Skp1	60 (75)	64 (78)	58 (73)	67 (82)
Saccharomyces cerevisiae	MCK1/MRK1	45−58 (62−76)	53 (68)	54−60 (73−77)	54−57 (72−76)
Plasmodium flaciparum	GSK-3	53 (74)	54 (72)	56 (76)	58 (76)
Trifolium repens	GSK-3	71 (84)	68 (81)	71 (84)	71 (85)
Ricinus communis	shaggy-like	74 (88)	70 (84)	74 (88)	74 (89)
Cicer arietinum	GSK-3	66 (77)	71 (82)	73 (85)	74 (85)

[a] GSK-3 homologues are represented as percentage identity and similarity (in parentheses) over the full-length and catalytic region of human GSK-3α and GSK-3β proteins. GSK-3 proteins share a remarkable degree of identity within the catalytic domain, and in vertebrates, the identity over this region is greater than 90%. GSK-3 family members have also been identified in numerous invertebrates (nematode, fruit fly, ascidians, ticks, sea urchin, and malaria parasite), plants (*Arabidopsis*, *Hydra*, garden petunia, rice, clover, sunflower, alfalfa, chickpea, and tobacco), and fungi (baker's yeast, fission yeast, and slime mold).

Table 2. Protein Substrates of GSK-3[a]

substrate	specific phosphorylation sequence	ref
acetyl CoA carboxylase	n.d.	154
adenomatous polyposis coli protein	FXVEXTPXCFSRXSSLSSLS	155
ATP-citrate lyase	LLNASGSTS**T**PAP**S**RTASFSESR	156
axin	**S**AND**S**EQQ**S**^{330}	90
	SDAD**T**LSL**T**^{341}	
	SLTD**S**^{343}	
β-catenin	D**S**GIH**S**GAT**T**TAP**S**	19
C/EBPa	**T**PPP**T**PVP**S**P	157
c-Jun	EEPQTVPEMPGE**T**PPL**S**PIDME**S**QER	158
c-Myb	APV**S**CLGEHHHC**T**P**S**PPVDH	159
c-Myc	DIWKKFELLP**T**PPL**S**PSRRSG	160
CREB	KRREIL**S**RRPSYR	161
cyclin D1	EEVDLAC**T**PTDVRDVDI	162
eIF-2B translation factor	DSEELD**S**RAGSPQLDDIKVF	163
G subunit of phosphatase 1	AIFKPGF**S**PQP**S**RRGSSESSEEVY	164
glycogen synthase	RPA**S**VPP**S**PSL**S**RHS**S**PHQSEDEE	161
heat shock factor-1	KEEPP**S**PPQSP	12
inhibitor-2	GLMKIDEPS**T**PYHSMIGDDDDAYSD	165
insulin receptor substrate 1	n.d.	166
JunD	**S**PPL**S**PIDME**T**QER	167
L-myc	DIWKKFELVP**S**PPT**S**PPWGL	168
NF-ATc	n.d.	169
RII subunit of cAMP-dependent protein kinase	LREAR**S**RA**S**TPPAAPPS	170
Tau	TPPK**S**PSAAK	
	SPVVSGDT**S**PR	171

[a] GSK-3 substrates are listed with the peptide sequences surrounding their phosphorylation sites. GSK-3-targeted serines and threonines are indicated in bold font. Sites of "priming" prephosphorylation required for GSK-3 phosphorylation (if known) are underlined. Some substrates have numerous GSK-3 phosphorylation sites in multiple regions and could all not be listed here. The notation "n.d." indicates that the phosphorylation sequence is undetermined.

the wingless signal.[26,27] Dishevelled lives two functional lives. In addition to modulating the wingless pathway, it is also a component in regulation of planar polarity in *Drosophila* which involves the JNK/SAPK pathway. Interestingly, DAK dramatically enhances the function of Dsh in the Wnt pathway while inhibiting in the establishment of planar polarity. Dsh thus appears to act as a teeter-totter in controlling flux through two distinct signaling systems.

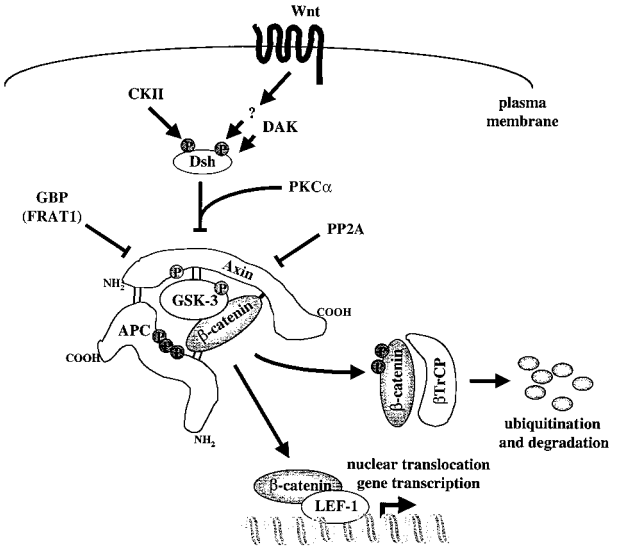

Figure 1. Role of GSK-3 in the Wnt signaling pathway. In the absence of a Wnt signal, GSK-3 in the cytoplasm interacts with β-catenin, axin, and APC. GSK-3 phosphorylates these proteins, leading to the Slimb/βTrCP-mediated ubiquitination and proteolytic degradation of β-catenin. Upon binding of Wnt by a seven-transmembrane domain receptor, dishevelled is activated resulting in the down regulation of GSK-3 kinase activity. These, and other, signaling events and interactions stabilize β-catenin, leading to the activation of LEF-1/Tcf-mediated transcription (see text for further details). Abbreviations: casein kinase-II (CKII), protein kinase C-α (PKCα), dishevelled (Dsh), GSK-3-binding protein (GBP), protein phosphatase-2A (PP2A), adenomatous polyposis coli protein (APC).

Under resting conditions, GSK-3/Zw3^{sgg} is highly phosphorylated at a tyrosine residue located within the phosphorylation loop proximal to the ATP binding site (in an analogous position to the activating threonine and tyrosine phosphorylation sites in MAPKs).[28,29] Constitutive phosphorylation of this tyrosine is important for kinase activity. The *Schizosaccharomyces pombe* homologue of GSK-3 termed Skp1 is also phosphorylated on a tyrosine residue in the P-loop, and this phosphorylation is required for its efficient activity.[30] In mammalian cells, GSK-3α is constitutively phosphorylated at tyrosine-279, GSK-3β at tyrosine-216.[28] Dephosphorylation of these residues is accompanied by kinase inactivation. Mitogens lead to inactivation of GSK-3 (see section V). Since this effect is reversible by treatment with serine/threonine-specific phosphatases, mitogen-induced inhibition is believed to be due to increased serine/threonine phosphorylation of GSK-3 rather than tyrosine dephosphorylation.[31] Indeed, serine 9 of GSK-3β (and serine 21 in GSK-3α) is phosphorylated upon insulin stimulation.[32−34] Several protein kinases target serine 9/21 including PKB/Akt, pp90 Rsk and cyclic AMP-dependent protein kinase (see section V).[33,35−37]

Inactivation of GSK-3 by phosphorylation at serine 9/21 or via the Wnt pathway invokes different cellular consequences. The mechanism underlying this signal-dependent regulation is likely via the existence of distinct pools of or complexes containing GSK-3. For example, in cells, cytoplasmic β-catenin is associated with a scaffold protein called axin, which also binds GSK-3. The population of GSK-3 molecules bound to axin is selectively sensitized to Wnt signaling. However, a different pool of GSK-3 appears to be responsible for transducing the effects of insulin/PI 3′ kinase signaling. The finding that axin-associated GSK-3 is insensitive to insulin-induced serine 9 phosphorylation but instead is coupled to Wnt activation further supports this notion.[38] Since GSK-3 has multiple substrates, the existence of distinct pools of responsive kinase molecules may allow specific responses to certain agonists, leaving other targets unaffected. This idea is further strengthened by the finding that inhibition of GSK-3 by certain Wnt signaling components (such as FRAT-1/GBP, see section III), leads to the inhibition of GSK-3-induced phosphorylation of β-catenin without effecting the phosphorylation of other substrates such as glycogen synthase.[39]

In mammalian cells, protein kinase C-like activity (PKC) has been implicated in Wg effects on GSK-3 activity.[40,41] Certain PKCs phosphorylate GSK-3 in vitro, resulting in inactivation. Wg-induced inactivation of GSK-3 is sensitive to both the PKC inhibitor Ro31−8220 and prolonged pretreatment of 10T1/2 fibroblasts with phorbol ester, suggesting a role for PKC in the regulation of GSK-3. In addition, it has been demonstrated that serum enhances the effect of certain GSK-3 inhibitors on the accumulation of cytoplasmic β-catenin through the activation of PKC.[42] However, since Wnt-induced accumulation of cytoplasmic β-catenin is only partially inhibited by PKC inhibitors, PKC likely serves to enhance the effects of Wnt signaling rather than being directly coupled to this pathway.

III. GSK-3 Homologues

The transmission of Wnt signal involves a cascade of events including modification and secretion of Wnt protein, its binding to cell surface frizzled receptors, activation of cytosolic intracellular components, and culminating in transcriptional activation of target genes in the nucleus. The Wnt signaling pathway is highly conserved and has been studied in a wide range of organisms. Likewise, GSK-3 homologues have been isolated from a number of organisms (Table 1), and roles have been discerned in early development as well as in cell fate decisions in *Dictyostelium*, *Drosophila*, Zebrafish, *Xenopus*, and mammals.[4] Besides its involvement in cell fate and establishment of bilateral symmetry in animal embryos, other roles for GSK-3 have also been implicated in lower eukaryotes. For example, the GSK-3 homologue in *Schizosaccharomyces pombe*, skp1, may play a role in cytokinesis.[30]

A. *Dictyostelium* gsk-A

The slime mold *Dictyostelium discoideum* harbors a single GSK-3-related gene termed gsk-A that shares over 70% identity with human GSK-3β.[43] Whereas in other systems the regulation of GSK-3 is inhibitory, *Dictyostelium* GSK-3 has been shown to be activated. Although gsk-A is not involved in the early development of *Dictyostelium*, it plays a critical role in specifying cell fate in a cAMP-dependent

process that controls differentiation into spore or stalk cells.[44] During normal development, aggregated progenitor cells differentiate into three cell types: prespore, prestalk-A, and prestalk-B. In *gskA*$^-$ mutant cells, differentiation proceeds with a reduction proportional to the prespore cell population and enhanced formation of prestalk-B cells. Since extracellular cAMP levels affect the differentiation process in a similar manner, it was hypothesized that gsk-A regulates cAMP responses in aggregated cells. Recent findings indicate that extracellular cAMP interacts with the cAR3 serpentine receptor to regulate the activity of a tyrosine kinase, ZAK-1.[45−47] In prespore cells, the activation of ZAK-1 increases tyrosine phosphorylation and the activity of gsk-A, resulting in spore cell differentiation, while inhibiting stalk cell formation. Cells mutant for cAR3 or ZAK-1 exhibit phenotypes similar to gsk-A null cells. Prestalk cells carry cell-type-specific cAR4 receptors in addition to cAR3. In these cells, interaction between the cAMP and cAR4 leads to the inhibition of gsk-A, resulting in the development of prestalk cells, while spore formation is inhibited due to lack of ZAK-1 activation. One of the targets of cAMP activation of gsk-A is a *Dictyostelium* STAT transcription factor.[48] GSK-A phosphorylation of this protein at a serine residue enhances nuclear export, causing functional inactivation of the STAT.[48] The physiological significance of STAT regulation by GSK-A with respect to cellular differentiation has yet to be addressed.

B. Sea Urchin GSK-3

Urchin GSK-3 is 88% similar to its vertebrate homologues (94% in the catalytic domain) and has been implicated in the establishment of animal-vegetal (A−V) axis in early development of sea urchin. During the embryogenesis, animal cells give rise to ectodermal tissues whereas vegetal cells carry a mesodermal and endodermal fate.[49] Overexpression of wild-type GSK-3 results in the animalization of the embryos by giving rise to ectodermal phenotype.[50] By comparison, inhibition of GSK-3 (by treating embryos with lithium, see section VI), ectopic expression of kinase-dead GSK-3 or mutant β-catenin, promotes vegetal cell fate. This implies that in animalized cells the constitutive activity of GSK-3 inhibits accumulation of β-catenin, thus promoting an ectodermal fate for these progenitors. Conversely, in the vegetal region, inhibition of GSK-3 by developmental cues induces an accumulation of β-catenin, resulting in transcriptional regulation of downstream genes required for establishing the endoderm and mesoderm lineages.[47] The similar effects of ectopic expression of GSK-3 homologues from both *Xenopus* and sea urchin illustrates the conserved function of the Wnt pathway in regulating the specification of bilateral symmetry in vertebrates and invertebrates.

C. *Caenorhabditis elegans* gsk-3

C. elegans gsk-3 displays a high degree of similarity to vertebrate GSK-3 homologue and has been assigned roles in endodermal induction and spindle orientation.[51,52] Initial endodermal induction occurs at the four-cell stage when the posterior blastomere (P2) induces a neighboring blastomere (EMS).[53] This polarization results in two differentiated daughter cells, E and MS. Eventually, E gives rise to endodermal structures while MS forms the mesoderm. One of the important genes that controls the endodermal induction is Pop-1, which has been shown to be a component of the Wnt signaling pathway. Unlike LEF-1/Tcf, which acts as transcriptional activator in the presence of upstream signals in other species, Pop-1 acts as a repressor for the endoderm induction. Previous findings have suggested that a Wnt signal from a P2 cell triggers gsk-3 activation. Activated gsk-3 then acts positively in the regulation of wrm-1/armadillo and, in turn, relieves the Pop-1-mediated transcriptional repression in the responding cell, named E. The inhibition of the repressor does not occur in the EMS cell; therefore, the specification of endoderm fate is limited to the E cell. While Wnt signaling in *C. elegans* does not reflect a typical Wnt pathway as established for vertebrates, another β-catenin homologue, Bar-1, interacts with Pop-1, induces LEF-1/Tcf promoter activity, and is a possible candidate in the regulation of endodermal induction.[54] Like β-catenin in higher vertebrates, Bar-1 contains putative GSK-3 phosphorylation sites in its N-terminal sequence that are possibly responsible for the transmission of Wnt signal. In response to a Wnt signal, gsk-3 also regulates spindle orientation via the cytoskeleton. However, the genes that regulate endodermal fate do not appear to play any role in the cytoskeletal-related processes.[52] Hence, it seems likely that gsk-3 serves as a branching point for selectively regulating two Wnt-dependent processes during nematode development.

D. *Drosophila* Shaggy/Zeste-White3

Drosophila represents the archetypal organism for understanding the Wnt/wingless pathway. In flies, the GSK-3 homologue, Zw3^{sgg}, was originally characterized as a segment-polarity.[55,56] Mutation of this gene in *Drosophila* embryos induces a phenotype in which the embryos lack most of the ventral denticles normally present in the anterior region of each segment. Sequence comparison (90% identity in the kinase domain) as well as genetic studies demonstrated remarkable similarity between GSK-3/Zw3^{sgg} in mammals and insects.[4,37−59] Zw3^{sgg} is essential for embryonic patterning as well as in events such as mesoderm formation and cardiogenesis in *Drosophila*.[60] Using genetic and biochemical approaches, various components of Wg signaling have been ordered within the pathway. For example, the embryonic phenotypes of armadillo and dishevelled mutants are very similar to the disruption of wingless, whereas Zw3^{sgg} has a mutant phenotype very similar to that of embryos in which wingless protein has been expressed in all cells. These data also support the notion that the functions of Zw3^{sgg} are antagonized by Wg signaling. Mutations in Wg and zw3^{sgg} have opposite effects on cell fate determination and armadillo protein levels.[20,61]

As described in section IV, the *Drosophila wingless* (wg) gene encodes a secreted glycoprotein homologous

to vertebrate Wnt proteins.[62] Wg protein is capable of inhibiting GSK-3 activity in rat 10T1/2 fibroblasts, inferring a high degree of conservation in Wnt/Wg signaling between species.[40] Wg has been assigned distinct roles in processes such as embryonic segmentation and imaginal disk patterning.[57,63] In *Drosophila* cultured cells, frizzled-2 (Dfz-2) was identified as a Wg receptor with a cysteine-rich extracellular domain (CRD) followed by seven transmembrane domains, similar in structure to G-protein-coupled serpentine receptors (direct evidence for a G-protein component in Wg signaling has yet to be demonstrated). Other well-studied components of the wingless pathway include dishevelled (dsh), a scaffold protein termed axin, and armadillo. The *armadillo* gene encodes the *Drosophila* homologue of β-catenin. As mentioned above, unlike the other components of the Wg signaling, Zw3^{sgg} plays an inhibitory role.[57] Secreted Wg protein is received by neighboring cells, and a signal transduction cascade is initiated.[62] The mechanism by which the Wg signal is transmitted across the membrane is incompletely understood. In addition to interaction of Wg with the extracellular CRD region of *D*fz-2, a single-pass transmembrane protein, arrow, has also been identified that can transduce a signal to Dsh.[64] In embryos and cultured cells, Wg signal leads to the hyperphosphorylation of Dsh protein. "Activated" Dsh acts to block the function of Zw3^{Sgg} and *D*-axin (the *Drosophila* homologue of axin), leading to decreased phosphorylation of armadillo (see section IV). Axin is a critical element in the regulation of β-catenin/armadillo levels in vertebrates.[65] *D*-Axin negatively regulates Wg signaling by suppressing intracellular levels of armadillo via promotion of phosphorylation of this protein by Zw3^{Sgg}. *D*-Axin acts as a scaffold protein and binds Zw3^{Sgg}, armadillo, and APC (adenomatous polyposis coli protein), forming a regulatory complex (see section IV).[25,66,67] Wg signaling reduces phosphorylation of armadillo, allowing this protein to accumulate in the cytoplasm, which, in turn, facilitates interaction with members of the LEF-1/Tcf transcription factor family. In cultured cells armadillo/β-catenin plays two roles. A membrane-associated population is involved in cell adhesion and participates in the formation of adherens junctions by binding to E-cadherin and α-catenin.[68] This function has been genetically isolated from the Wg signaling events using mutants of armadillo that impact adhesion but not the Wg pathway. Only soluble, cytoplasmic armadillo appears to be relevant to Wg responses.[69] Another zinc finger protein, teashirt, has also been implicated in the transmission of wingless signals.[70] Wg signaling promotes the phosphorylation and nuclear accumulation of teashirt, a process dependent upon binding of teashirt to the C-terminal portion of armadillo. Zw3^{sgg} is also associated with teashirt in a complex, and nuclear levels of teashirt are influenced by Zw3^{sgg}. Since teashirt expression is limited to embryonic central segments, it has been speculated that it regulates trunk formation.[71]

E. Zebrafish *Z*GSK-3

A crucial step during early vertebrate development is establishment of dorso-ventral polarity. A combination of maternally derived gene products as well as activation of the zygotic genome regulates early embryogenesis.[72] Evidence for the involvement of Wnt pathway during zebrafish development derives from microinjection experiments that demonstrate a requirement for the Wnt receptor, frizzled, in the specification of dorsal mesoderm.[73] Coexpression of GSK-3 reverses the effect of frizzled overexpression. At the protein level the two zebrafish GSK-3 homologues, *Z*GSK-3α and *Z*GSK-3β, exhibit 90% similarity with mammalian and *Xenopus* homologues.[74]

F. *Xenopus* XGSK-3

Transcripts encoding the known components of Wnt pathway are present in the *Xenopus* embryo as maternal products.[75−77] After fertilization, cortical rotation relocalizes these molecules by the movement of outer cytoplasm with respect to the inner. This rotation brings the dorsalizing components to the future dorsal side of the embryo. Thus, by the morula stage of development the decision for specifying the dorsal and ventral symmetry has already been established within the developing embryo. The dorsal side, which eventually gives rise to ectodermal cells, seems to be relatively abundant in β-catenin and Dsh.[47,78] However, the relative levels of GSK-3 are low in this region. One of the major advantages in using *Xenopus* embryos is that microinjection is relatively facile due to the size of the oocytes. Using this approach, misexpression of a dominant-negative form of GSK-3β in the ventral side of embryo resulted in the formation of secondary dorsal axis.[22] Consistent with this finding, ectopic expression of wild-type GSK-3 within the dorsal side caused ventralization. Results from ectopic expression of β-catenin are also in agreement with the canonical model of Wnt/Wg pathway.[79] These experiments clearly show the importance of the regulation of GSK-3 activity in the establishment of the embryonic polarity. Inhibition of GSK-3 in the dorsal region leads to accumulation of β-catenin, which then can activate the transcription of dorsal-specific genes, while active GSK-3 in the ventral side destabilizes β-catenin leading to ventral fates. A similar role for β-catenin during development is also observed during avian development where bilaterally symmetric distribution of nuclear β-catenin coincides with the process of gastrulation.[80] The involvement of GSK-3 in spatial determination is consistent with the role of Wnt signaling in the control of the neural plate and neural crest formation during the development of the avian embryonic nervous system.[81]

In *Xenopus* oocytes, a novel role of GSK-3 in the release of cell cycle arrest has recently been uncovered.[82] Prior to maturation the arrested oocytes contain constitutively active GSK-3; progesterone-mediated inhibition of this activity appears to contribute to meiotic maturation.

IV. Mammalian GSK-3

A. Wnt Signaling

Studies on the physiological functions of mammalian GSK-3 have taken important cues from the

role of the proteins in simpler organisms, especially with respect to its regulation by the Wnt pathway. In contrast, most of our knowledge of the regulation of GSK-3 by the phosphatidylinositol (PI) 3′ kinase pathway has derived from studies in mammals. That is not to say that this mode of regulation is not conserved in invertebrates, since $Zw3^{sgg}$ is inactivated by insulin signaling in a PI3′ kinase-dependent manner in *Drosophila* (Ali, A.; Woodgett, J. R. Manuscript in preparation).

In mammals, the Wnt signaling pathway is not only important in early embryonic development (see section III), but also a target for tumorigenesis.[83] Inappropriate activation of Wnt signaling has been observed in various human cancers, including hepatomas, colon carcinomas, melanomas, and uterine and ovarian cancers. The first member of the Wnt family, Wnt-1, was originally identified as an oncogene activated by the insertion of mouse mammary tumor virus in virus-induced mammary adenocarcinomas.[84] In addition to Wnts, the downstream signaling components β-catenin and FRAT (frequently rearranged in advanced T-cell lymphomas, also known as GSK-3 binding protein, GBP), both positive effectors of the pathway, have been identified as proto-oncogenes. The two negative regulators of the pathway, APC (adenomatous polyposis coli) protein and axin, function as tumor suppressors. Mutations of these Wnt signaling pathway components have a similar consequence—accumulation of β-catenin in the cytoplasm. Increased cellular levels of β-catenin induce activation of downstream genes, some of which, such as cyclin D1,[85] WISP-1,[86] and c-Myc,[87] have been implicated in cancer.

The molecular cascade of events in mammalian Wnt signaling is very similar to that in simpler organisms (see section III). In the absence of a Wnt signal, cytoplasmic β-catenin levels are kept low via GSK-3 phosphorylation, which targets β-catenin for ubiquitin-mediated degradation by Slimb/βTrCP (Figure 1).[88,89] As in invertebrates, axin also negatively regulates Wnt signaling and forms a complex with GSK-3, β-catenin, and APC.[90] Binding of axin to GSK-3 promotes GSK-3-dependent phosphorylation of both β-catenin and axin, the latter of which is necessary for axin stability. GSK-3 also phosphorylates APC, and this helps promote binding of APC to β-catenin.[91] This interaction is facilitated by axin, which associates with APC via a RGS (regulators of G-protein signaling) domain. Whether the interaction between APC and axin is critical for Wnt signaling remains to be established: although deletion of this domain has a dominant-negative effect on Wnt signaling in *Xenopus*, it is dispensable in *Drosophila*.[76,92]

In the presence of a Wnt signal, the protein complex containing GSK-3, axin, APC, and β-catenin is disrupted (possibly by recruiting the GSK-3 inhibitor GBP/FRAT1) and GSK-3 kinase activity is downregulated. GSK-3 phosphorylation of β-catenin is thus prevented, leading to accumulation of β-catenin and subsequent interaction with HMG-box transcription factors of the LEF-1/Tcf (leukemia enhancer factor-1/T cell factor) family and hence activation of specific target genes.[93]

B. Inhibition of GSK-3 by GBP/FRAT

A novel GSK-3-binding protein (GBP) was identified in *Xenopus*, and it functions in vivo to stabilize β-catenin and is required for the formation of the endogenous dorsal axis.[94] Injection of GBP antisense RNA prevents correct axis formation, while injection of GBP mRNA into the ventral region of the embryo leads to the formation of a second axis and therefore produces a tadpole with two heads. It has been shown, both in vivo and in vitro, that GSK-3 cannot bind GBP and axin simultaneously. When a limiting amount of GSK-3 is coexpressed with an excess of axin, addition of GBP reduces the amount of GSK-3 bound to axin.[95] Axin does not bind GBP in vitro, either directly or in the presence of GSK-3, and moreover, when axin, GSK-3, and GBP are coexpressed at comparable levels, immunoprecipitation of GBP brings down GSK-3 but not axin, demonstrating that these proteins cannot form a trimolecular complex. Li et al. (1999) detected a quaternary complex of overexpressed GSK-3, axin, Dsh, and GBP by immunoprecipitation in mammalian cells, but this quaternary complex was less stable than either the GSK-3/axin/Dsh or GSK-3/GBP complexes and possibly represents a transition state.[96] GBP has been demonstrated to inhibit GSK-3-mediated phosphorylation of protein substrates without eliminating the kinase activity of GSK-3 toward small peptides. Taken together, this leads to a model in which GBP functions in part to prevent GSK-3 binding to the axin/APC/β-catenin complex. However, a molecular mechanism to explain how GBP itself is regulated during Wnt signaling remains to be determined.

GBP is related to a mammalian protein termed frequently rearranged in advanced T-cell lymphoma 1 (FRAT1).[97] GBP and FRAT1 share three highly conserved domains, the most C-terminal of which interacts with GSK-3. The other two domains may interact with other proteins, such as Dsh. FRAT1 is an oncogene that is upregulated during infection with Moloney murine leukemia virus. FRAT1 expression confers a selective advantage to tumor cells that overexpress the oncogenes Myc and Pim1.[97] Expression of a 39 amino acid peptide corresponding to residues 188−226 of FRAT1 (containing the GSK-3 interaction domain) blocks the interaction of GSK-3 with axin and prevents GSK-3-catalyzed phosphorylation of axin and β-catenin, hence allowing accumulation of β-catenin. A second GBP homologue, FRAT2, mimics the effect of GBP on secondary axis formation.

GBP homologues have yet to be identified in *Drosophila* or *Caenorhabditis elegans*, and given the complete sequencing of these genomes, they are therefore unlikely to exist. This suggests that GBP may have evolved as a selective component of the Wnt pathway that acts as a further layer in the control of axin and GSK-3.

C. GSK-3 Phosphorylation of β-Catenin and APC

GSK-3 can phosphorylate β-catenin on serine and threonine at positions 33, 37, 41, and 45 (see Table

2).[98] An N-terminally truncated form of β-catenin that lacks the GSK-3 phosphorylation sites and thereby circumvents the normal requirement for Wnt signaling is stabilized constitutively in vivo and is not impaired in its ability to bind E-cadherin, α-catenin, and LEF-1/Tcf members. Mutations in β-catenin that impair or block phosphorylation of these sites have been identified in certain colon cancers (particularly those in which APC is wild type). These β-catenin mutants have been useful in delineating the physiological outcome of GSK-3-mediated β-catenin phosphorylation. For instance, when an N-terminally truncated human β-catenin mutant (DN87-β-catenin) is placed under the control of the keratin K14 promoter and expression is driven in the basal layer of the mouse epidermis, these cells become capable of inducing follicle morphogenesis normally occurring only in embryogenesis.[99] In addition, these mice develop hair follicle tumors such as trichofolliculomas and pilomatrichomas, which are often found in certain human familial polyposis syndromes.[100] An approach relying on Cre recombinase excision of β-catenin exon 3, which contains the GSK-3-phosphorylated residues, in the intestinal epithelium provided evidence for intestinal adenomatous polyps in mice at young ages.[101] Interestingly, the intestinal and colonic tumors in these mice resembled those of the APC^{D716} knockout.[102]

Mammalian studies have also suggested that a more complex mechanism for the regulation of β-catenin levels by GSK-3 involves APC.[91] APC is directly phosphorylated by GSK-3 via axin, which increases binding of APC to β-catenin and its subsequent degradation. β-Catenin contains a critical sequence, made up of the N-terminal armadillo repeats, that provides binding sites for the cytoplasmic fragment of E-cadherin, the 15- and 20-amino acid repeats of APC, the N-terminal region of LEF-1/Tcf, and a central domain of axin. E-cadherin, LEF-1/Tcf, and APC compete for binding to this region. Deletion mutagenesis indicates that all binding sites are located in armadillo repeats 3−8 of β-catenin, which form a tightly packed superhelix.

Xenopus extracts contain an activity that promotes the binding of β-catenin to axin at low concentrations.[103] This activity is inhibited by expression of RGS domain of axin, suggesting that it is either APC or APC complexed to other proteins. Also, axinDRGS (which cannot bind APC) does not bind β-catenin in extracts. Also, with purified components in vitro, APC accelerates the binding of β-catenin to axin, thereby recapitulating the effect of Wnt-stimulated extracts on binding between the two proteins. Taken together, these results identify APC as the activity stimulating the axin−β-catenin interaction. As a caveat, mutation of a *Drosophila* APC homologue does not affect Wnt/Wg function;[104] however, additional APC-like molecules exist in flies and there may be some functional redundancy between them.[105]

These general results are supported by experiments performed using SW480 colon carcinoma cells, which contain a truncated nonfunctional APC, showing that endogenous β-catenin is stabilized.[106] If APC is reintroduced in these cells, β-catenin is degraded. While endogenous axin (and a related protein conductin) is present in these cells, it is presumably incapable of regulating β-catenin phosphorylation in the absence of wild-type APC. In this way, overexpression of the axin-related protein, conductin, can induce β-catenin degradation in SW480 cells, while a conductin mutant which lacks the β-catenin binding domain cannot.[67] More recently, SW480 cells have been successfully utilized to further examine the functional interaction and stability of various β-catenin mutants.[107] These experiments showed that β-catenin mutants that do not interact with conductin (and were therefore resistant to degradation induced by ectopic expression of conductin) were, however, effectively degraded in the presence of wild-type APC. A double point mutant of β-catenin that bound to neither 15- nor 20-amino acid repeats of APC was still degraded by exogenous wild-type APC or a fragment of APC containing only the 20-amino acid and SAMP (for Ser, Ala, Met, and Pro) repeats, indicating that APC indeed does not need to bind directly to β-catenin to induce β-catenin degradation. This is consistent with previous findings that APC and β-catenin can interact indirectly via conductin/axin's binding of the APC SAMP repeats.[67] Finally, only a mutant β-catenin incapable of binding either the 15- and 20-amino acid repeats of APC or conductin binding was fully stable in the presence of exogenous wild-type APC. Thus, all possible interactions between β-catenin and APC appear functionally equivalent in the in vitro assays, namely, the direct interaction via either the 15- or 20-amino acid repeats or the indirect interaction via conductin/axin. However, it was found that APC with mutated SAMP repeats did not induce degradation of β-catenin in SW480 cells. The stability of β-catenin can thus be regulated if a degradation complex with conductin and APC is formed, that is, if β-catenin makes at least one interaction with either conductin or APC. However, direct interaction between conductin/axin and APC is required for this complex to be regulatory. This is supported by sequence data from human tumors, which showed that the majority of mutations in APC result in deletion of the SAMP repeats that provide an axin interaction domain.[108] Many of these same mutants retain 15- and 20-amino acid repeat units that allow β-catenin binding. Thus, the tumorigenic potential of mutated APC correlates with the loss of binding to axin rather than to β-catenin.

V. Regulation of GSK-3 by Other Signaling Pathways

Glycogen synthase contains four GSK-3-targeted phosphorylation sites.[4] Inhibition of GSK-3 reduces the level of phosphorylation of glycogen synthase, which then becomes active for converting glucose into glycogen. Stimulation of glycogen synthesis by insulin also involves the dephosphorylation of serine residues in glycogen synthase.

Several signal-dependent mechanisms have been proposed to lead to inhibition of GSK-3 without involvement of the Wnt pathway. Although some studies support the involvement of mitogen-activated protein kinase (MAPK) in GSK-3 regulation, other

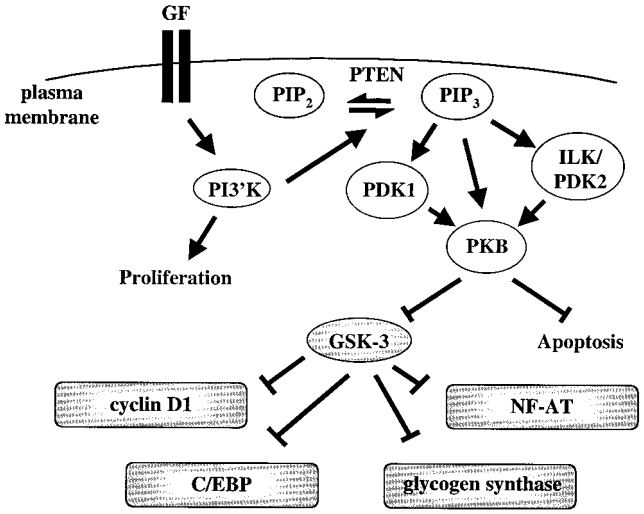

Figure 2. Role of GSK-3 in phosphatidylinositol 3-kinase signaling. Upon receptor tyrosine kinase activation, PI3′ kinase is recruited to the plasma membrane and phosphorylates phosphoinositides at the 3′-position of their inositol ring. This, in turn, recruits PH-domain-containing proteins such as PKB and the PDKs. Once phosphorylated by the PDKs, PKB is activated and phosphorylates GSK-3 leading to its inhibition. See text for details. Abbreviations: growth factor (GF), phosphatidylinositol 3-kinase (PI3′K), PI3′K-dependent protein kinase-1/2 (PDK1/2), protein kinase B (PKB), phosphoinositide (P_i), phosphoinositides(4,5)P_2 (PIP_2), phosphoinositides(3,4,5)P_3 (PIP_3), phosphatase and tensin homologue (PTEN), integrin-linked kinase (ILK).

data do not. For example, in rat adipocytes EGF activates MAPK without effecting GSK-3 activity.[109] Similarly, the inhibitors of MAPK have little effect on GSK-3 kinase activity. In vitro, the MAPKAPK-1 (MAPK-activated protein kinase-1)-activated ribosomal S6 kinases p70 (p70S6k) and p90 (p90rsk) inhibit GSK-3; however, specific inhibitors of these protein kinases fail to block signal-induced inhibition of GSK-3 in vivo.[33,35]

Many of the agonists that lead to suppression of GSK-3, including mitogens and insulin, do so in a wortmannin-sensitive manner, implicating PI3′K in the pathway. One of the most important physiological mediators of PI3′K signaling is the protein−serine kinase PKB, also termed AKT, which exists in three forms in mammalian cells (α, β, and γ). Mitogenic stimulation results in the phosphorylation of PKBa at two conserved sites: threonine 308 (T308) and serine 473 (S473)[110,111] These phosphorylation events are dependent on PI3′ kinase activity and are required for full activation of PKB (see Figure 2). In vitro, activated PKB phosphorylates serine 21 of GSK-3α and serine 9 of GSK-b, both modifications causing inactivation. Antibodies that selectively bind the phosphorylated sites on both GSK-3 subtypes have demonstrated a good correlation between PI3′K activity and phosphorylation of serine 21/9, although there are exceptions (see below).

Elevation of cyclic AMP within cells causes rapid activation of cyclic AMP-dependent protein kinase (PKA). Recently, PKA has been shown to directly bind and phosphorylate GSK-3 at serines 21 (GSK-3α) and 9 (GSK-3β), respectively.[36,37] Another enzyme implicated in GSK-3 inactivation is integrin-linked kinase (ILK) which, like PKB, is also regulated by PI3′K signaling. Human ILK phosphorylates and inhibits GSK-3 activity in vitro at an as yet undetermined site.[112] We have found that expression of *Drosophila D*ILK induces inactivation of $zw3^{sgg}$ by inducing serine phosphorylation. In cultured *Drosophila* cells, insulin induces serine phosphorylation of $zw3^{sgg}$, which is enhanced by overexpression of wild-type but not by kinase-deficient forms of *D*ILK (Ali, A.; Woodgett, J. R. Unpublished data). Whether this effect of *D*ILK is mediated directly or via a third-party protein kinase (such as PKB) is unclear.

Although GSK-3 can be inactivated by at least two distinct molecular mechanisms within cells, the relevant pathways appear to be independently insulated from each other to the point that effects on GSK-3 substrates are dependent upon the pathway by which the kinase is inactivated. This distinction is illustrated by genetic studies in *Drosophila* where overexpression of activated PKB fails to generate a naked cuticle phenotype, which is typical of activation of the wingless pathway or disruption of $zw3^{sgg}$.[113] Furthermore, unlike injection of GSK-3, microinjection of activated PKB does not result in formation of a secondary axis in *Xenopus*. These data might suggest that distinct populations of GSK-3 exist within cells that are somehow attentive to certain signals but not others. It is tempting to speculate that GSK-3 molecules sequestered into the axin/β-catenin complex have adapted to a specific function that is entirely independent of the bulk of the GSK-3 molecules within a cell.

VI. GSK-3β in Inflammatory Signaling

A. NF-κB Pathway

The Rel family of proteins represents a large group of trans-acting transcription factors that have been implicated in development, differentiation, and oncogenesis. The founding member of the family *v-rel* is the oncogene of reticuloendotheliosis virus strain T, which causes rapid and fatal leukemia in juvenile birds.[114] Cellular counterparts of *v-rel* have been cloned in numerous other organisms. The most highly characterized member of this family is NF-κB, which plays an important role as a regulator of the immune response and was first discovered as a constitutively nuclear transcription factor in mature B cells that bound to an element in the kappa immunoglobulin light-chain enhancer. NF-κB is, in fact, a group of binary complexes of proteins with related promoter-binding and transactivation activities. All members of this family contain a 300 amino acid N-terminal DNA-binding and dimerization domain, known as the Rel-homology domain, and most combinations of NF-κB homo- and heterodimers can be found in vivo. The prototypical NF-κB complex consists of a p65−p50 heterodimer. p65/RelA, RelB, and c-Rel stimulate transcription, whereas p50/NF-κB1 and p52/NF-κB2 serve primarily to bind DNA. Involvement of NF-κB in the immune system was confirmed by the discovery that treatment with inflammatory cytokines, such as tumor necrosis factor-α (TNF-α) and interleukin-1 (IL-1), facilitated its release from cytoplasmic I-κB inhibitor proteins, which resulted in trans-

location to the nucleus, binding of DNA, and induction of gene expression. In addition to the regulation of NF-κB activity at the level of subcellular localization, new information has emerged regarding the role of NF-κB phosphorylation in transactivation of the p65/RelA subunit and proteolytic processing of p105, the precursor for the p50 subunit.

An important physiological function for NF-κB was revealed by studies that interfered with NF-κB-induced gene expression (for instance, using actinomycin D or cycloheximide). Treatment of cells with TNF-α in the presence of such inhibitors potently induced cell death.[115] This effect was traced to an anti-apoptotic function for NF-κB. Thus, under normal circumstances, TNF-α induces both pro- and anti-apoptotic pathways. Inhibition of the latter switches the balance toward cell death. The consequences of suppression of this protective role is most strikingly manifested in the developing mouse embryo. Mouse embryos lacking the p65 subunit of NF-κB die during days 13−15 of gestation due to massive apoptosis of their developing hepatocytes.[116] However, p65-deficient mice that also lack TNF-α or one of its receptors (TNFR1) are viable.[117,118] Embryonic fibroblast cells from these mice have defects in NF-κB activation and heightened sensitivity to TNF-α-mediated apoptosis.[119] Several other mutations in components of the NF-κB pathway also exhibit this tell-tale phenotype (such as IKKb and T2K, two protein kinases involved in the phosphorylation of I-κB).[120,121]

Unexpectedly, mice lacking GSK-3β suffer from defects similar to those of mice that are mutant for essential components of the NF-κB pathway.[122] Hence, GSK-3β-deficient mice are morphologically normal up to approximately day 12 of embryonic development but die between days 13.5−14.5 due to massive liver degeneration and hepatocyte apoptosis. This death can be prevented by blocking the function of TNF-α. Embryonic fibroblast cells from GSK-3β mutant mice are highly sensitive to TNF-α-mediated apoptosis but not to other apoptotic stimuli. In addition, GSK-3β-deficient fibroblasts show a significant reduction in NF-κB DNA binding and reporter gene response following treatment with TNF-α or IL-1.

The mechanism by which GSK-3β impacts NF-κB activity is not known, but the kinetics of NF-κB nuclear translocation and the half-life of the regulatory protein I-κB-α is unaffected in GSK-3β mutant cells (see Figure 3). These data rule out an effect on the cascade of proteins that culminates in phosphorylation of I-κB and its degradation. Presumably then, GSK-3β is required for a later step in the signaling pathway, such as a direct or indirect requirement for phosphorylation of the NF-κB subunit, p65, or an as yet unidentified transcriptional co-activator of p65. Whatever the molecular mechanism, it is clear that this effect is specific for GSK-3β since GSK-3α is unable to compensate. This is in stark contrast to the lack of effect of loss of GSK-3β on the Wnt pathway. GSK-3β mutant mice do not exhibit overt disregulation of this pathway as might have been predicted, indicating that the Wnt pathway does not discriminate between these two GSK-3 isoforms. Indeed, to date, NF-κB activation is the only known differentiator between these two enzymes. While the finding that GSK-3β is required for NF-κB activation was surprising, there are other clues in the literature.

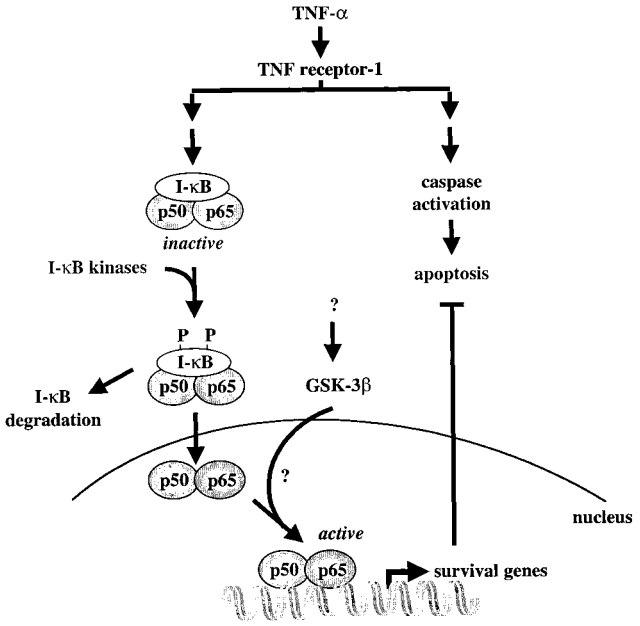

Figure 3. Model of NF-κB regulation by GSK-3β. GSK-3β is required for NF-κB transactivation independent of I-κB-α degradation and NF-κB nuclear translocation. See text for details. Abbreviations: nuclear factor-κB (NF-κB), tumor necrosis factor (TNF), inhibitor of NF-κB (I-κB).

B. *Xenopus* rel

Additional support for a connection between GSK-3 and NF-κB during vertebrate development has emerged from studies of *Xenopus laevis* development. The mRNA of at least one *Xenopus* member of the rel family, XrelA, is expressed in oocytes and early embryos. XrelA impacts the dorsoventral patterning process.[123] XrelA induces a ventralizing effect early in embryonic development and attenuates morphogenetic movements characteristic of dorsal mesoderm. XrelA RNA was also shown to reverse the strong dorsal axis-promoting effects of a dominant mutant of *Xenopus* GSK-3β. This complementation effect argues in favor of a p65 NF-κB homologue being downstream of GSK-3β, at least in some systems.

C. SCF Ubiquitin Ligase Complex

Recently, components of the ubiquitin proteasome pathway have been implicated as a common link between the Wnt and NF-κB signal transduction pathways. Ubiquitin-dependent proteolysis by the proteasome plays an essential role in a number of key biological processes, including cell-cycle progression, transcription, and signal transduction.[124,125] Frequently the target protein is first marked for degradation or processing by phosphorylation. The phosphorylated protein is then recognized and ubiquitinated in a process that requires three proteins: a ubiquitin-activation enzyme (E1), a ubiquitin-conjugating enzyme (E2), and a ubiquitin ligase (E3).

Ubiquitin is first attached to an E1 protein in an ATP-dependent reaction to form a high-energy thioester bond. The ubiquitin is then transferred from the E1 to an E2 enzyme, which functions in conjunction with an E3 protein to link ubiquitin to lysine residues in the targeted protein. A specific lysine residue in the conjugated ubiquitin can then attach to a second ubiquitin, and reiteration of this process results in the assembly of a polyubiquitin chain. The polyubiquitinated protein is recognized by the 26S proteasome and is subsequently degraded. Ubiquitination of both β-catenin and I-κB is targeted by F-box/WD40 repeat-containing proteins, such as *Drosophila* Slimb (supernumerary limbs) and its mammalian homologue β-TrCP, that are components of a class of E3 ligases, termed the Skp1/Cullin1/F-box (SCF) complex.[88,126−128]

A common structural feature between I-κB and β-catenin is that phosphorylation occurs on two closely located serines at positions 32/36 (I-κB-α) and 33/37 (β-catenin). This suggests that Slimb/β-TrCP recognizes a DS_PGXXS_P amino acid motif. The association between Slimb/β-TrCP and the substrates is specific because other F-box proteins neither interact nor promote ubiquitination. Since phosphorylation and degradation of β-catenin is mediated by a constitutively active GSK-3β and IkB is inducibly phosphorylated by IKKs in response to various extracellular stimuli, what then distinguishes I-κB and β-catenin? Perhaps it is only the kinases that are responsible for phosphorylation of these substrates: phosphorylated DSGXXS motifs on these substrates might be the signal for the common ubiquitination pathway through Slimb/β-TrCP. Another possible explanation is the differential use of E2 ubiquitin-conjugating enzymes.

Overexpression of a stabilized β-catenin mutant in which the GSK-3-targeted serines were altered to alanine induces activation of the NF-κB pathway.[129] The proposed mechanism of cross-talk between the GSK-3/β-catenin and NF-κB pathways has been suggested to occur via LEF-1/Tcf-dependent upregulation of βTrCP levels and facilitated ubiquitination of phosphorylated I-κB, since β-catenin signaling augments NF-κB activation by a constitutively active IKKb.

VII. Small Molecule Inhibitors of GSK-3

A. Lithium

The alkali metal lithium was first discovered in 1817, and over the ensuing period of time, lithium has been utilized in various formulations as a remedy for a multitude of human maladies.[130] With the seminal work of Australian physician/scientist, John Cade, and subsequent clinical studies by Mogens Schou in the early 1950s, lithium was introduced as an effective therapy for manic-depressive illness (bipolar affective disorder).[131,132]

More recently, lithium has also been shown to perturb the development of diverse organisms, including *Xenopus*, zebrafish, sea urchins, and *Dictyostelium* (see section III). For instance, in *Dictyostelium*, lithium alters cell fate determination, blocking spore cell formation and promoting stalk development.[133] In *Xenopus* embryos, lithium treatment causes an expansion of dorsal mesoderm, leading to formation of a second dorsal axis.[134] Treatment of sea urchin animal blastomeres with lithium causes them to display a morphology resembling that of isolated vegetal blastomeres.[135] Importantly, these effects have since been shown to be similar to embryonic patterning defects resulting from either disruption of the GSK-3 gene or overexpression of inactive forms of GSK-3 that have a dominant-negative activity.

The developmental and neuropsychiatric effects of lithium have been attributed to a variety of biochemical processes including modulation of G proteins and inhibition of inositol monophosphatase (IMPase), an enzyme important for recycling of myo-inositol in the phosphatidylinositide (PI) pathway.[136] Exposure to lithium reduces the level of myo-inositol in the rat brain[137] via noncompetitive inhibition of IMPase[138] and to a lesser extent inositol polyphosphate-1-phosphatase (INPP-1).[139] However, an alternative target of lithium was suggested by the close similarity between lithium action[133,140] and the effect of either GSK-3 disruption in *Dictyostelium*[43] and ectopic expression of Wnt genes in *Xenopus* embryos.[141] While examining lithium-mediated effects on dorsal/ventral patterning in Xenopus embryos, Klein and Melton (1996) observed that injection of a potent and selective IMPase inhibitor (L690,330)[142] did not phenocopy lithium. The nature of the connection between lithium and the Wnt pathway was revealed by the finding that lithium inhibits the activity of purified GSK-3α and β.[143] This finding was extended to GSK-3 function in intact cells.[144] These results directed attention toward GSK-3 as an important cellular target for lithium action.

Lithium is highly selective for GSK-3. While one report suggested that lithium could activate PKB/AKT,[145] this result has not been confirmed. Lithium has thus been employed as a tool to study the role of GSK-3 in various cellular processes. Although the pleiotropic nature of the ion on cells means that if a lithium effect is observed this may not be mediated by GSK-3, if lithium has no effect on a process, a role for GSK-3 can be excluded.

In the case of the reduction in NF-κB activity in cells lacking GSK-3β (see section V), the results obtained using fibroblasts lacking functional GSK-3β through genetic disruption were reproduced using lithium, a potent inhibitor of GSK-3. Lithium inhibited the induction of NF-κB transactivation by 70% relative to a potassium control in HEK293 cells, and lithium-treated wild-type fibroblasts were sensitized to TNF-induced killing, similar to GSK-3β null cells. These results are consonant with those of Beyaert et al. (1989), who first reported that lithium causes a dose-dependent enhancement of TNF cytotoxicity in human and murine cell lines.[146] Lithium also enhanced the in vivo antitumor action of TNF. Similar results have been obtained when using interleukin-2 (IL-2), another NF-κB agonist.[147] IL-2 in combination with lithium demonstrated a stronger inhibitory effect on tumor growth than IL-2 alone,

as determined by reduction of tumor size and prolongation of survival in tumor-bearing mice.

B. Other Inhibitors of GSK-3

Hymenialdisine (HD) and related compounds, such as debromohymenialdisine (DBH), were originally purified from the marine sponge *Axinella verrucosa* and have recently been identified in a screen for antiinflammatory compounds. For example, DBH exhibits antiinflammatory activity in a model of adjuvant-induced arthritis in the rat.[148]

HD was evaluated for its effects on the activation of NF-κB using electrophoretic mobility shift assay (EMSA) and luciferase reporters under the control of either the HIV-LTR or IL-8 promoter. Similar to lithium treatment or genetic disruption of GSK-3β, DH inhibited NF-κB DNA binding and NF-κB-regulated gene expression without affecting I-κB degradation.[149,150] In particular, HD caused a concentration-dependent inhibition of luciferase production regardless of the stimulus used (TNF-α, LPS, or PMA), and HD-inhibited DNA binding in U937 cells was most evident at 1.0 mM HD, commensurate with 50% inhibition of TNF-stimulated DNA binding.

The striking similarities between HD effects and GSK-3β inhibition were noted by Dr. Michel Roberge, who had been working with HD. Both HD and DBH have been demonstrated as potent in vitro inhibitors of GSK-3β (Hoeflich, Roberge, and Woodgett, unpublished observations).[151] HD and DBH also interfere with in vivo phosphorylation of specific proteins by GSK-3β. In particular, phosphorylation of Tau[151] and β-catenin (Hoeflich, Roberge, and Woodgett, unpublished observations) are completely inhibited by HD and DBH in vivo. These effects occur at two-orders of magnitude lower concentrations of HD/DBH than lithium.

Although HD is also known to inhibit PKC,[148] the selective PKC inhibitor RO 32−0432[152] has no effect on TNF-α-stimulated luciferase reporter activity or IL-8 production at low concentrations. As expected, RO 32−0432 only inhibited luciferase production in response to PMA stimulation (with IC_{50} values of 0.2 mM), demonstrating the ability of this compound to inhibit PKC. These data support the idea that the antiinflammatory activity of HD compounds may be related to their ability to block the activity of the transcription factor NF-κB via inhibition of GSK-3β.

Additional ATP competitor molecules specific for GSK-3 (K_i 10−30 nM) have been recently reported to stimulate glycogen synthesis in liver cell lines and to induce transcription of β-catenin-regulated genes in epithelial cells.[153] The availability of these inhibitors will greatly assist dissection of the physiological processes influenced by GSK-3.

VIII. Concluding Remarks

Since its inauspicious discovery over 20 years ago, considerable progress has been made in understanding the regulation, signaling mechanisms, and physiological effects of glycogen synthase kinase-3. Progress was fueled by research using genetically tractable organisms by many different groups, most of whom had no interest in glycogen metabolism! Despite these advances, much is still unclear. The means by which cells effectively sequester different populations of GSK-3 that are responsive to distinct pathways and that culminate in specific responses is poorly understood. Some data point to differential requirements for substrate priming. Specifically, some GSK-3 substrates do not require prephosphorylation whereas others, like glycogen synthase, require prephosphorylation at a serine−threonine just C-terminal to the GSK-3 site.[39] Thus, different substrate phosphorylation requirements might allow GSK-3 to affect one substrate but not another. This idea is supported by effects of a 39 amino acid peptide corresponding to the GSK-3-binding domain of FRAT, which prevents the GSK-3-catalyzed phosphorylation of axin and β-catenin but not other substrates, such as glycogen synthase and eIF2B. Phosphorylation of these latter targets is independent of axin but instead is dependent on priming prephosphorylation.

Among the targets of GSK-3 regulation is a who's who list of transcription factors including β-catenin, NF-AT, C/EBP, c-Jun, c-Myc, NF-κB, and CREB, most of which are negatively regulated in mammalian cells. As such, GSK-3 carries significant responsibility in regulating gene expression, and it is therefore perhaps not too surprising that its regulation is complex and redundant. With the recent development of small molecule inhibitors of the enzyme, additional targets are sure to be revealed. More importantly, new biological functions will be discerned, possibly leading to new therapies. There is considerable concern about the consequences of interfering with such a pleiotropic enzyme. Indeed, predicted effects on β-catenin disregulation raise the spectre of inhibitors being tumor promoters. However, the history of this enzyme is replete with surprises, and it would be foolish to discount useful therapeutic consequences of its careful modulation.

IX. Acknowledgments

We apologize to the authors whose original work is not included in the references owing to space limitations. Special thanks to current and former lab members who have contributed to the work on GSK-3, particularly Jing Jin, Elizabeth Rubie, Laurent Ruel, and Vuk Stambolic. During part of this study, A.A. was a recipient of an NSERC postdoctoral fellowship and K.P.H. was supported by a Canadian Institutes of Health Research (CIHR) studentship. J.R.W. is supported by grants from the CIHR and Howard Hughes Medical Institute and is a CIHR Senior Scientist.

X. Note Added in Proof

Recently, two groups have solved the crystal structure of GSK-3β.[172,173] The most striking aspect of the structure was insight into the mechanism by which the protein kinase activity is inhibited upon phosphorylation of Serine 9. As mentioned in section II and Table 2, several GSK-3 targets require prior phosphorylation to be recognized by GSK-3. When Serine 9 is phosphorylated, the phosphoserine binds

to a region within the catalytic lobe that normally binds the prephosphorylated residue of the substrate, thus acting as an intramolecular pseudosubstrate inhibitor. This same conclusion was also arrived at by biochemical studies in which Arginine 96 in GSK-3β lies in the active site as coordinates phosphorylated Serine 9. This mechanism does not impact substrates that do not require prior phosphorylation (e.g., β-catenin) and provides a means by which cells may regulate phosphorylation of some GSK-3 without affecting others.

XI. References

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CR000110O