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Review Article

GSK-3β and its Unexpected Role in Immunity, Inflammation and Cancer

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Abbreviations GSK-3, Glycogen Synthase Kinase-3; NF-κB, Nuclear transcription Factor κB; S, Serine; PKA, Protein Kinase A; PKC, Protein Kinase C; S6K, p70 S6 Kinase; Y, Tyrosine; CREB, CAMP Response Element-Binding Protein; GATA 4, GATA binding protein 4; HIF-1, Hypoxia-Inducible Factor 1; NFAT, Nuclear Factor of Activated T-cells; ROS, Reactive Oxygen Species; CKI, Casein Kinase I; APC, Adenomatous Polyposis Coli protein; FRAT, Frequently Arranged in T cell lymphomas; Dvl, Disheveled; TLR, Toll-Like Receptor; LPS, Lipo Poly Saccharide; TNF, Tumor Necrosis Factor; IFN, Interferon; mTORC1, mammalian Target Of Rapamycin Complex 1; TCF/LEF, T-Cell Factor/Lymphoid Enhancer-binding Factor; MHC, Major Histocompatibility Complex; CCR7, C-C chemokine Receptor type 7; mtDNA, mitochondrial DNA; UTR, UnTranslated Regions; NBR, Nucleotide Binding Region; ABD, ATP Binding Domain; AS, protein Active Site.

Abstract

Glycogen Synthase Kinase-3β (GSK-3β) is a key component of a complex array of cellular processes. Several mechanisms are involved in controlling its activity, including phosphorylation, protein complex formation and sub cellular distribution. Aberrant GSK-3β action has been implicated in many diseases and disorders, such as cancer, heart disease, metabolic and neurological disorders. More recently, GSK-3β has been identified as a crucial regulator of the balance between pro and anti-inflammatory cytokine production. This review will highlight the immunological importance of GSK-3β and the latest discoveries that led to the identification of a new central role of GSK-3β in tumor immunity.

Introduction

Glycogen Synthase Kinase-3 (GSK-3) is a serine/threonine protein kinase that plays an important role in different biological processes, including early embryo development, oncogenesis, neurodegenerative disease, diabetes, inflammatory conditions and cell death [1-3]. Farther, this kinase has been reported to phosphorylate more than 50 proteins, including different transcription factors as nuclear transcription factor κB (NF-κB), p53 and β-catenin [2, 4]. Molecular cloning showed two genes encoding different kinase isoforms, GSK-3α and GSK-3β, ubiquitously expressed in mammalian tissues [5, 6]. GSK-3α presents an 85% amino acid identity to GSK-3β and differs from the other isoform of an N-terminal glycine rich extension [5]. GSK-3β is the most studied form of GSK-3; its gene including 12 exons is located on the chromosome 3 (q13.3) and produces a 7134 bp mRNA (NM_002193.3). Human GSK-3β is a 47 kDa protein with a small N-terminal domain, a kinase domain, presenting an ATP binding site and a protein active site and, finally, a C-terminal domain (Figure 1). Mukai et al. documented also an alternative splice variant of GSK-3β, GSK-3β2, with a 13 amino acid insert in the catalytic domain [7].

Regulation of GSK-3β

Two key functional domains of GSK-3β have been identified (Figure 2), a primed-substrate binding domain that recruits substrates to the protein, and a kinase domain with phosphorylation activity [8-10]. GSK-3β has two kinds of target proteins: primed and unprimed substrates. Primed substrates consist of proteins that are pre-phosphorylated at a "priming" site located at C-terminus of the consensus sequence: S/T (target residue)-X-X-X-S/T (priming residue). The priming phosphorylation allows the substrate to bind the primed-substrate binding domain and places the target serine/threonine adjacent to the kinase domain of GSK-3β. This event greatly increases the efficiency of substrate phosphorylation by 100-1000 fold [11]. However, some GSK-3β substrates lack a priming site. These unprimed proteins often display negatively charged residues in place of the priming residue that contribute to optimize the orientation of the kinase domain and to place the substrate at the correct position within the catalytic pocket.

Phosphorylation

GSK-3β is inactivated by diverse stimuli and signaling pathways. In particular, phosphorylation at the N-terminal Serine 9 (S9) residue is the most frequently examined mechanism that negatively regulates the activity of the kinase. This modification induces the interaction between the S9 and the substrate docking motif in the binding domain, generating a pseudo substrate that inhibits the substrate access to the catalytic groove of the kinase [12] (Figure 3). Several kinases can phosphorylate this serine, including Akt, Protein Kinase A (PKA), Protein Kinase C (PKC), p70 S6 Kinase (S6K) and p90 ribosomal S6 kinase [13-15] (Figure 4). Thus, many signaling pathways that activate these kinases can inhibit GSK-3β by S9 phosphorylation. A consequence of the GSK-3β inhibition is that concentration of primed substrates increases sufficiently to compete with the pseudo substrate [8]. However, it should always be borne in mind that the serine-phosphorylation inhibitory mechanism does not necessarily regulate the phosphorylation of non-primed substrates by GSK-3β. Therefore, if a non-primed substrate is under investigation, examining changes in the serine-phosphorylation of the kinase should be interpreted cautiously.

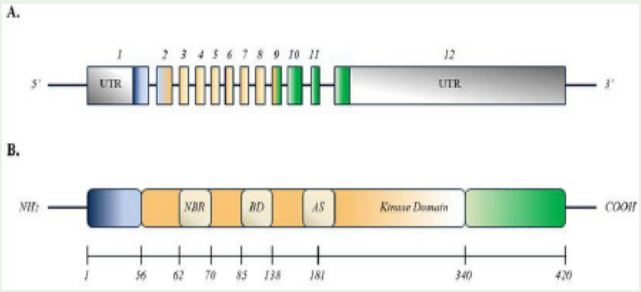

Figure 1: The GSK-3*β* gene and protein structure.
A. GSK-3*β* gene with its 12 exons; the grey boxes are Untranslated Regions (UTR).
B. Protein structure from the amino to the carboxy-terminal region. The Kinase Domain presents, starting from the left side, a Nucleotide Binding Region (NBR), an ATP Binding Domain (ABD) and a protein Active Site (AS). The amino acid number that outlines the different domains is shown.

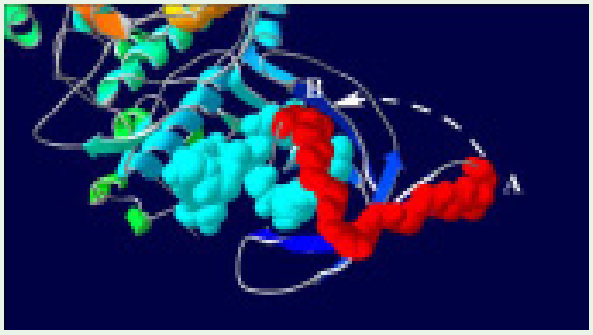

Figure 3: Conformational change after S9 phosphorylation. Before the phosphorylation (A) the conformation of the *β*-strand containing S9 allows the substrate access to the catalytic groove of the kinase (light blue). After phosphorylation (B) S9 residue interacts with the substrate docking motif in the binding domain, generating a pseudo substrate.

In opposition to this inhibitory regulation, GSK-3β is activated by phosphorylation of Tyrosine 216 (Y216) residue that is located in the "activation loop" of the enzyme (Figure 5). Y216 might act as an autophosphorylation site or as a substrate for other protein tyrosine kinases, such as Pyk2, MEK1 and SRC-family tyrosine kinases [16-19]. Several proapoptotic stimuli were also demonstrated to increase the activity and Y216 phosphorylation of GSK-3*β* [20], but the kinases mediating this modification remain unclear. Overall, studies of the tyrosine phosphorylation of GSK-3*β* are relatively sparse. In particular, the finding that Fyn, a member of the src tyrosine kinase family [21], and calcium [22] participate in regulating the activating phosphorylation of GSK-3*β* indicates that this modulatory mechanism may be involved in many intracellular signaling cascades.

Cellular Localization and Complex Formation

In addition to phosphorylation, mechanisms that regulate the intracellular localization of GSK-3*β* control its access to substrates. Although GSK-3*β* is traditionally considered a cytosolic protein, it is also located in nuclei and mitochondria, where it is highly activated compared with its cytosolic counterpart [23, 24]. Nuclear GSK-3*β* is particularly interesting because it regulates many important transcription factors, such as cAMP Response Element-Binding protein (CREB), GATA binding protein 4 (GATA4), Hypoxia-Inducible Factor 1 (HIF-1), Nuclear Factor of Activated T-cells (NFAT), NF-*κ*B, Notch and p53. Meares et al. reported the existence of a bipartite nuclear localization sequence in GSK-3*β*, consisting of residues 85-103, that were identified by assessing the sub cellular localization of mutants created by site-directed mutagenesis [25]. The nuclear level of GSK-3*β* is not static but changes dynamically in response to intracellular signals; in particular, kinase levels fluctuate during the cell cycle and can rapidly increase during the apoptotic process, enabling GSK-3*β* to modulate gene expression [23, 26]. In opposition to the nuclear level of GSK-3*β*, which is decreased by activated Akt [24], mitochondrial GSK-3*β* is inhibited by activated Akt without affecting its protein levels [27]. Moreover, a recent study has shown that mitochondrial translocation of GSK-3*β*, triggered by exogenous hydrogen peroxide, induced enhanced Reactive Oxygen Species (ROS) production and that both mitochondrial translocation of GSK-3*β* and ROS production were dependent on GSK-3*β* kinase activity [28]. Further studies will be needed to better understand the regulation of the nuclear and mitochondrial localization of GSK-3*β*.

Complexes that contain GSK-3*β* are very important in regulating its actions. It is also intriguing to note that its activity can also regulate the actions of some GSK-3*β*-inhibiting kinases. This bi-directionality has been studied particularly in the Akt–GSK-3*β* interaction, in which Akt not only inhibits GSK-3*β* but GSK-3*β* can also regulate Akt [29]. The best characterized protein complex system that involves the kinase is the Wnt signalling pathway [27]. In absence of the

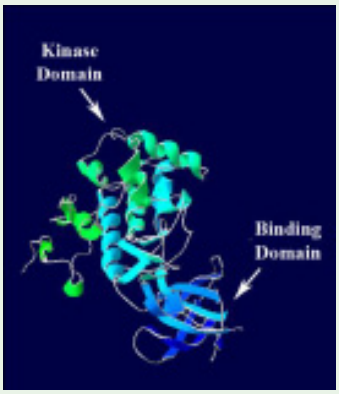

Figure 2: The structure of GSK-3*β*. The N-terminal domain (blue) corresponds to the *β*-strand domain that recruits substrates to the protein. The *α*-helices (green and light blue) correspond to the kinase domain.

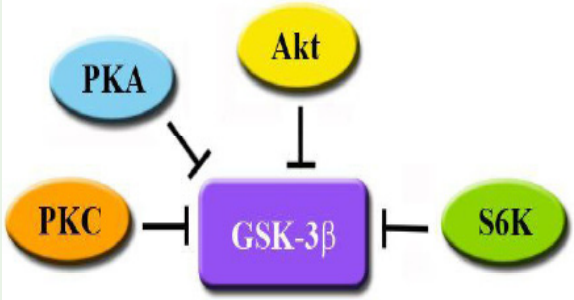

Figure 4: Kinases primarily involved in GSK-3*β* inhibition. Several kinases can phosphorylate GSK-3*β* at S9, including Akt, Protein Kinase A (PKA), Protein Kinase C (PKC), p70 S6 Kinase (S6K) and p90 ribosomal S6 kinase.

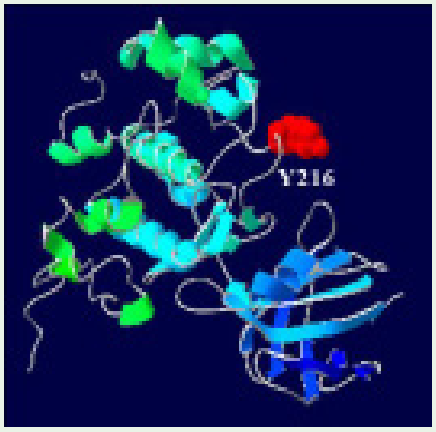

Figure 5: GSK-3β activation by of Y216 phosphorylation. Y216 (red residue) is located in the "activation loop" of the enzyme, between the kinase domain and the binding domain.

Wnt ligand, GSK-3β forms a complex with Axin, β-catenin, Casein Kinase I (CKI) and Adenomatous Polyposis Coli protein (APC). CKI phosphorylates β-catenin to prime it for phosphorylation by GSK-3β. These two events induce the proteasomal degradation of β-catenin. After Wnt stimulation, Frequently Arranged in T cell lymphomas (FRAT) and Disheveled (Dvl) are recruited into the GSK-3β complex, preventing β-catenin phosphorylation and enabling its translocation into the nucleus. Similarly, GSK-3β plays a role in the regulation of the Hedgehog pathway [30].

Finally, GSK-3β is regulated by additional post-translational mechanisms, such as cleavage by calpain [31, 32] and by matrix metalloproteinase-2 [33], which may affect its selection of substrates, acetylation [34], mono-ADP-ribosylation [35,36] and citrullination [37]. These and other phosphorylation-independent post-translational mechanisms seem likely to contribute in regulating the multiple actions of GSK-3β.

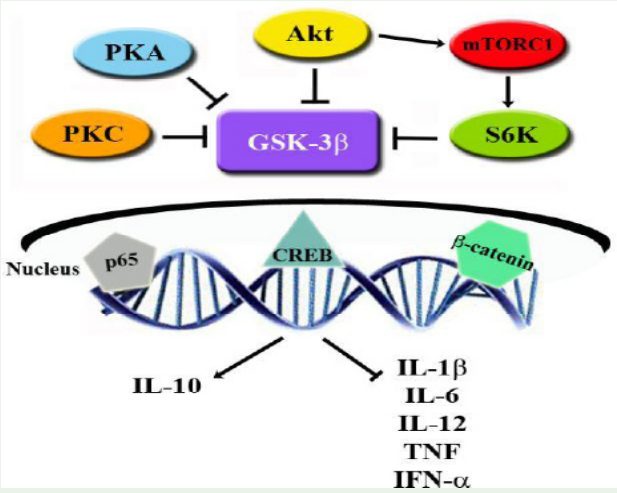

Figure 6: Role of GSK-3β in the innate immune response. After GSK-3β inhibition, many important transcription factors can migrate into the nucleus and regulate the production of several pro and anti-inflammatory cytokines.

GSK-3β and Immunity

Innate Immune Response

Inflammation represents a primary response to infection and it is critical for both innate and adaptive immunity.

Recently, it has been documented that GSK-3β activity is crucial to regulate inflammatory response either promoting or inhibiting the process through the expression of pro or anti-inflammatory cytokines [38].

Several studies have demonstrated that inflammation is regulated by the Toll-Like Receptor (TLR)-dependent activation of PI3K/Akt signaling pathway [39-42]. Martin et al. [43] established that the PI3K/Akt-dependent inhibition of GSK-3β activity in human monocytes, stimulated with Lipo Poly Saccharide (LPS), differentially affected the nature and magnitude of the inflammatory response through the activation of TLR2. This resulted in the production of the anti-inflammatory cytokine IL-10 and in a strong reduction of pro-inflammatory cytokines IL-1β, IL-6, Tumor Necrosis Factor (TNF), IL-12 and Interferon (IFN)-α (Figure 6). The GSK-3β inhibition negatively modulated the inflammatory response because it differentially affected the nuclear amounts of the transcription factors NF-κB (p65 subunit) and CREB, interacting with the co activator CBP. In a recent study it has been also demonstrated that the mammalian Target Of Rapamycin Complex 1 (mTORC1) negatively regulates the activity of GSK-3β through the activation of S6K, conditioning the inflammatory response in LPS-stimulated human monocytes [44]. Furthermore, the inhibition of GSK-3β by mTORC1 affected the association of p65 subunit and CBP. GSK-3β activity induced a decrease of the anti-inflammatory cytokine IL-1Rα levels and increased the levels of the inflammatory cytokine IL-1β, confirming the model proposed by Martin et al., in which GSK-3β in its active form acts as a positive regulator of inflammation. Moreover, GSK-3β inactivation might be able to modulate the transcription of specific pro-inflammatory genes containing a T-Cell Factor/ Lymphoid Enhancer-binding Factor (TCF/LEF) binding site in their promoter. Indeed, it was recently demonstrated that β-catenin induces pro and anti-inflammatory responses simultaneously as a result of differential gene expression carried out by Wnt/β-catenin signaling through a TCF/LEF consensus sequence and NF-κB modulation in the context of liver cancer related inflammation [45].

Adaptive Immune Response

The adaptive immune response depends on successful antigen presentation by Major Histocompatibility Complex (MHC) and MHC-like molecules, and recent findings raise the possibility that GSK-3 is involved in antigen presentation by antigen-presenting cells. Maintenance of GSK-3β inhibition is critical for CD4+ and CD8+ T cell survival after activation [46]. However, memory CD4+ T cells are less dependent than naive CD4+ cells on inhibition of GSK-3β for proliferative responses [47]. Expression of constitutively active GSK-3β decreased proliferation of CD8+ cells and suppressed TCR-induced IL-2 production [48], whereas inhibition of GSK-3 increased IL-2 production in both CD4+ and CD8+ T cells [46-50]. Similar to the innate immune system, GSK-3 inhibition reduced the production of several pro-inflammatory cytokines by splenocytes stimulated by myelin oligodendrocyte glycoprotein peptide after isolation from

Table 1: Clinical trials with GSK-3β inhibitors (Clinical Trials.gov).

Inhibitor Name	Therapeutic application	Ref/Clinical Trials
Tideglusib	Alzheimer's Dementia Cerebrovascular Diseases	NCT00948259 NCT01049399
LY-2090314	Advanced/metastatic cancer Leukemia	NCT01632306 NCT01287520 NCT01214603
Valproic acid sodium salt	Epilepsy, Mania, Bipolar disorder Alzheimer's Dementia	NCT00088387
Indirubin	Myeloid Leukemia	Damiens E and Meijer L [65]

Identifier:
NCT00948259: Phase II of Tideglusib used for treating patients with Alzheimer's disease.
NCT01049399: Phase II of Tideglusib for progressive supranuclear palsy.
NCT01632306: Phase I/II of LY-2090314 used in combination with different chemotherapies in treating advanced/metastatic pancreatic cancer.
NCT01287520:Phase I of LY-2090314 in combination with pemetrexed and carboplatin in patients with advanced/metastatic cancer.
NCT01214603: Phase II study of intravenous LY-2090314 in acute leukemia patients.
NCT00088387: Valproic acid sodium salt is used in clinical trial in combination with lithium for treatment of patients suffering from Alzheimer's disease.

experimental autoimmune encephalitis-induced mice [51] and increased the production of anti-inflammatory IL-10 by memory CD4+ T cells and by B cells.

Inflammation and Cancer

Cytokines generated by activated immune cells are considered important components in orchestrating the relationship between inflammation and cancer. Studies conducted over the last several years have elucidated the molecular mechanisms of intracellular signaling pathways of inflammatory cytokines for tumor development [52, 53]. GSK-3β has been identified by recent findings as vital factor in the inflammation process [43].

GSK-3β is mostly known as a pro-inflammatory agent and drugs that inhibit its activity are being developed for diseases such as Alzheimer's, cancer, diabetes and immune disorders. Indeed, Balamurugan et al. demonstrated that GSK-3β can act in cooperation with the protein FBXW7 as inhibitor of the inflammatory response [54]. In this work, authors showed a dual role of the kinase that might complicate clinical applications of drugs targeted at inhibiting GSK-3β.

Another study has pointed out how the over expression of the C-C chemokine Receptor type 7 (CCR7), involved in the development and progression of chronic inflammatory diseases and cancer, was partly mediated by the Akt/GSK-3β signaling pathway in colon cancer [55]. The inhibition of the Akt/GSK-3β cascade may emerge as potential therapeutic strategy to reduce CCR7 expression in this neoplasm.

Moreover, the ability of GSK-3β inhibition to differentially regulate pro and anti-inflammatory cytokine production and its functional role in adaptive immune responses might play an important role in the progression of esophageal cancer [56].

It has also been demonstrated that some inflammatory mediators in tumor microenvironment, including TGF-β and IL-6, contributed to cancer invasion and metastasis [57]. In particular, Salim et al. suggested a direct effect of the pro-inflammatory mediator leukotriene D4, a component of the tumor microenvironment, in regulating the proliferation and migration of colon cancer cells, most likely via a GSK-3β/β-catenin signaling pathway [58].

In a recent study, authors investigated the effect of flavonoid apigenin treatment on the expression of genes involved in inflammation and cancer in human pancreatic cancer cells [59]. The results showed that apigenin inhibited the GSK-3β/NF-κB signaling pathway, leading to an induction of the mitochondrial pathway of apoptosis in cell lines. Moreover, gene expression analysis revealed apigenin treatment up regulated 59 genes and down regulated 63 genes related to inflammation and cancer.

It is currently recognized that chronically elevated TNFα, a major pro-inflammatory cytokine, in tissues may promote tumor growth, invasion and metastasis [60]. Michalaki et al. demonstrated that TNFα expression is significantly increased in the serum of prostate cancer patients and associated with tumor metastasis [61]. Some authors demonstrated that Akt/GSK-3β-mediated stabilization of Snail is required for TNFα-induced epithelial-mesenchimal transition in colorectal and prostate cancer cells [62, 63].

Furthermore, Vadrot et al. reported that GSK-3β was involved in TNFα-induced mitochondrial DNA (mtDNA) depletion and that p53 was necessary for the recovery of mtDNA content [64]. They suggested that p53 binding to GSK-3β, TFAM and mtDNA regulatory region D-loop could participate in this recovery by stimulating mtDNA repair.

Given the central role of GSK-3β in all the aforementioned pathways, the therapeutic potential of its inhibitors has become an important area of investigation (Table 1).

Conclusions

GSK-3β, identified initially as a kinase involved in the glycogen metabolism, has been recognized as an important mediator of the innate and adaptive immune systems. The regulatory effects of GSK-3β and its involvement in the inflammatory processes may have strong implications in cancer development. Based on this hypothesis, the discovery of selective GSK-3β inhibitors could have an important role in giving new therapeutic alternatives in cancer treatment. Nevertheless many questions remain unanswered and the role of GSK-3β and its potential application in tumor immunity become an interesting aspect to clarify.

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