Regulation of the Interaction between Glycogen Synthase Kinase 3 and the Kaposi's Sarcoma-Associated Herpesvirus Latency-Associated Nuclear Antigen

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The Kaposi's sarcoma-associated herpesvirus (KSHV)-encoded latency-associated nuclear antigen (LANA) protein stabilizes β -catenin by the novel mechanism of binding to the negative regulator, glycogen synthase kinase 3 (GSK-3), and depleting cytoplasmic GSK-3 levels. The two domains of LANA required for interaction with GSK-3 were further characterized. Evidence for similarity between the C-terminal LANA interaction domain and the axin GSK-3 interaction domain was obtained using GSK-3 and LANA mutants. GSK-3(F291L), which does not interact with axin, also failed to bind to LANA, and a mutation in the axin homology domain of LANA, L1132P, destroyed binding to GSK-3. The N-terminal LANA interaction domain was found to mediate interaction by acting as a substrate for GSK-3. GSK-3(R96A), a priming pocket mutant, did not bind to LANA, suggesting that LANA was a primed GSK-3 substrate. Phosphorylation of endogenous LANA precipitated from primary effusion lymphoma cells was inhibited by the GSK-3 inhibitor LiCl. GST-LANA(1-340) was phosphorylated by GSK-3, and mitogen-activated protein kinase (MAPK) and casein kinase I functioned as priming kinases in vitro. Mutation of consensus GSK-3 sites revealed that sites between LANA amino acids 219 and 268 were important for GSK-3 phosphorylation. Immunoprecipitation assays revealed that loss of GSK-3 phosphorylation of this N-terminal domain correlated with loss of GSK-3 interaction. Although LANA-associated GSK-3 actively phosphorylated LANA, GSK-3 coprecipitated with LANA was unable to phosphorylate an exogenous peptide substrate. LANA sequestration of GSK-3 may explain the ability of KSHV-infected cells to tolerate increased levels of nuclear GSK-3.

The latency-associated nuclear antigen (LANA) is encoded by Kaposi's sarcoma-associated herpesvirus (KSHV) ORF73 and is a multifunctional protein. LANA is essential for the replication and maintenance of KSHV episomal genomes during latency (4, 11). LANA binds to two adjacent sites in the latency origin of replication (10, 25), and the relative importance of individual nucleotides in the binding site has been defined by mutagenesis (51). The latency origin of replication is located within the terminal repeats of the KSHV genome (5, 15, 26) and, hence, is present in multiple copies. One copy is sufficient to enable DNA LANA-mediated replication of a terminal repeat-containing plasmid, but a minimum of two copies are required for episomal maintenance (31). The LANA C terminus contains an oligomerization domain and the DNA binding domain, and LANA binds DNA as an oligomer (33, 48). DNA binding is required for LANA-mediated DNA replication (30), as is chromosomal association (6). LANA-mediated chromosomal association is also essential for long-term maintenance of terminal repeat-containing episomes (6, 50). LANA has an N-terminal chromatin binding domain that is required for LANA association with chromosomes (6, 43, 59). A second C-terminal chromosome associa-

tion domain is unmasked when the C terminus of LANA is expressed independently, and this domain likely functions as an ancillary tethering region (34). The N-terminal chromatin binding domain interacts with the cellular protein methyl CpG binding protein 2, and the C-terminal domain interacts with cellular DEK (34). Chromosome tethering is believed to facilitate KSHV maintenance by ensuring segregation of episomal genomes to daughter cells during cell division.

LANA also affects cell growth and cell gene expression in ways that are likely to contribute to the genesis of KSHV-associated malignancies. Cell growth is influenced by stimulation of E2F-regulated genes through interactions with pRb (44), stimulation of S-phase entry (22), and protection from p16 INK4A-induced cell cycle arrest (2), stimulation of the telomerase reverse transcriptase promoter (54), and abrogation of p53-mediated apoptotic activity (20). LANA has global positive and negative effects on cell gene expression. Direct binding of LANA to DNA appears to mediate transcriptional repression (24, 25, 35, 48), whereas positive transcriptional regulation appears to be mediated indirectly. Genes regulated by the transcription factors Sp1, Ap1, RBP-J κ , ATF4, CBP, and Id-1 have been found to be modulated by LANA (3, 36–38, 52, 54). A major impact on cell gene regulation occurs through LANA-mediated stabilization of β -catenin (2, 22). LANA binds to glycogen synthase kinase 3 (GSK-3) and mediates a cell cycle-regulated nuclear relocalization of GSK-3. This results in cytoplasmic accumulation of β -catenin, presumably

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because there is a depletion of GSK-3 from the cytoplasmic β -catenin destruction complex. The elevated β -catenin levels in turn lead to increased nuclear transcriptional activity of β -catenin-Tcf/Lef-regulated genes as measured by activation of a Tcf/Lef reporter and higher levels of the β -catenin-regulated genes. All of these outcomes are dependent on LANA binding to GSK-3, and LANA deletion variants that are unable to bind GSK-3 are defective for GSK-3 relocalization, β -catenin accumulation, increased β -catenin gene regulatory activity, and stimulation of S-phase entry (21, 22).

There are two separate GSK-3 genes, GSK-3 α and GSK-3 β , and a splice variant of GSK-3 β has also been described (41, 42). Both GSK-3 α and GSK-3 β can participate in Wnt signaling, and LANA binds to both GSK-3 α and GSK-3 β (21). Interaction with GSK-3 requires two separate regions of LANA, an N-terminal region between amino acids (aa) 241 and 275 and a C-terminal domain that was mapped using C-terminal deletions and was recognized to contain a low level of amino acid homology to the GSK-3 interaction domain (GID) of axin (21).

A small proportion of the total GSK-3 accumulates in the nucleus during S phase (13) and in senescent cells (65), and a number of transcription factors are substrates for GSK-3 (32). GSK-3 also enters the nucleus in response to apoptotic stimuli and binds to and stimulates the transcriptional activity of p53 (55). β -Catenin accumulation and GSK-3 nuclear relocalization have also been described in Epstein-Barr virus-infected B cells (14, 49). In Epstein-Barr virus-infected lymphoblastoid cell lines and in nasopharyngeal carcinoma tissue, nuclear GSK-3 was present in the phosphorylation-inactivated form (14, 40). Nuclear GSK-3 must also be regulated in such a way as to avoid a negative outcome in KSHV-infected cells. In view of the central role played by LANA-mediated nuclear relocalization of GSK-3 in KSHV-infected cells, we sought to better understand the factors that regulate this event. Mutagenesis data strengthened the prediction that LANA contains an axin-like GID. LANA, like axin, was found to be a substrate for GSK-3. However, unlike axin, GSK-3 phosphorylation of LANA was dependent on the activity of priming kinases. We also show that GSK-3 phosphorylation regulates the affinity of binding of LANA to GSK-3 and GSK-3 activity appears to be sequestered by the association with LANA.

MATERIALS AND METHODS

Plasmids and antibodies. For eukaryotic expression, LANA, GSK-3 β , and mutant derivatives were cloned into SG5 (Stratagene)-based vectors. The parental vectors were Flag-LANA(pDY52), Flag-dCR (pMF24), and HA-GSK-3 β (obtained from F. McCormick). S-GSK-3 β is a 2x S-peptide (KETAAAKFER QHMDS)-tagged, full-length GSK-3 β -expressing plasmid in the pCI-neo vector (Promega). The luciferase reporter plasmids (pGL3-OT), Tcf-4, and β -catenin expression vectors were obtained from K. W. Kinzler (Johns Hopkins School of Medicine). Bacterially expressed glutathione S-transferase (GST) fusions GST-LANA(1-340) (pGL13) and GST-LANA(936-1162) (GL12) were generated in pGEX2T (Invitrogen).

The molecular mass standards, pre-stained protein ladder and BenchMark protein ladder, were purchased from Invitrogen. Antibodies used for immunoblotting were anti-GSK-3 β and mouse monoclonal (Transduction Laboratories); anti-LANA rat monoclonal (Advanced Biotechnologies Inc.); and antihemagglutinin (anti-HA) rabbit polyclonal (Santa Cruz Biotechnology). Anti-Flag and anti-HA mouse monoclonal antibodies were obtained from Sigma.

Immunoprecipitation assays. HeLa cells were seeded at 2×10^5 per well of a six-well plate and transfected using the calcium phosphate procedure with 3 μ g of Flag-LANA and 2 μ g of HA-GSK-3 β (or S-GSK-3 β). Cells were harvested

48 h after transfection, resuspended in 1 ml of ice-cold lysis buffer containing 50 mM Tris-HCl (pH 7.8), 0.5% Nonidet P-40, 5% glycerol, 1 mM dithiothreitol (DTT), 0.5 mM EDTA, 50 mM NaCl, 0.5 mM phenylmethylsulfonyl fluoride, 1 μ g/ml pepstatin, and 5 μ g/ml aprotinin and sonicated for 5 seconds. Lysates were centrifuged at 15,000 rpm for 15 min. The cell extract was incubated with 3 μ g of anti-HA monoclonal antibody or 10 μ l of S-protein-agarose beads (Novagen) for 2 h at 4°C followed by incubation with protein G-Sepharose beads (30 μ l). Beads were washed with ice-cold lysis buffer six times. The beads were then resuspended in sample buffer (30 μ l), and samples (15 μ l) were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) on a 9% polyacrylamide gel followed by Western blotting analysis using anti-LANA rat-monoclonal antibody or anti-Flag monoclonal antibody.

GSK-3 kinase assays. To obtain GST-LANA fusion proteins, expression plasmid, pGL12 (GST-LANA 936-1162), pGL13 (GST-LANA 1-340), and pGST-2T (GST) were transfected into BL21 bacteria. Fusion proteins were bound to glutathione-Sepharose 4B beads (Pharmacia) and eluted with 15 mM glutathione in phosphate-buffered saline. For experiments examining the effects of priming kinases, GST-fusion proteins were washed two times with kinase buffer (50 mM Tris-HCl [pH 7.9], 10 mM MgCl₂, 2 mM DTT, 100 mM NaCl, 20 μ M cold ATP). The resulting beads were resuspended in 20 μ l of kinase buffer containing 0.1 U of the priming kinases (mitogen-activated protein kinase [MAPK], casein kinase I [CKI], or CKII) and 2 mM cold ATP for 30 min at 30°C for priming phosphorylation. After the reaction, the beads were washed with washing buffer (50 mM Tris-HCl [pH 7.9], 10 mM MgCl₂, 2 mM DTT, 50 mM NaCl) (three times) and kinase buffer (two times) and resuspended in 20 μ l of kinase buffer containing 0.1 U of GSK-3 β , 2 μ M of U0126 (for MAPK inhibition), 2 μ M of IC261 (for CKI inhibition), 20 μ g/ml of heparin (for CKII inhibition), and 0.08 MBq of [γ -³²P]ATP. Reaction mixtures were incubated for 20 min at 30°C and then washed with ice-cold washing buffer two times and subjected to SDS-PAGE on a 9% polyacrylamide gel. Radiolabeled polypeptides were detected by autoradiography.

For experiments examining phosphorylation of LANA expressed in eukaryotic cells, HeLa cells were seeded at 2×10^5 per well in six-well plates and transfected with 5 μ g of Flag-LANA plasmid. After 48 h, cells were resuspended in 1 ml of ice-cold lysis buffer (50 mM Tris-HCl [pH 7.8], 0.4% Nonidet P-40, 5% glycerol, 1 mM DTT, 0.5 mM EDTA, 50 mM NaCl, 0.5 mM phenylmethylsulfonyl fluoride, 1 mg/ml pepstatin, 5 mg/ml aprotinin) and sonicated for 5 seconds. Cell extracts were incubated with 4 μ g of anti-Flag monoclonal antibody (M2; Sigma) for 2 h at 4°C and incubated with protein G-Sepharose beads (30 μ l) for 1 h at 4°C. Beads were washed with ice-cold lysis buffer three times and were then washed three times with kinase reaction buffer (50 mM Tris-HCl [pH 7.9], 10 mM MgCl₂, 2 mM DTT, 50 mM NaCl, 20 μ M cold ATP, 20 μ g/ml heparin, 2 μ M U0126, 2 μ M IC261, 2 μ M 8-Br-cyclic AMP). The washed beads were resuspended in 20 μ l of kinase reaction buffer containing 0.1 U of GSK-3 β (Cell Signaling) and 0.08 MBq [γ -³²P]ATP for 20 min at 30°C. After the reaction, the mixture was washed with ice-cold lysis buffer two times and subjected to SDS-PAGE followed by autoradiography.

For phosphorylation assays using endogenous LANA, nuclei from BC3 cells (5×10^6) were prepared as previously described (22). The nuclei were suspended in 3 ml of lysis buffer (50 mM Tris-HCl [pH 7.8], 0.4% Nonidet P-40, 5% glycerol, 1 mM DTT, 0.5 mM EDTA, 50 mM NaCl, 0.5 mM phenylmethylsulfonyl fluoride, 1 mg/ml pepstatin, 5 mg/ml aprotinin) and homogenized in a glass Dounce homogenizer. The lysates were precleared with Sepharose beads and centrifuged at 12,000 rpm for 5 min. Cell extracts were incubated with 4 μ g of anti-LANA rat monoclonal antibody for 2 h at 4°C and then incubated with 30 μ l of protein G-Sepharose beads for 2 h. Beads were washed with ice-cold lysis buffer three times and were washed with kinase reaction buffer (50 mM Tris-HCl [pH 7.9], 10 mM MgCl₂, 2 mM DTT, 50 mM NaCl, 10 μ M cold ATP) three times. The resulting beads were resuspended in 20 μ l of kinase reaction buffer containing 50 μ M [γ -³²P]ATP and LiCl for 20 min at 30°C. After the reaction, the mixture was washed with ice-cold lysis buffer two times and subjected to SDS-PAGE followed by autoradiography.

For phosphorylation of peptide substrates, 5 picomoles of each peptide was incubated for 20 min at 30°C in 20 μ l of reaction buffer containing 50 mM Tris-HCl (pH 7.8), 10 mM MgCl₂, 5% glycerol, 2 mM DTT, 0.5 mM EDTA, 50 mM NaCl, 2 μ M cold ATP, and 0.08 MBq [γ -³²P]ATP. The reaction was stopped by the addition of SDS sample buffer. Samples were subjected to Tricine-SDS-PAGE (47) using a 16.1% polyacrylamide gel followed by autoradiography.

Reporter assays. HeLa cells (2×10^6) cultured in six-well plates were transfected with 1 μ g of luciferase reporter (pGL3-OT), 0.75 μ g of Myc-TCF4, 0.75 μ g of β -catenin, 1.5 μ g of HA-GSK-3 β , 1.0 μ g of Flag-LANA, and 0.5 μ g of simian virus 40 promoter- β -gal plasmid using the calcium phosphate method.

FIG. 1. Evidence for an axin-like GID in LANA. (A) Western blot showing equal expression of HA-GSK-3 β and the HA-GSK-3 β mutants R96A and F291L in transfected HeLa cells. (B) Western blot to detect coprecipitation of LANA with HA-GSK-3 β proteins. HA-GSK-3 proteins were immunoprecipitated with anti-HA antibody from extracts of cotransfected HeLa cells, and the presence of coprecipitated LANA was detected by probing the blot with anti-LANA antibody. Control, nonspecific Ig. The extract lanes contain 5% of the extract used for immunoprecipitation. (C) Alignment of the human axin GID with a potential GID domain in LANA. *, identity; †, similarity. Arrowheads indicate the positions of previously described truncations that did not (black) or did (gray) disrupt binding to GSK-3. The position of the L1132P mutation is shown by an arrow. (D) Western blots probed with anti-Flag antibody (upper) or anti-HA antibody (lower). Flag-LANA or Flag-LANA(L1132P) was detected in extracts of HeLa cells cotransfected with HA-GSK-3 β (upper, lanes 1 and 5); HA-GSK-3 β was detected in the same extracts (lower, lanes 1 and 5). Immunoprecipitates generated with anti-HA antibody detected coprecipitating Flag-LANA but not L1132P (upper, lanes 2 and 6) and directly precipitated HA-GSK-3 (lower, lanes 2 and 6). Precipitates generated with anti-Flag antibody detected directly precipitated Flag-LANA (upper, lanes 3 and 7) and HA-GSK-3 β coprecipitated only with wild-type Flag-LANA (lower, lanes 3 and 7). Lanes 4 and 8, precipitation with control Ig. The extract lanes contained 5% of the amount used for immunoprecipitation.

Total transfected DNA was kept constant (5 μ g) in each sample by using vector (SG5) DNA.

RESULTS

Interaction between LANA and GSK-3 mutant proteins. We had previously shown that both N-terminal and C-terminal regions of LANA are required for efficient binding to GSK-3 (21). The LANA C-terminal region that is required for interaction with GSK-3 β was recognized to have low-level homology with the axin GID (22). Two HA-GSK-3 mutants with

known properties were generated and shown to be expressed comparably to wild-type HA-GSK-3 (Fig. 1A). The GSK-3(F291L) mutation causes a 90% loss of GSK-3 binding to axin and to Frat-1, which binds to the same domain of GSK-3 as axin (12). In a precipitation assay performed on extracts of cotransfected HeLa cells, precipitation of HA-GSK-3 wild type with anti-HA antibody resulted in coprecipitation of LANA as expected (Fig. 1B). However, HA-GSK-3(F291L) was significantly impaired for interaction with LANA, and only trace amounts of LANA were detected coprecipitating with

HA-GSK-3(F291L) (Fig. 1B). An alignment between the GID of human axin and the C-terminal domain of LANA is shown in Fig. 1C. The LANA GID had previously been identified using C-terminal truncations of LANA (Fig. 1C). LANA truncated at amino acid 1147 retained GSK-3 interaction, while LANA truncated at amino acid 1133 had lost the ability to bind GSK-3 (22). To validate the C-terminal boundary of this domain, a mutation was introduced at LANA position 1132 (L1132P). The corresponding amino acid is a glutamine in human axin but is a leucine in *Xenopus laevis* axin. An immunoprecipitation assay performed on extracts from cotransfected cells revealed that, unlike wild-type Flag-LANA, Flag-LANA(L1132P) did not bind HA-GSK-3 and was not detected in HA-GSK-3 precipitates (Fig. 1D, upper, lane 2 versus lane 6). LANA(L1132P) was present in the cotransfected cell extract (Fig. 1D, upper, lane 5) and could also be directly precipitated using anti-Flag antibody (Fig. 1D, upper, lane 7). The Flag-LANA proteins were not detected in immunoprecipitates generated with control immunoglobulin (Ig) (Fig. 1D, upper, lanes 4 and 8). Similar results were obtained in the complementary immunoprecipitations (Fig. 1D, lower), where HA-GSK-3 was detected coprecipitating with wild-type Flag-LANA (Fig. 1D, lower, lane 3) but not with Flag-LANA(L1132P) (Fig. 1D, lower, lane 7). The behavior of the GSK-3 and LANA mutant proteins reinforced the similarities between the axin and LANA GIDs.

The second GSK-3 mutant, HA-GSK-3(R96A), is mutated in the binding pocket for the priming phosphate residue. GSK-3(R96A) is unable to phosphorylate primed substrates such as β -catenin but retains the ability to bind to and phosphorylate axin (17, 19, 28). An examination of the ability of HA-GSK-3(R96A) to bind to LANA revealed that this mutation also had severely impaired interaction with LANA in a coprecipitation assay (Fig. 1B, IP: α -HA). This result implies that efficient interaction between GSK-3 and LANA may require coordinate binding to the LANA GID and to phosphorylated priming residues and that, unlike axin, LANA might be a primed substrate for GSK-3.

KSHV LANA is a substrate for GSK-3. An inspection of the LANA amino acid sequence revealed seven potential GSK-3 phosphorylation sites in the N terminus of LANA, including sites in the region of aa 241 to 275 required for GSK-3 binding (aa 116-PSHPVSPGTTDTHS-129, 165-PSQQTTPPHS-174, 185-KSSPD~~S~~LAP~~S~~TLRS-198, 218-QSPVVS-213, 249-SSDGD T-254, 260-PTSPISIGSS-269, and 267-GSSSPS-272 [underlining indicates a consensus GSK-3 site; boldface indicates a priming kinase site]). No GSK-3 consensus sites were present in the C terminus of LANA. Evidence that LANA is a substrate for GSK-3 in KSHV-infected primary effusion lymphoma (PEL) cells was obtained using LANA immunoprecipitated with anti-LANA antibody from nuclear extracts of BC3 PEL cells. The immunoprecipitate was washed, and an in vitro phosphorylation assay was performed in the presence of [32 P]ATP plus 20 μ g/ml heparin, which was added to block the extensive phosphorylation of the LANA C terminus mediated by CKII. Immunoprecipitated LANA was phosphorylated in a manner that was sensitive to the GSK-3 inhibitor LiCl (Fig. 2A). No phosphorylation was observed at the position of the LANA protein in the control Ig immunoprecipitation (Fig. 2A,

FIG. 2. LANA is a substrate for GSK-3. (A) Phosphorylation assay in which LANA immunoprecipitated from BC3 PEL cells with anti-LANA antibody was washed with kinase buffer containing cold ATP followed by resuspension in kinase buffer containing [32 P]ATP and heparin (to inhibit CKII). Aliquots were incubated without (0) or with the GSK-3 inhibitor LiCl (50 or 100 mM). Ig, kinase assay performed with an immunoprecipitate generated with nonspecific Ig. (B) GSK-3 β phosphorylation assay in which GST-LANA(1-340) was incubated with commercial GSK-3 β alone (-) or after preincubation with MAPK, CKI, or CKII in the presence of cold ATP. Subsequent incubation with GSK-3 β was performed in the presence of [32 P]ATP and inhibitors for MAPK (U0126), CKI (IC261), or CKII (heparin). Phosphorylated products were separated by gel electrophoresis and detected by autoradiography. (C) GSK-3 β phosphorylation assay using GST-LANA(dCR) containing both LANA N (1-340) and C (936-1162) domains (lanes 1), GST (lanes 2), or GST-LANA(936-1162) (lanes 3). No phosphorylation was seen in the absence of priming (-). GSK-3 phosphorylation of GST-LANA(dCR) was detected after priming with MAPK (lane 1) or CKI (lane 1). CDC2 did not serve as a priming kinase (lane 1).

IP:Ig). These data show that LANA is associated with enzymatically active GSK-3 in PEL cells and is a substrate for GSK-3.

GSK-3 phosphorylates substrates containing the motif S/TxxxS/Tp, where the +4 position serine or threonine has

been phosphorylated by another kinase. An exception to the need for a primed substrate is axin, which has a separate GSK-3 interaction domain and can be phosphorylated by GSK-3 variants such as GSK-3(R96A). LANA did not bind to GSK-3(R96A) (Fig. 1B), suggesting that LANA might be a primed substrate. To show that it was the LANA N terminus that was the GSK-3 substrate and to examine the requirement for priming phosphorylation, GST-LANA(1-340) was incubated in kinase buffer containing [32 P]ATP with a commercial preparation of purified GSK-3 β . No phosphorylation of the LANA N terminus by GSK-3 β was observed (Fig. 2B, –). There are consensus sites for the kinases CKI, CKII, and MAPK in LANA(1-340), and the ability of these enzymes to act as priming kinases for GSK-3 phosphorylation was tested. Pretreatment of GST-LANA(1-340) with MAPK in kinase buffer containing cold ATP followed by incubation with purified GSK-3 β in the presence of the MAPK inhibitor U0126 and [32 P]ATP led to the incorporation of 32 P into GST-LANA(1-340) (Fig. 2B). This result indicates that GSK-3 β phosphorylation of LANA requires priming and that MAPK can function as a priming kinase. Detectable, but reduced, GSK-3 β phosphorylation of GST-LANA(1-340) also occurred when CKI was the preincubated kinase and GSK-3 β was added in the presence of the CKI inhibitor IC261 and [32 P]ATP (Fig. 2B). Thus, CKI can also act as a priming kinase for LANA(1-340). The lower level of phosphorylation supported by CKI may reflect fewer CKI priming sites in LANA(1-340) than for MAPK or, alternatively, CKI priming may be underestimated. CKI phosphorylates in the context of D/ExxS/T but also S/TpxxS/T, where the –3 position S/T is phosphorylated by a priming kinase. These latter sites would not have been phosphorylated under the experimental conditions used in this assay. Preincubation with CKII did not support subsequent GSK-3 β phosphorylation of LANA(1-340) (Fig. 2B).

LANA contains a GSK-3 binding domain in the C terminus, and it was possible that the N-terminal GSK-3 LANA sites requires priming in the absence of the C-terminal binding domain but would not require priming in the presence of the LANA C terminus. To address this aspect of the requirement for priming, the phosphorylation assay was repeated using GST-LANA(dCR) (Fig. 2C, lane 1), which has the central repeat region deleted but contains both N- and C-terminal domains. [Note that the GST-LANA(dCR) protein is unstable and, consequently, the amount of intact GST-LANA(dCR) protein present in these assays was less than the amount of GST-LANA(1-340) protein used in the assays shown in Fig. 2B.] Phosphorylation of GST-LANA(dCR) was compared to that of the control proteins GST (Fig. 2C, lane 2) and GST-LANA(936-1162) (Fig. 2C, lane 3). Again, no GSK-3 β phosphorylation of any of the substrates was observed in the absence of priming kinases (Fig. 2C, –). GSK-3 β phosphorylation of GST-LANA(dCR) was detected after preincubation with MAPK and CKI (Fig. 2C, MAPK and CKI, lane 1), and this phosphorylation was specific for the N-terminal LANA sequences, as phosphorylation was not detected with GST or with GST-LANA(936-1162) (Fig. 2C, lanes 2 and 3). No GSK-3 β phosphorylation of any of the substrates was seen after addition of cdc2 (Fig. 2C, lanes 1 to 3), suggesting that cdc2 was not an effective priming kinase. Thus, despite the fact

that LANA contains a separate GSK-3 interaction domain, GSK-3 still requires priming activity to phosphorylate LANA.

Multiple sites in the LANA N terminus are phosphorylated by GSK3 β . A series of Flag-LANA N-terminal deletions and Flag-LANA variants containing point mutations in consensus GSK-3 phosphorylation sites were generated in the background of a construction with the LANA central repeat domain deleted. The deleted region contains no consensus GSK-3 binding sites. The GSK-3 phosphorylation sites present in the individual Flag-LANA variants are shown diagrammatically in Fig. 3A. The Flag-LANA variants were immunoprecipitated with anti-Flag antibody from transfected HeLa cells, and the immunoprecipitates were washed and then incubated with commercial GSK-3 β and [32 P]ATP in the presence of heparin to eliminate CKII activity. The relative incorporation of radiolabel into the different N-terminal-deleted LANA proteins was suggestive of phosphorylation by GSK-3 β at multiple sites (Fig. 3B). A gradient of labeling was observed from LANA(dCR) and LANA(N93), which contain all seven consensus GSK-3 sites, through LANA(N155), LANA(N175) (with the aa 117 and 166 sites deleted), LANA(N204) (with the 117, 166, and 186 sites deleted), LANA(N241) (with the 117, 166, 186, and 219 sites deleted), and LANA(N204, m268) (with the 117, 166, 186, 219, and 268 sites deleted). No phosphorylation was observed with Flag-LANA substrates that lacked GSK-3 consensus sites. This includes LANA(N275), where the N terminus commences at aa 275 (Fig. 3B, lane 7), and LANA(N204, m219, 250, 261, 268) and LANA(N241, m250, 261, 268), which have N termini at aa 204 and 241, respectively, and in which all consensus GSK-3 sites have been mutated (Fig. 3B, lanes 9 and 10). A Western blot assay showing expression of the mutant LANA proteins in transfected HeLa cells is presented in Fig. 3C.

Examination of additional mutated Flag-LANA derivatives provided specific evidence for phosphorylation at sites 219 and 250 by GSK-3 (Fig. 3B). LANA(N241,m261,268) contains only the 250 consensus site intact, while LANA(N204,m250,261,268) contains only the 219 consensus site intact. Both of these proteins were phosphorylated by GSK-3 β (Fig. 3B, lanes 8 and 11).

The effect of mutation of individual consensus phosphorylation sites in the background of Flag-LANA with the central repeat domain deleted (dCR) was also examined (Fig. 4). A diagram of the individual Flag-LANA variants showing the consensus GSK-3 sites retained in each mutant is presented in Fig. 4A. Individual mutation of the N-terminal three GSK-3 consensus sites at positions 117, 166, and 186 resulted in a relatively small and equal decrease in overall phosphorylation compared to the parental construction, Flag-LANA(dCR) (Fig. 4B, lanes 2 to 4 versus lane 1). Mutation of any of the next four GSK-3 consensus sites at 219, 250, 261, and 268 had a more dramatic effect on GSK-3 phosphorylation of Flag-LANA (Fig. 4B, lanes 5 to 8), suggesting that sites in this region may be particularly important for LANA to serve as a substrate for GSK-3. Expression of the mutant LANA proteins in transfected HeLa cells was demonstrated by Western blot analysis (Fig. 4C).

Efficient binding of GSK-3 β to LANA requires GSK-3 phosphorylation. We had previously found that an N-terminal region between LANA amino acids 241 and 275 was needed for

FIG. 3. GSK-3 phosphorylation of N-terminally deleted LANA variants. (A) Diagram showing the location of consensus GSK-3 phosphorylation sites in LANA and the relative positions of the N-terminal deletions in the Flag-LANA variants. (B) Phosphorylation of the Flag-LANA variants precipitated from transfected HeLa cells with anti-Flag antibody and incubated with commercial GSK-3 β in kinase buffer containing [32 P]ATP and the CKII inhibitor heparin. The electrophoretically separated, phosphorylated proteins were detected by autoradiography. (C) Western blot probed with anti-Flag antibody showing expression of the Flag-LANA proteins in transfected HeLa cells.

efficient interaction between LANA and GSK-3 (21). This region contains the consensus GSK-3 phosphorylation sites at positions 250, 261, and 268 that when individually mutated significantly reduce the ability of LANA to serve as a substrate for GSK-3 (Fig. 4B). To determine whether the reduction in GSK-3 phosphorylation seen with mutation of these sites was related to a reduction in binding affinity for GSK-3, the ability of Flag-LANA variants carrying mutations in specific GSK-3 sites to interact with S-tag GSK-3 β was tested. Complexes containing S-GSK-3 β were precipitated from extracts of cotransfected HeLa cells using S-peptide-Sepharose beads. The presence of coprecipitated Flag-LANA was detected by Western blotting with anti-Flag antibody. Flag-LANA commencing at aa 204 (N204) or 241 (N241) coprecipitated with S-GSK-3 β (Fig. 5B, group 1 IP and group 5 IP), whereas the N204 and N241 variants in which all the GSK-3 sites were mutated (N204m219,250,261,268 and N241m250,261,268) and which were not substrates for GSK-3 (Fig. 3B) did not interact (Fig. 5B, group 4 IP and group 6 IP). These data indicate that GSK-3 phosphorylation is required for efficient GSK-3 β interaction with LANA. Flag-LANA was present in the extracts of the transfected cells (Fig. 5B, groups 1 to 6, lane E), and immunoprecipitation of Flag-LANA was not observed with control Ig (Fig. 5B, groups 1 to 6, lane C).

To determine whether GSK-3 interaction was dependent on the number of phosphorylation sites or on specific sites, the

effect of mutating individual GSK-3 sites in the N204 or N241 backgrounds was examined. Flag-LANA N204 variants in which only the 219 and 250 phosphorylation sites remained intact (N204m261,268) retained interaction with GSK-3 β (Fig. 5B, group 2 IP). However, a variant in which only the 219 site remained (N204m250,261,268) did not interact (Fig. 5B, group 3 IP). These data suggested either that a minimum of two sites was required for interaction or that the 250 site, which was present in all the interacting proteins, was particularly important. To investigate this aspect, another mutant was generated that contained only the intact 250 site in an N241 background (N241m219,261,268). A coprecipitation assay was performed using the interacting (N204m261,268) and noninteracting (N204m250,261,268) variant LANA proteins as positive and negative controls and extracts from cells cotransfected with HA-GSK-3 β and the Flag-LANA variants (Fig. 5C). As expected, N204m261,268 coprecipitated with GSK-3 β (Fig. 5C, group 2 IP) and N204m250,261,268 did not (Fig. 5C, group 3 IP). LANA(N241m219,261,268) also coprecipitated with HA-GSK-3 (Fig. 5C, group 7 IP), indicating that not all consensus sites are functionally equivalent and that a single GSK-3 phosphorylation site at the 250 position was sufficient for stable binding of LANA to GSK-3 β .

GSK-3 phosphorylation of a LANA(246-258) peptide. To further establish that the consensus site S (250) xxxT was a substrate for GSK-3, a 13-amino-acid peptide was synthesized

FIG. 4. GSK-3 phosphorylation of LANA(dCR) carrying mutations in consensus GSK-3 sites. (A) Diagram showing the location of the mutations introduced into consensus GSK-3 phosphorylation sites in Flag-LANA. (B) GSK-3 phosphorylation assay in which the Flag-LANA mutants immunoprecipitated from transfected HeLa cells were incubated with commercial GSK-3 β in the presence of [32 P]ATP and the CKII inhibitor heparin. The electrophoretically separated, phosphorylated proteins were detected by autoradiography. (C) Western blot probed with anti-Flag antibody showing expression of the Flag-LANA proteins in transfected HeLa cells.

that contained LANA sequences from amino acids 246 to 258 (Fig. 6A). Addition of purified GSK-3 β alone did not result in phosphorylation of this peptide in an in vitro kinase assay (Fig. 6B, lane 1). The peptide was a substrate for CKI (Fig. 6B, lane 2), while phosphorylation by MAPK when added alone was below the level of detection (Fig. 6B, lane 3). The GSK-3 phosphorylation assays were then repeated after priming of the peptide with CKI, MAPK, or CKI plus MAPK. The priming reactions were carried out for 10 min at 30°C in kinase buffer containing unlabeled ATP. The activity of the priming kinases was then blocked by the addition of the MAPK inhibitor U0126 (10 μ M), the CKI inhibitor IC261 (10 μ M), or both

inhibitors. These inhibitors were sufficient to prevent subsequent phosphorylation of the peptide upon the addition of kinase buffer containing [32 P]ATP (Fig. 6B, lanes 4, 6, and 8). The ability of GSK-3 β to phosphorylate the primed peptide was then examined. Priming by either CKI or MAPK resulted in phosphorylation of the peptide by GSK-3 β (Fig. 6B, lanes 5 and 7). Concurrent preincubation with CKI and MAPK did not enhance GSK-3 phosphorylation activity (Fig. 6B, lane 9). This is consistent with the presence of a single GSK-3 consensus site in the peptide and independent priming by CKI and MAPK at the -4 position threonine 254 (Fig. 6A). The peptide was a better substrate for CKI priming than MAPK priming in vitro,

FIG. 5. Phosphorylation by GSK-3 modulates interaction with LANA. (A) Diagram showing the GSK-3 sites present in the LANA variants used in the interaction assays. (B) Western blot probed with anti-Flag antibody to determine the ability of the Flag-LANA variants to coprecipitate with S-GSK-3 β . Extracts of HeLa cells cotransfected with S-GSK-3 β and Flag-LANA variants were immunoprecipitated with S-peptide-Sepharose beads, and the precipitated proteins were separated by SDS-PAGE. E, extract; IP, immunoprecipitated with S-peptide-Sepharose; C, immunoprecipitated with control Ig. The extract lane contains 5% of the protein used in the immunoprecipitation reactions. (C) Western blot comparing the ability of Flag-LANA variants containing consensus GSK-3 sites at 219 and 250 versus 219 or 250 to coprecipitate with HA-GSK-3 β .

FIG. 6. GSK-3β phosphorylation of a LANA(246-258) peptide. (A) Amino acid sequence of LANA(246-258) highlighting S/T residues that are the consensus sites for phosphorylation. S, GSK-3 site; T, MAPK [↓] or CKI [^] site. (B) In vitro phosphorylation of the LANA(246-258) synthetic peptide. The peptide was incubated with the indicated kinases in the presence or absence of GST-3β. MAPK and CKI priming reactions were performed in kinase buffer containing cold ATP. The priming kinase reaction was stopped by the addition of the inhibitors U0126 (MAPK) or IC261 (CKI), and [³²P]ATP was then added. The phosphorylated products were electrophoretically separated and detected by autoradiography. The aa 246 to 258 peptide was a substrate for GSK-3β only after priming by CKI or MAPK.

as independent MAPK phosphorylation was below the level of detection (Fig. 6B, lane 3). Thus, GSK-3 is able to phosphorylate a peptide containing the serine 250 consensus site in vitro.

Multiple LANA GSK-3 phosphorylation sites are required for efficient LANA-mediated activation of a β-catenin-responsive reporter. LANA overcomes GSK-3-mediated degradation of β-catenin by binding to GSK-3 in the nucleus and depleting cytoplasmic levels of GSK-3 (22). The ability of LANA variants carrying mutations in the GSK-3 phosphorylation sites to overcome GSK-3 repression and activate expression of a β-catenin-responsive Tcf-luciferase reporter was examined in transfected HeLa cells. The location of the GSK-3 consensus sites in the parental N204, N241, and N275 LANA constructions is shown diagrammatically in the inset in Fig. 7. Transfected GSK-3 repressed expression of the pGL3-OT reporter (Fig. 7, lane 2 versus lane 1). LANA(N204) overcame this repression and activated expression from the pGL3-OT reporter 30-fold (Fig. 7, lane 3 versus lane 2). Mutation of either the 261 or 268 phosphorylation sites individually (N204m261 and N204m268) had no effect on activation of the reporter (Fig. 7, lanes 4 and 5 versus lane 3), and mutation of both sites resulted in only a small decrease in activity (Fig. 7, lane 6 versus lane 3). LANA(N241), which lacks the 219 site, was almost as active as LANA(N204), which retains this site (Fig. 7, lane 7 versus lane 3). Mutation of either the 261 or 268 sites in the N241 background (N241m261 and N241m268) had a more significant effect than in the N204 background, and these LANAs activated reporter expression approximately twofold less effectively (Fig. 7, lanes 8 and 9 versus lanes 4 and 5). Retention of only the 250 site in the N241 background (N241m261,268)

reduced activation of the reporter to fivefold (Fig. 7, lane 10 versus lane 7) and LANA N275, lacking any GSK-3 consensus sites, was significantly impaired in its ability to activate reporter expression (Fig. 7, lane 11 versus lane 3). Overall, LANA with a single GSK-3 site at aa 250 had the ability to activate the β-catenin-responsive reporter but was less effective than LANA containing three GSK-3 sites, while LANA retaining two GSK-3 sites gave an intermediate activation of the reporter. Thus, multiple phosphorylation sites seem to be required for full activation of a β-catenin-responsive reporter.

LANA binding sequesters GSK-3 activity. LANA mediates a nuclear accumulation of GSK-3 that occurs during S phase in KSHV-infected cells. Nuclear GSK-3 is normally present at low levels, and phosphorylation of nuclear proteins by GSK-3 has a negative effect on cell proliferation. For example, GSK-3 phosphorylation of cyclin D1 and c-Myc targets these proteins for export into the cytoplasm, while phosphorylation increases the transcriptional activity of p53. The ability of PEL cells to tolerate increased accumulation of nuclear GSK-3 implies that a mechanism exists to control the activity of LANA-associated nuclear GSK-3. LANA is a substrate for phosphorylation by GSK-3, indicating that LANA-associated GSK-3 is enzymatically active. The presence of both a GSK-3 binding domain and multiple consensus GSK-3 phosphorylation sites in LANA raised the possibility that LANA might sequester GSK-3 in a manner that limited access to other substrates. To test this possibility, the effect of LANA association on the ability of GSK-3 to phosphorylate a primed peptide substrate was examined using an extract of unsynchronized HeLa cells that had been transfected with Flag-LANA and HA-GSK-3β. The pres-

FIG. 7. Multiple GSK-3 sites are necessary for maximal LANA-mediated activation of a β -catenin-responsive reporter. Results are from a transient expression assay in which HeLa cells were transfected with the pGL3-OT luciferase reporter (lane 1) plus GSK-3 β (lanes 2 to 11) and the indicated Flag-LANA variants (lanes 3 to 11). (Inset) Diagram of the parental N204, N241, and N275 Flag-LANA proteins showing the location of consensus GSK-3 sites.

ence of Flag-LANA and HA-GSK-3 β in the extract was demonstrated by Western blotting using anti-LANA and anti-HA antibodies (Fig. 8, upper and middle, lane 1). A glycogen synthase-derived primed substrate peptide was phosphorylated by GSK-3 in the transfected cell extract, as shown by incorporation of 32 P (Fig. 8, lower, lane 1). No phosphorylation of the peptide was seen with an immunoprecipitate generated with control Ig (Fig. 8, lower, lane 2), and neither Flag-LANA nor HA-GSK-3 β was present in this immunoprecipitate (Fig. 8, upper and middle, lane 2). GSK-3 in unsynchronized cells is predominantly cytoplasmic, even in the presence of LANA. HA-GSK-3 β precipitated from the total extract with anti-HA antibody was not highly associated with LANA (Fig. 8, upper, lane 3) and was capable of phosphorylating the peptide substrate (Fig. 8, lower, lane 3). In contrast, nuclear HA-GSK-3 associated with Flag-LANA in a precipitate generated with anti-Flag-Sepharose beads (Fig. 8, upper and middle, lane 4) did not phosphorylate the added peptide substrate. (Fig. 8, lower, lane 4). (Note that the use of Flag-Sepharose beads eliminates the Ig band in the precipitates in lane 4, middle.) Thus, the phosphorylation sites in LANA may serve two purposes: increasing the affinity of binding for GSK-3 and providing a competitive substrate that minimizes phosphorylation of other noncomplexed substrates.

Overall, these results suggest that, while the relocation of GSK-3 to the nucleus by LANA provides a novel mechanism for stabilizing β -catenin, GSK-3-mediated regulation of protein-protein interactions and sequestration of GSK-3 activity are common features of both the cytoplasmic β -catenin destruction complex and the nuclear LANA-GSK-3 complex (Fig. 9).

FIG. 8. LANA-bound GSK-3 β does not phosphorylate an exogenous peptide substrate. (Upper) Western blot probed with anti-Flag antibody to detect the presence of Flag-LANA in extracts from HeLa cells cotransfected with Flag-LANA plus HA-GSK-3 β . Lane 1, extract; lane 2, immunoprecipitate generated with control Ig; lanes 3 and 4, immunoprecipitates generated with anti-HA antibody (lane 3) or anti-Flag-Sepharose beads (lane 4). (Middle) Western blot of the same extract probed with anti-HA antibody to detect HA-GSK-3 β . Lane 1, extract; lane 2, immunoprecipitates generated with control Ig; lanes 3 and 4, anti-HA antibody (lane 3) or anti-Flag-Sepharose beads (lane 4). (Lower) In vitro GSK-3 β phosphorylation assay using the same transfected cell extract showing incorporation of 32 P into a primed glycogen synthase substrate peptide in the presence of extract (lane 1) or GSK-3 β directly precipitated with anti-HA antibody (lane 3). No GSK-3 activity was detected in the presence of an immunoprecipitate generated with control Ig (lane 2) or in the presence of GSK-3 β coprecipitated with LANA in an immunoprecipitate generated with anti-Flag-Sepharose beads (lane 4).

FIG. 9. GSK-3 activity in the β -catenin destruction complex and in LANA-expressing cells. (A) Axin binds to GSK-3, APC, β -catenin, and CKI in a cytoplasmic complex. The apposition of these proteins allows GSK-3 to phosphorylate β -catenin that has been primed by phosphorylation with CKI. Phosphorylated β -catenin is targeted for proteasomal destruction, thus regulating β -catenin levels. Phosphorylation of axin and APC by GSK-3 regulates protein interactions and axin stability. (B) Wnt signaling leads to dissociation of the β -catenin destruction complex. GSK-3 is displaced from axin by Frat/GBP, and GSK-3 activity is repressed through the action of the protein phosphatase 1 inhibitor 2 complex. β -Catenin is no longer targeted for degradation and accumulates, allowing nuclear entry and activation of promoters containing Tcf/Lef binding sites. (C) LANA binds to GSK-3 in the nucleus. Binding of GSK-3 to LANA depletes the levels of cytoplasmic GSK-3 and results in cytoplasmic β -catenin accumulation. The bound GSK-3 phosphorylates LANA using MAPK and CKI as priming kinases. GSK-3 phosphorylation of LANA is necessary for high-affinity interaction with GSK-3. Nuclear GSK-3 bound to LANA is sequestered, and its activity is limited to participants in the LANA–GSK-3 complex.

DISCUSSION

The key to LANA-mediated dysregulation of β -catenin is the binding of LANA to nuclear GSK-3 and consequent nuclear accumulation and cytoplasmic depletion of GSK-3. The experiments described here provide additional insight into the mechanisms that regulate this process. We had previously recognized that the C terminus of LANA contained a region with a low level of homology to the GID of axin (22). Axin and Frat/GBP each interact with the same domain on GSK-3, and crystallographic studies have revealed that the axin and Frat GSK-3-interacting regions adopt a very similar tertiary structure, even though these domains have little primary sequence identity (12). The F291L GSK-3 mutant is unable to bind efficiently to either Frat-1 or axin. In our experiments, GSK-3(F291L) was also unable to bind to LANA, suggesting that the GID of LANA adopts a similar structure to that of axin and Frat-1. The GID of human axin has been defined as a 24-amino-acid region (aa 380 to 403) that inhibits GSK-3 activity in *Xenopus* oocytes and embryos and in mammalian cells, resulting in activation of Wnt signaling (29, 64). C-terminal deletions previously introduced into LANA showed that deletion to LANA aa 1147 did not affect binding to GSK-3, whereas removal of C-terminal sequences to position 1133 resulted in loss of GSK-3 interaction (22). This boundary has now been

confirmed by introduction of a mutation at LANA aa 1132. LANA(L1132P) also does not interact with GSK-3 in immunoprecipitation assays. In the alignment between the 25-amino-acid axin GID and the LANA C terminus, L1132 is equivalent to aa 22, which is a glutamine residue in human and mouse axin but a leucine in *Xenopus* axin.

The fact that LANA uses an axin-like interaction domain to bind to GSK-3 is likely to have additional functional consequences. LANA mediates a nuclear accumulation of GSK-3 that is dependent on binding to GSK-3 (21). Nuclear accumulation of proteins often reflects a balance between nuclear entry and nuclear export. Apoptotic stimuli and S phase of the cell cycle are known to be factors that stimulate nuclear entry of GSK-3 (7, 13), although how these stimuli are transmitted has not been defined. LANA-mediated nuclear accumulation of GSK-3 is cell cycle dependent and is most dramatically demonstrable in S phase (22). Nuclear export of GSK-3 is regulated by Frat/GBP (18). GSK-3 accumulates in the nucleus in the presence of leptomycin B, and a Frat peptide from the GSK-3 interaction domain that blocks binding of endogenous Frat was shown to mediate nuclear accumulation of GSK-3. Axin has also recently been shown to shuttle between the nucleus and cytoplasm and has been implicated in the nuclear export of β -catenin (9, 57). Frat/GBP orthologs have not been

identified in *Drosophila melanogaster* or worms, raising the possibility that other proteins, potentially axin, could also contribute to GSK-3 nuclear export. Although mutations can be introduced into GSK-3 that differentially affect binding of axin and Frat-1, their binding domains on GSK-3 overlap to the extent that binding of GSK-3 by axin and Frat-1 is mutually exclusive (16). By binding to the same region of GSK-3 as axin and Frat-1, LANA would prevent Frat-1- or axin-mediated nuclear export of GSK-3 and foster nuclear accumulation.

We have now shown that, in addition to binding to GSK-3, LANA is a substrate for GSK-3 both in vitro and in KSHV-infected PEL cells. LANA has seven separate consensus GSK-3 phosphorylation sites within the LANA N terminus. In vitro phosphorylation assays on wild-type and mutant and deleted LANA proteins produced results consistent with GSK-3 phosphorylation of the LANA N terminus at multiple sites, but evidence for phosphorylation of specific sites was limited to the sites at LANA amino acids 219 and 250. In addition to the LANA C-terminal axin-like GSK-3 interaction domain, efficient binding of LANA to GSK-3 also requires an N-terminal region of LANA located between amino acids 241 and 275 (21). This region contains three consensus GSK-3 phosphorylation sites. Examination of LANA derivatives carrying mutated GSK-3 consensus sites in the aa 241 to 275 region revealed that mutation of all of the sites abolished GSK-3 interaction. Thus, the interaction between LANA and GSK-3 is regulated by GSK-3 phosphorylation of LANA. Mutation of individual GSK-3 consensus sites in this region indicated that LANA serine 250 was particularly important for GSK-3 binding to LANA.

In the cytoplasmic β -catenin destruction complex, phosphorylation of β -catenin by GSK-3 requires priming of the +4 position by casein kinase I α (1, 39, 63) and, in the phosphorylation of APC, GSK-3 and casein kinase I ϵ mutually prime each other's activity (23, 27, 46). On the other hand, axin is one of the few GSK-3 substrates that is phosphorylated by GSK-3 in the absence of priming (8, 17). LANA, like axin, has a separate C-terminal GSK-3 interaction domain, and yet LANA requires priming phosphorylation to become a substrate for GSK-3. Further, GSK-3 R96A, which is mutated in the GSK-3 priming pocket, is impaired for binding to LANA. Two kinases that can function as priming kinases for LANA in vitro are p38 MAPK and CKI α . If the presence of a GSK-3 interaction domain in axin were sufficient to allow nonprimed phosphorylation of axin, then why would LANA require priming? In KSHV-infected cells, nuclear accumulation of GSK-3 occurs predominantly during S phase and then subsequently reverts to a more normal cytoplasmic distribution. Cell cycle-regulated recruitment of the priming kinases to LANA might provide a mechanism for modulating GSK-3 phosphorylation of LANA and hence the affinity of the LANA-GSK-3 interaction and GSK-3 intracellular distribution.

While the presence of a single N-terminal GSK-3 consensus site was sufficient to promote LANA binding to GSK-3, activation of β -catenin-regulated gene expression in reporter assays was more effective in the presence of LANA derivatives retaining multiple GSK-3 consensus sites. This may merely reflect differences in the assays. The coprecipitation assay for interaction is either positive or negative and does not provide the more quantifiable readout of the reporter assay. Thus, the

increased response in the reporter assay to LANA carrying multiple GSK-3 consensus sites may reflect a gradient of binding affinity. On the other hand, the presence of multiple GSK-3 phosphorylation sites on LANA may serve an additional purpose. Many nuclear proteins are substrates for GSK-3, and GSK-3 is recruited into the nucleus in response to apoptotic stimuli, where it binds to p53 and activates p53-mediated transcription and apoptotic activity (7, 55, 56). Thus, if unregulated, LANA-mediated accumulation of GSK-3 could have drastic consequences for the cell. LANA is phosphorylated by GSK-3, indicating that LANA-bound GSK-3 is enzymatically active. However, the ability of GSK-3 coprecipitated with LANA to phosphorylate a primed peptide substrate in vitro was severely impaired. This suggests that GSK-3 bound to LANA is sequestered in a way that makes it unavailable to phosphorylate other substrates. The combination of a GID and multiple primed GSK-3 phosphorylation sites on LANA may make LANA an effective competitive inhibitor. As a consequence, other nuclear GSK-3 substrates that are not participants in the LANA-GSK-3 complex may be spared from the consequences of increased nuclear GSK-3 levels.

Despite the novel nature of LANA-mediated dysregulation of β -catenin, there are proving to be some commonalities with natural Wnt signaling (Fig. 9). There is now increased support for the similarity between the GIDs of axin and LANA. We propose that GSK-3 phosphorylation regulates the affinity of the LANA-GSK-3 interaction and GSK-3 phosphorylation also modulates the affinity of protein-protein interactions in the cytoplasmic β -catenin destruction complex. GSK-3 phosphorylation of axin increases axin stability and affinity for β -catenin (53, 58, 62), while GSK-3 phosphorylation of APC increases APC binding to the axin interaction domain on β -catenin (27, 45, 61). CKI can function as a GSK-3 priming kinase in both complexes. Further, GSK-3 appears to be sequestered in both complexes. GSK-3 associated with LANA did not phosphorylate an exogenous primed peptide, and insulin-mediated signaling that leads to inactivation of GSK-3 through Akt does not affect GSK-3 activity in the β -catenin destruction complex (60). As the participants in the LANA-GSK-3 complex are further characterized, it is possible that additional consequences of the LANA-GSK-3 interaction will become apparent.

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