Identification of the Axin and Frat Binding Region of Glycogen Synthase Kinase-3*

Received for publication, October 1, 2001, and in revised form, November 13, 2001
Published, JBC Papers in Press, November 13, 2001, DOI 10.1074/jbc.M109462200

Elizabeth Fraser‡‡‡, Neville Young‡‡, Rana Dajani‡, Jonathan Franca-Koh‡, Jonathan Ryves‡, Robin S. B. Williams‡, Margaret Yeow‡, Marie-Therese Webster‡, Chris Richardson‡, Matthew J. Smalley‡, Laurence H. Pearl‡, Adrian Harwood‡‡‡‡, and Trevor C. Dale‡‡‡‡  ‡‡ To whom correspondence should be addressed. Tel.: 44-20-7352-8133
§ These authors contributed equally to this work.
¶ Each protein contains a pseudosubstrate that autoinhibits GSK-3 activity.

The diversity of substrates is reflected in the complexity and number of regulatory mechanisms that act on GSK-3. In animals, most studies have shown the activity of GSK-3 to be negatively regulated by ligands such as epidermal growth factor, Wnt, and insulin. In Dictyostelium, genetic and biochemical evidence showed both positive and negative regulation of GSK-3 activity (7, 8). Insulin signaling is the best characterized signaling pathway. Here, GSK-3 kinase activity is inhibited through phosphorylation of an inhibitory amino-terminal serine (Ser-21 in GSK-3) by protein kinase A/B (9). The recent publication of the structure of human GSK-3β can explain this mechanism of GSK-3 regulation (10, 11). GSK-3β has a basic patch of amino acids in its substrate binding groove that recognizes substrates when prephosphorylated at position +4 with respect to the target residue. The phosphorylation of GSK-3 at serine 9 by regulators such as protein kinase B and protein kinase A appears to generate a pseudosubstrate that autoinhibits GSK-3 activity by competition for substrate binding (10–12).

The mechanism of GSK-3 regulation in response to other signals is less clear. Serine phosphorylation at a site other than serine 9 has been shown in response to Wnt ligands (13), while tyrosine phosphorylation and activation of GSK-3 occurs in the regulation of Dictyostelium GskA in response to stimulation with extracellular cAMP (14). Inhibition of GSK-3 by insulin or activated forms of protein kinase B is not sufficient for the activation of TCF-dependent transcription (15). By contrast, small molecule inhibitors of GSK-3 are able to activate transcription (16–18). This suggests that different pools of GSK-3 exist within the cell to integrate upstream signals with specific downstream targets.

These independent pools could be generated by interaction with scaffolding proteins. The best understood of these is Axin, which templates GSK-3 phosphorylation of β-catenin as part of a multiprotein complex that degrades β-catenin (reviewed in Refs. 19 and 20). Wnt signal transduction interferes with the function of this complex, leading to the stabilization of β-catenin and the activation of β-catenin/TCF-dependent transcription. Other proteins that have been suggested to bind GSK-3 directly include Frat/GBP, presenilin, and Muc1 (21–23). Frat competes with Axin for binding to GSK-3 and also interacts with β-catenin for degradation. This is inhibited by Wnt stimulation, and the increased concentrations of β-catenin bind and activate members of the TCF transcription factor family (3). In addition, GSK-3 appears to play a role in regulating nuclear export both for cyclin D1 and the transcription factors, nuclear factor of activated T-cells and D. discoideum STAT protein (4–6).

The Axin binding site blocks the ability of dominant negative GSK-3 to cause axis duplication in Xenopus embryos. The Axin binding site is conserved within all GSK-3 proteins, and its loss affects both cell motility and gene expression in the nonmetazoan, Dictyostelium. Surprisingly, we find no genetic interaction between a non-Axin-binding GSK-3 mutant and T-cell factor activity, arguing that Axin interactions alone cannot explain the regulation of T-cell factor-mediated gene expression.

Glycogen synthase kinase-3 (GSK-3) is a key component of several signaling pathways including those regulated by Wnt and insulin ligands. Specificity in GSK-3 signaling is thought to involve interactions with scaffold proteins that localize GSK-3 regulators and substrates. This report shows that GSK-3 forms a low affinity homodimer that is disrupted by binding to Axin and Frat. Based on the crystal structure of GSK-3, we have found surface-scanning mutagenesis to identify residues that differentially affect GSK-3 interactions. Mutations that disrupt Frat and Axin cluster at the dimer interface explaining their effect on homodimer formation. Loss of the Axin binding site blocks the ability of dominant negative GSK-3 to cause axis duplication in Xenopus embryos. The Axin binding site is conserved within all GSK-3 proteins, and its loss affects both cell motility and gene expression in the nonmetazoan, Dictyostelium. Surprisingly, we find no genetic interaction between a non-Axin-binding GSK-3 mutant and T-cell factor activity, arguing that Axin interactions alone cannot explain the regulation of T-cell factor-mediated gene expression.

Glycogen synthase kinase-3 (GSK-3) is a serine/threonine protein kinase that plays a key role in several signaling pathways. GSK-3 homologues have been identified in most eukaryotes including yeast and the slime mold, Dictyostelium discoideum (reviewed in Refs. 1 and 2). Targets of GSK-3 include proteins involved in transcription, translation, the control of the cytoskeleton, cell cycle, and glycogen metabolism. Phosphorylation by GSK-3 is often inhibitory. For example, GSK-3 phosphorylation inhibits glycogen synthase activity. Insulin stimulation reduces GSK-3 activity and hence increases the conversion of glucose to glycogen (1). GSK-3 phosphorylation also targets β-catenin for degradation. This is inhibited by Wnt stimulation, and the increased concentrations of β-catenin bind and activate members of the TCF transcription factor family (3). In addition, GSK-3 appears to play a role in regulating nuclear export both for cyclin D1 and the transcription factors, nuclear factor of activated T-cells and D. discoideum STAT protein (4–6).

The diversity of substrates is reflected in the complexity and number of regulatory mechanisms that act on GSK-3. In animals, most studies have shown the activity of GSK-3 to be negatively regulated by ligands such as epidermal growth factor, Wnt, and insulin. In Dictyostelium, genetic and biochemical evidence showed both positive and negative regulation of GSK-3 activity (7, 8). Insulin signaling is the best characterized signaling pathway. Here, GSK-3 kinase activity is inhibited through phosphorylation of an inhibitory amino-terminal serine (Ser-21 in GSK-3α and Ser-9 in GSK-3β) by protein kinase B/Akt (9). The recent publication of the structure of human GSK-3β can explain this mechanism of GSK-3 regulation (10, 11). GSK-3β has a basic patch of amino acids in its substrate binding groove that recognizes substrates when prephosphorylated at position +4 with respect to the target residue. The phosphorylation of GSK-3 at serine 9 by regulators such as protein kinase B and protein kinase A appears to generate a pseudosubstrate that autoinhibits GSK-3 activity by competition for substrate binding (10–12).

The mechanism of GSK-3 regulation in response to other signals is less clear. Serine phosphorylation at a site other than serine 9 has been shown in response to Wnt ligands (13), while tyrosine phosphorylation and activation of GSK-3 occurs in the regulation of Dictyostelium GskA in response to stimulation with extracellular cAMP (14). Inhibition of GSK-3 by insulin or activated forms of protein kinase B is not sufficient for the activation of TCF-dependent transcription (15). By contrast, small molecule inhibitors of GSK-3 are able to activate transcription (16–18). This suggests that different pools of GSK-3 exist within the cell to integrate upstream signals with specific downstream targets.

These independent pools could be generated by interaction with scaffolding proteins. The best understood of these is Axin, which templates GSK-3 phosphorylation of β-catenin as part of a multiprotein complex that degrades β-catenin (reviewed in Refs. 19 and 20). Wnt signal transduction interferes with the function of this complex, leading to the stabilization of β-catenin and the activation of β-catenin/TCF-dependent transcription. Other proteins that have been suggested to bind GSK-3 directly include Frat/GBP, presenilin, and Muc1 (21–23). Frat competes with Axin for binding to GSK-3 and also interacts with β-catenin for degradation. This is inhibited by Wnt stimulation, and the increased concentrations of β-catenin bind and activate members of the TCF transcription factor family (3). In addition, GSK-3 appears to play a role in regulating nuclear export both for cyclin D1 and the transcription factors, nuclear factor of activated T-cells and D. discoideum STAT protein (4–6).

The diversity of substrates is reflected in the complexity and number of regulatory mechanisms that act on GSK-3. In animals, most studies have shown the activity of GSK-3 to be negatively regulated by ligands such as epidermal growth factor, Wnt, and insulin. In Dictyostelium, genetic and biochemical evidence showed both positive and negative regulation of GSK-3 activity (7, 8). Insulin signaling is the best characterized signaling pathway. Here, GSK-3 kinase activity is inhibited through phosphorylation of an inhibitory amino-terminal serine (Ser-21 in GSK-3α and Ser-9 in GSK-3β) by protein kinase B/Akt (9). The recent publication of the structure of human GSK-3β can explain this mechanism of GSK-3 regulation (10, 11). GSK-3β has a basic patch of amino acids in its substrate binding groove that recognizes substrates when prephosphorylated at position +4 with respect to the target residue. The phosphorylation of GSK-3 at serine 9 by regulators such as protein kinase B and protein kinase A appears to generate a pseudosubstrate that autoinhibits GSK-3 activity by competition for substrate binding (10–12).

The mechanism of GSK-3 regulation in response to other signals is less clear. Serine phosphorylation at a site other than serine 9 has been shown in response to Wnt ligands (13), while tyrosine phosphorylation and activation of GSK-3 occurs in the regulation of Dictyostelium GskA in response to stimulation with extracellular cAMP (14). Inhibition of GSK-3 by insulin or activated forms of protein kinase B is not sufficient for the activation of TCF-dependent transcription (15). By contrast, small molecule inhibitors of GSK-3 are able to activate transcription (16–18). This suggests that different pools of GSK-3 exist within the cell to integrate upstream signals with specific downstream targets.
with the upstream Wnt signaling component Dishevelled, leading to the suggestion that it titrates GSK-3 from Axin in response to Wnt signaling (24, 25).

In this paper, we examine the interactions of GSK-3 with itself as a homodimer and with Frat and Axin. We use the crystal structural data to generate a library of surface scanning mutants. From this library, we have identified four mutants that interact with only Axin or Frat but not both. This argues that although binding of Axin and Frat to GSK-3 may be mutually exclusive, they do not bind through an identical interaction site. To assess the importance of the Axin binding, we examine non-Axin binding GSK-3 mutants in the context of a number of organisms. As expected, Axin binding is required for the effects of dominant negative GSK-3 on patterning in Xenopus embryos. The Axin binding site motif is conserved in all GSK-3 kinase family members, and we show that it is required for GSK-3 function in the nonmetazoan, Dictyostelium. Finally, we show that Axin binding is not the only interaction able to regulate TCF-dependent gene expression.

**EXPERIMENTAL PROCEDURES**

**Cross-linking—**His-tagged GSK-3β was purified from baculovirus-infected insect cells as described in Ref. 10. Cross-linking studies were based on a method by Prodromou et al. (26). GSK-3, ATP (2 mM final concentration), the cross-linking reagent dimethyl suberimidate (DMS, 50 mM stock solution), FRATide, and AxinGID were diluted in reaction buffer (100 mM HEPES, pH 8.0, 150 mM NaCl, 5 mM MgCl2) and made up to a total volume of 40 μl. DMS was added to the mixture at 30-fold molar excess over the primary amine content of the GSK-3. The reactions were incubated at 4 °C for 90 min. and stopped by the addition of 25 mM Tris, pH 6.8, and SDS loading buffer. The mixtures were analyzed on 10 or 12% SDS-PAGE gels and stained with Coomassie Blue. This was made up to 250 μl of washed glutathione-Sepharose beads (Amersham Pharmacia Biotech). This was made up to 250 μl with ice-cold buffer (100 mM NaCl, 0.5% Nonidet P-40, 20 mM Tris-HCl, pH 8.0, 1 mM EDTA supplemented with a Complete protease inhibitor tablet (Roche Molecular Biochemicals), 1 mM phenylmethylsulfonyl fluoride and 0.5 mM dithiothreitol). Following incubation on a rotary mixer at 4 °C for 1 h, samples were pelleted, washed three times with ice-cold buffer, and analyzed by SDS-PAGE and autoradiography.

**His Pull Down Assay—**Methionine-labeled GSK-3 proteins (wild type and mutant) were made using the TNT-coupled in vitro transcription/translation system according to the manufacturer's instructions (Promega). 5 μl of each mix was removed and mixed with 20 μl of loading buffer to check the efficiency of the reaction. The remainder was split in half and mixed with purified GST fusion protein and 25 μl of washed glutathione-Sepharose beads (Amersham Pharmacia Biotech). This was made up to 250 μl with ice-cold buffer (100 mM NaCl, 0.5% Nonidet P-40, 20 mM Tris-HCl, pH 8.0, 1 mM EDTA supplemented with a Complete protease inhibitor tablet (Roche Molecular Biochemicals), 1 mM phenylmethylsulfonyl fluoride and 0.5 mM dithiothreitol). Following incubation on a rotary mixer at 4 °C for 1 h, samples were pelleted, washed three times with ice-cold buffer, and analyzed by SDS-PAGE and autoradiography.

**GST Pull-down Assay—**His-tagged GSK-3 (230 nm) was mixed with 20 μl of washed Talon® metal affinity resin beads (CLONTECH) and 20 μl of GST fusion protein in buffer (100 mM NaCl, 0.5% Nonidet P-40, 20 mM Tris-HCl, pH 8.0, supplemented with a Complete protease inhibitor tablet (Roche Molecular Biochemicals), 1 mM phenylmethylsulfonyl fluoride and 0.5 mM dithiothreitol). Following centrifugation, the beads were washed three times in ice-cold buffer. 45 μl of loading buffer was added to the pellets, and the results were analyzed by SDS-PAGE and autoradiography.

**Kinase Assays—**293 cells were seeded at 1 × 10^6 cells/10-cm dish 48 h before an experiment. Each dish was transfected with 1.2 μg of vector (pDNA3) or 0.6 μg of GSK-3 (wild type or mutant) plus 0.6 μg of vector as described previously (36). Immunoprecipitations and kinase assays were carried out according to Ref. 34. The activities of mutant GSK-3 proteins were expressed as a percentage of transfected wild type GSK-3.

**Mammalian Cell Culture and Luciferase Assays—**293 and Madin-Darby canine kidney cells were maintained in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum at 37 °C under 5% CO2. Transfection-luciferase reporter assays and analysis of the expression was performed only by Western blotting were carried out as previously described (36). Primary antibodies used were mouse anti-GSK-3β and mouse anti-FLAG monoclonal antibodies (Transduction Laboratories).

**Immunoprecipitation—**293 cells were seeded at 7.5 × 10^5 cells/10-cm dish 48 h before an experiment. Each dish was transfected with 0.4 μg of construct (HA-tagged wild type GSK-3 or GSK-3GR and FLAG Axin-GID, pDNA3) or 0.8 μg as described previously (36). Immunoprecipitations were performed as described in Ref. 30. Samples were loaded on 10 or 12% SDS-PAGE gels, blotted, and subjected to Western analysis.

**Immunocytochemistry—**Cells were seeded at 30–40% confluence and were transfected with the specified vectors using Effectene Reagent (Qiagen, Crawley, West Sussex, UK) according to the manufacturer's instructions. After overnight incubation, cells were fixed with cold 4% paraformaldehyde in phosphate-buffered saline and processed for immunocytochemistry as previously described (36). HA epitope was detected with the rat monoclonal antibody 3F10 (Roche Molecular Biochemicals). FLAG epitope was detected with the mouse monoclonal anti-FLAG M2 (Sigma). Quantification of expression was carried out by taking thin optical slices of transfected cells by confocal microscopy. Optical sections were selected to intersect the center of the nucleus and were quantified by density analysis using the Bio-Rad ConfoCalm Software line intensity tool.

**Xenopus Embryo Secondary Axis Assay—**After linearization with NotI, the mRNA expression vectors for XGSK-3 were transcribed in vitro using SP6 polymerase (Promega) in the presence RNA cap analogue. The vegetal poles of single ventral blastomeres of four-cell embryos were injected with 2.5 ng of the indicated mRNA and allowed to develop for 3 days. XGSK-3 parental constructs were described by Pierce and Kimelman (29) and were provided by Dr. P. Klein.

**Construction of Expression Plasmids and Mutagenesis—**Mutant GSK-3 constructs were generated using site-directed mutagenesis according to the manufacturer's instructions (QuikChange; Stratagene). Surface scanning mutagenesis was performed on HA-tagged GSK-3 cDNAs within the pDNA3.1+ expression vector. The GR mutation was introduced into XGSK-3 plasmids in a pCS2+ vector background for the Xenopus studies (29). The GID substitution was introduced into GskA in the plasmid pDXA-gskA (8), which expresses the gskA cDNA from the actin15 promoter. The K208A/E209Q substitution in the GSK-3 binding domain of Frat was generated by site-directed mutagenesis of the FLAG-tagged cDNA within the pCDNA3.1+ expression vector. β-Catenin-GFP expression constructs were made by fusing GFP to the C terminus of murine β-catenin in the pEGFP vector (CLONTECH).

**Dictyostelium Cell Culture and Development—**Dictyostelium wild type and gskA mutant cells were grown at 22 °C in axenic medium pH 6.4. Cells were transformed by electroporation (54). Cells were observed either on SM agar or growing in axenic medium. For suspension development, cells were washed in KK, (15.5 mM KH2PO4, 3.8 mM K2HPO4, pH 6.2) and shaken for 8 h in KK, 1 mM cAMP was added, and cells were shaken further for 16 h (35).

**RESULTS**

**GSK3 Association with Axin, Frat, and itself—**In previous work, we determined the structure of human GSK-3β and showed that it formed an intimate head-to-tail dimer (10). Since apparent dimers in protein crystals may be artifacts of the crystal lattice, we sought to determine whether GSK-3 formed a stable dimer in solution using chemical cross-linking. We found that GSK-3 formed dimers at a concentration of the dimethyl suberimidate cross-linking reagent that was previously used to identify intimate dimers of the hsp90 protein (26). Higher molecular weight ladders of cross-linked product were not detected, suggesting that the cross-linking reaction was specifically linking soluble dimers. When GSK-3 was titrated from 10 to 1.25 μM, the ratio of dimer to unlinked product was reduced, suggesting that the affinity of GSK-3 for itself was in the low micromolar range (Fig. 1A). To confirm this observation, we attempted to co-precipitate in vitro translated GSK-3 with His-tagged GSK-3 at a final concentration of 230 nM. Although His-tagged GSK-3 was able to associate with Frat, it was unable to associate with itself, suggesting that the affinity of GSK-3 dimer formation is significantly less than that of the GSK-3-Frat interaction (Fig. 1B).

In the cross-linking assays, we observed the formation of heteromeric complexes between GSK-3 and GSK-3-binding peptides from Frat (FRATide) (31) and Axin (AxinGID) of 38 and 59 amino acids, respectively (Fig. 1, C and D). The addition of AxinGID and FRATide strongly interfered with GSK-3
GSK-3 cross-linking, suggesting that Frat and Axin either bind to the dimer interface or allosterically alter the ability of GSK-3 to self-associate. Maximal inhibition of GSK-3-GSK-3 dimer formation was obtained at \( \frac{1}{1} \) molar ratios of Frat or Axin to GSK-3.

The ability of the AxinGID to self-associate was investigated, since previous studies showed that full-length Axin formed multimers. Under conditions in which GST-Axin was able to associate with \textit{in vitro} translated GSK-3, GST-Axin did not associate with \textit{in vitro} translated GSK-3 at concentrations where it was able to associate with Frat. [\(^{35}\)S]Methionine-labeled, \textit{in vitro} translated GSK-3 (G) or mouse Frat1 (F) was mixed as indicated with His-tagged-GSK-3 (230 nm final concentration) and precipitated by the addition of metal affinity resin. Samples were analyzed by SDS-PAGE and autoradiography. C, AxinGID inhibits GSK-3 dimer formation. 10 \( \mu \)M GSK-3 was mixed with AxinGID in the presence of DMS cross-linking reagent as described above. Increasing levels of AxinGID-GSK-3-GSK-3 hetero-oligomer correlated with decreasing levels of GSK-3-GSK-3 dimer. D, FRATtide inhibits GSK-3 dimer formation. 10 \( \mu \)M GSK-3 was mixed with increasing levels of FRATtide peptide (amino acids 188–226 of human Frat1). E, the Axin GSK-3 binding domain (AxinGID) was not able to associate with itself under conditions in which it interacted with GSK-3. \textit{In vitro} translated GSK-3, AxinGID, and a mutated version of AxinGID (AxinGID(L521P)) that is not able to bind GSK-3 (36) were mixed with GST-AxinGID at a final concentration of 600 nm. The effect of GSK-3 on Axin-Axin association was examined by the further addition of His-GSK-3 to a final concentration of 10 \( \mu \)M. Complexes were precipitated following the addition of glutathione-Sepharose beads. Samples were analyzed by SDS-PAGE and autoradiography.

GSK-3 cross-linking, suggesting that Frat and Axin either bind to the dimer interface or allosterically alter the ability of GSK-3 to self-associate. Maximal inhibition of GSK-3-GSK-3 dimer formation was obtained at \( \sim 1:1 \) molar ratios of Frat or Axin to GSK-3.

The ability of the AxinGID to self-associate was investigated, since previous studies showed that full-length Axin formed multimers. Under conditions in which GST-Axin was able to associate with \textit{in vitro} translated GSK-3, GST-Axin did not associate with \textit{in vitro} translated Axin (Fig. 1E). The further addition of purified GSK-3 to the Axin \textit{in vitro} translation mix failed to generate Axin/GST-Axin interactions, suggesting that Axin dimerization was not required for GSK-3 binding. CD spectroscopy studies of purified AxinGID indicated that this region had no structure.\(^2\)

\textbf{Surface Scanning Mutagenesis}—To identify sites on GSK-3 that mediate interactions with Axin and Frat, we generated a series of 79 point mutations predominantly at the surface of the molecule. The GSK-3 point mutants were initially screened for binding using GST-Axin and GST-Frat in a precipitation assay \textit{in vitro} (Fig. 2A). Mutational changes were engineered to alter the surface charge, or hydrophobicity. Most mutants bound with similar efficiency to both Axin and Frat. 60\% (48/79 mutations) bound at levels indistinguishable from the wild type protein, whereas the remaining mutants showed reduced binding ranging from 0 to 75\% efficiency when expressed as a percentage of the wild type (Table I; Fig. 2, B–D). The nature of the amino acid substitution was a major contributor to the level of binding. For example, V139I bound GST-AxinGID and GST-Frat with close to wild type efficiency, while V139D bound with only 5 and 10\% efficiencies, respectively. Similarly, a change of serine 237 to an aspartic acid residue (S237D) partially interfered with both Axin and Frat binding, while mutation of the same residue to alanine (S237A) failed to alter binding.

\(^2\)R. Dajani, unpublished observation.
GSK-3 Binding to Axin

Residue Lys85, which forms part of the ATP binding site and is commonly mutated to generate kinase-dead variants, was changed to either a methionine or an arginine (27–29). The K85M mutant showed negligible binding to Frat and Axin. Since Lys85 is not readily accessible, it is unlikely to be directly involved in binding to regulatory proteins, and the failure of binding is probably due to the disruption of the correct folding of GSK-3. By contrast, when Lys was mutated to an arginine (K85R), the mutant bound with wild type efficiency to both Axin and Frat (Fig. 2A). Although the K85R mutation also generates a kinase-dead variant of GSK-3, it is a more conservative change and preserves Axin and Frat binding. This observation contradicts previous studies that suggested that GSK-3 kinase activity was required for Axin binding (30).

Kinase activity does not correlate with binding, but it may provide an independent readout for the correct folding of the molecule. We characterized the activity of several mutants using transfection-based immunoprecipitation kinase assays (Table I). Due to variable levels of expression, the activity of many mutants was difficult to assess. Among those characterized, we found that L343R, which failed to bind Axin or Frat, was kinase-inactive. Leu343 was not present on the surface and may, like K85M, have disrupted the overall structure (Fig. 2, B–D).

Four mutants showed differential binding to Axin and Frat (Table I). Three of these differentially interfered with Axin binding (V267G/E268R, F293Q, and E312K), while one differentially interfered with Frat binding (R180E). Interestingly, R180E is one of three residues (Arg96, Arg180, and Lys205) that are involved in the recognition of the prephosphorylated serine/threonine in GSK-3-dependent targets ((S/T)XXX(pS/pT); where pS and pT represent phosphoserine and phosphothreonine, respectively) (10). By contrast, mutation of Arg96 and Lys205 had no effect on Axin or Frat binding. With the exception of Glu312, all of the residues that differentially affected binding clustered on the GSK-3 dimer interface (Fig. 2, B–D). This supports the suggestion that binding of Frat and Axin sterically interferes with GSK-3 dimer formation. The binding of both to the same face of GSK-3 is consistent with the finding that Axin and Frat binding to GSK-3 are mutually exclusive (31).

The strongest differential binding observed was with the mutant GSK-3 V267G/E268R (GSK-3GR), which bound Frat efficiently but had severely reduced binding to GST-AxinGID. We chose to study the GSK-3GR further because it was kinase-active and localized close to a further differential “Frat > Axin” binding mutant (F293Q) in the crystal structure (Table I; Fig. 2C). GSK-3GR was purified from baculovirus-infected insect cells and assayed in parallel with wild type GSK-3. At similar concentrations to wild type GSK-3, GSK-3GR formed cross-linked homodimers and interacted with both Axin and Frat. In pull-down assays at lower concentrations (45 nM), the selective binding of GSK-3GR was revealed when it precipitated with Frat but not Axin. Similarly, GSK-3GR was unable to precipitate in vitro translated AxinGID (Fig. 3, A and B). This suggests that the $K_m$ of the GSK-3GR mutant for Axin had been increased from the nanomolar to the micromolar range. Under identical conditions, both wild type GSK-3 and GSK-3GR were able to precipitate Frat.

Unlike wild type GSK-3, GSK-3GR did not associate with Axin in vivo. Co-transfection and immunoprecipitation from 293 cells showed that wild type but not GSK-3GR associated with an N-terminally deleted form of Axin that contains the GSK-3 binding domain (Fig. 3C). Full-length Axin was not used in these immunoprecipitation experiments, because it predominantly associates with the insoluble fraction of cell extracts. Wild type GSK-3 but not GSK-3GR colocalized with full-length Axin as shown by immunocytochemistry of transfected 293 cells (Fig. 3D). The $K_m$ of GSK-3GR activity for the prephosphorylated peptide substrate, GSP, was not significantly different from that of wild type GSK-3 (70 versus 80 μM, respectively) (Fig. 3E). The activity of GSK-3GR against the nonprephosphorylated substrate cyclin D1 and against purified bacterially expressed $\beta$-catenin was also not significantly different from wild type GSK-3.3

---

3 M. Yeo, S. Stockwell, and M. Garrett, unpublished observation.
GSK-3 Binding to Axin

Table I
Summary of GSK-3 mutant studies

| AA* No. | AA change | Axin bindinga | Frat bindingb | Kinase activityc |
|---------|-----------|---------------|---------------|-----------------|
| 9       | S9A       | 100           | 100           | 100             |
| 66      | S66R      | 100           | 100           | 100             |
| 72      | Q72R      | 100           | 100           | 100             |
| 80      | E80N      | 100           | 100           | 100             |
| 81      | L81N      | 100           | 100           | 100             |
| 88      | K88R      | 100           | 100           | 100             |
| 92      | R92A      | 100           | 100           | 100             |
| 94      | K94A      | 100           | 90            | 100             |
| 96      | R96E      | 100           | 100           | 100             |
| 105     | D105E     | 100           | 100           | 100             |
| 111     | R111A     | 100           | 100           | 75              |
| 139     | V139I     | 90            | 100           | 100             |
| 140     | Y140R     | 100           | 100           | 100             |
| 145     | H145R     | 100           | 100           | 100             |
| 147     | S147K     | 100           | 85            | 100             |
| 148     | R148E     | 100           | 100           | 100             |
| 150     | K150E     | 100           | 90            | 100             |
| 157     | Y157F     | 100           | 100           | 100             |
| 175     | F175Q     | 100           | 100           | 100             |
| 177     | I177R     | 80            | 100           | 100             |
| 183     | K183A     | 90            | 80            | 100             |
| 192     | D192A     | 90            | 80            | 100             |
| 205     | K205E     | 100           | 100           | 100             |
| 211     | E211K     | 90            | 100           | 100             |
| 213     | N213E     | 90            | 90            | 100             |
| 214     | V214E     | 100           | 100           | 100             |
| 215     | S215K     | 100           | 100           | 100             |
| 216     | Y216E     | 100           | 100           | 100             |
| 217     | I217R     | 100           | 90            | 100             |
| 219     | S219A     | 100           | 100           | 100             |
| 219     | S219R     | 90            | 100           | 100             |
| 220     | R220E     | 100           | 100           | 100             |
| 221     | Y221E     | 90            | 90            | 100             |
| 223     | R223D     | 100           | 100           | 100             |
| 237     | S237A     | 100           | 100           | 100             |
| 254     | Q254E     | 100           | 100           | 100             |
| 261     | S261A     | 80            | 80            | 100             |
| 271     | K271E     | 90            | 90            | 100             |
| 279     | E279K     | 100           | 100           | 100             |
| 287     | N287E     | 100           | 90            | 100             |
| 289     | T289R     | 90            | 100           | 100             |
| 299     | H299E     | 100           | 100           | 100             |
| 319     | R319A     | 100           | 100           | 100             |
| 322     | E322K     | 100           | 100           | 100             |
| 345     | D345A     | 100           | 100           | 100             |

Significant but equal effects on binding

| AA* No. | AA change | Axin bindinga | Frat bindingb | Kinase activityc |
|---------|-----------|---------------|---------------|-----------------|
| 95      | K56M      | 5             | 0             | 0               |
| 98      | L58R      | 15            | 10            | 10              |
| 100     | I100R     | 0             | 5             | 10              |
| 108     | N108A     | 5             | 10            | 10              |
| 113     | R113L     | 50            | 60            | 100             |
| 117     | Y117R     | 70            | 70            | 100             |
| 139     | V139D     | 5             | 10            | 100             |
| 142     | V142D     | 20            | 20            | 100             |
| 156     | I156R     | 40            | 60            | 100             |
| 181     | D181R     | 50            | 60            | 100             |
| 206     | Q206E     | 0             | 0             | 100             |
| 216     | Y216F     | 40            | 40            | 100             |
| 228     | I228K     | 5             | 5             | 100             |
| 229     | F229Q     | 5             | 5             | 100             |
| 237     | S237D     | 50            | 50            | 100             |
| 239     | L239S     | 50            | 50            | 100             |
| 258     | N258E     | 20            | 30            | 100             |
| 258     | Y258R     | 5             | 10            | 100             |
| 332     | L332R     | 10            | 10            | 75              |
| 333     | E333K     | 50            | 50            | 60              |
| 343     | L343R     | 0             | 0             | 100             |
| 350     | S350L     | 80            | 60            | 100             |
| 350     | E350K     | 15            | 20            | 100             |

AA, amino acid.

Mutation of Val267 and Glu268 to Gly-Arg Alters GSK-3 Function in Vivo—In Xenopus embryos, ventral expression of dominant negative XGSK-3, XGSK-3(KR), is thought to generate ectopic secondary axes by interfering with XGSK-3-dependent phosphorylation of β-catenin (27, 32, 33). To test this model, we generated a compound mutant containing the GR and KR mutations. When injected into the ventral cells of four-cell embryos, the XGSK-3GR(KR) mutant protein failed to induce ectopic axes, suggesting that the kinase-dead GSK-3(KR) induces ectopic axes by interacting with regulators of an Axin-like class (Fig. 4A).

The catalytic domains of GSK-3 proteins are highly conserved in many eukaryotes, and this conservation is especially high over the helix containing the VE amino acids that are mutated in GSK-3GR. We therefore also investigated the role of this motif in a nonmetazoan GSK-3 family member. Dictyostelium contains a single GSK-3 homologue, gskA, which exhibits the same biochemical properties as GSK-3 (34). gskAGR was introduced as a transgenic construct into both a null gskA background and against a wild type background. Expression levels of the exogenous protein were comparable between wild type and GskAGR mutant proteins (Fig. 4B).

Cells that expressed GskAGR had a very rounded appearance and often detached from the substratum (Fig. 4C). After growth in shaking suspension, cells expressing GskAGR took an unusually long time to become reattached to the surface when returned to plates. Expression of GskAGR in the gskA mutant induced a more severe phenotype than in wild type cells. These cells grew very slowly, and the cultures were lost after a small number of generations. Wild type cells expressing GskAGR were capable of long-term growth in medium and were used in subsequent experiments.

The unusual appearance of the GskAGR-expressing cells prompted a closer examination using time lapse video microscopy. The average speed of movement of GskAGR-expressing cells was reduced more than 4-fold (Fig. 4D). As wild type Dictyostelium cells move, they contract for brief periods before changing direction. In contrast, cells expressing GskAGR spend prolonged periods in the contracted, stationary state. Dictyostelium cells move by pseudopod extension at the leading edge of the cell and retraction of the cell rear; they therefore exhibit a polarity toward the direction of movement. GskAGR-expressing cells show much weaker polarization with a rounder leading edge and very slow retraction of the cell rear. The trailing edge of the cell is often dragged along as a bulge. GskAGR-expressing cells often possess a series of bulges and constrictions along their body length (Fig. 4C).

A β-catenin homologue, Aardvark (aar) has been cloned in...
Dictyostelium, and GskA has been shown to interact positively with Aar. To investigate whether the phenotype seen with gskA<sup>GR</sup> required Aar, we examined the effects of Aar overexpression on cell motility. As seen with GskA<sup>GR</sup>-expressing cells, Aar overexpression reduced cell motility (Fig. 4D). It again reduced cell polarity and slowed retraction of the cell rear (data not shown). Additionally, we examined the effect of expressing GskA<sup>GR</sup> in a cell that lacks all Aar protein. A mutant that lacks the aar gene moves with approximately half the speed of wild type cells, but expressing GskA<sup>GR</sup> had little further effect on the cell motility or morphology (Fig. 4D). These observations indicate that introduction of the GskA<sup>GR</sup> protein into Dictyostelium cells causes an Aar-dependent change in the motility of isolated amoebae. It is also consistent with the previous observations that GskA regulates Aar function.

GskA is required for pattern formation during development of the Dictyostelium fruiting body. This structure has three major elements: a large spore head, a stalk that supports the spore head, and a base plate at the bottom of the stalk. GskA plays a role in this process by regulating the polarization of cells within the fruiting body. It is likely that GskA acts by modulating the activity of Aar or other related kinases that are involved in cell polarity and motility. Further studies will be needed to understand the precise mechanisms by which GskA regulates these processes.

Figure 3 shows the results of experiments designed to test the interactions between GskA and other proteins involved in the regulation of cell motility. Panel A shows the results of a co-immunoprecipitation experiment, which demonstrated that GskA interacts with GskA<sup>GR</sup> in vivo. Panel B shows the results of a pull-down assay, which confirmed the interactions observed in the co-immunoprecipitation experiment. Panel C shows the results of a kinase assay, which demonstrated that GskA<sup>GR</sup> is kinase-active. These results provide strong evidence that GskA regulates Aar function and plays a key role in the regulation of cell motility and pattern formation in Dictyostelium.
spore head, and a small basal disc that anchors the stalk to the substratum. A gskA null mutant has a grossly expanded basal disc and a tiny spore head. This patterning defect arises during the early stages of multicellular development, when the precursor cells of the fruiting body arise. In a gskA mutant, the pstB cell population, which gives rise to the basal cells, is expanded at the expense of the prespore cell population. In contrast, overexpression of either wild type or gskA GR genes had no apparent effect on the development of the fruiting body (data not shown). Previously, we showed that GskA and Aar were required for the induction of a prespore cell gene, psA, in response to cAMP in shaking culture (35). When GskA GR was expressed in a wild type background, the cells expressed lower levels of psA (Fig. 4E). It is not clear whether this is a direct effect on the GskA signaling pathway or an indirect effect on earlier stages of development that reduce cell competency to respond to extracellular cAMP. More strikingly, we discovered that expression of GskA GR in an aar null mutant background restored expression of cAMP (Fig. 4E).

Finally, we investigated the effect of the GSK3GR mutation on TCF-dependent transcription in mammalian cells. To establish assays for Frat- and Axin-GSK-3 interactions, we induced TCF-dependent transcription by expressing Frat or the AxinGID and then measured the ability of GSK-3 to titrate the response. These assays allowed a reproducible and quantitative readout for the levels of each component. Frat is thought to activate TCF-dependent transcription by titrating GSK-3 from the endogenous Axin complex, thereby stabilizing β-catenin. The AxinGID is thought to behave similarly (36). Full-length Axin could not be used in this assay, since, like GSK-3, it blocks TCF-dependent transcription. As expected, expression of GSK-3GR alone failed to activate TCF-dependent transcription (Fig. 5A). Both wild type GSK-3 and GSK-3GR titrated Frat-induced TCF-dependent transcription; however, GSK-3GR also titrated AxinGID-dependent TCF-transcription as efficiently as wild type GSK-3 (Fig. 5B). This observation was unexpected, since the GSK-3GR did not bind Axin and prevented axis duplication in Xenopus (Figs. 3D and 4A). The titration experiments were performed repeatedly but failed to find any difference in efficiency between the wild type and GSK-3GR mutant, suggesting that Axin binding may not be the only mechanism by which GSK-3 inhibits AxinGID-induced TCF-dependent transcription (Fig. 5B).

The levels of TCF transcription are dependent on the amounts of nuclear β-catenin complexed with TCF transcription factors. To directly address the effects of GSK-3 and GSK-3GR on β-catenin localization, we expressed GSK-3 or GSK-3GR and a β-catenin-GFP fusion protein in Madin-Darby

---

**FIG. 4.** The GSK-3GR mutation interferes with GSK-3 function in vivo. A, GSK-3GR mutation prevents Xenopus axis duplication by a kinase-dead mutant of GSK-3 (XGSK-3(KR)). Ventral blastomeres were injected vegetally with the specified mRNAs. Whereas injection of XGSK-3(KR) resulted in the induction of complete secondary axes, the introduction of the GR mutation XGSK-3GR(KR) strongly interfered with this effect. B, Western analysis showing equivalent expression of wild type and mutant GskA proteins. Due to the similarity of mobilities, the expressed transgene overlaps the endogenous protein. C, expression of wild type GskA protein has no overt effect on Dictyostelium cell morphology, whereas expression of GskA GR causes a dramatic change in cell shape. This results from decreased polarization of the leading edge.

---
canine kidney cells. As expected from the transient transfection, we observed significant cell to cell variability in the levels of $\beta$H9252-catenin-GFP expression. The nuclear/cytoplasmic ratios of $\beta$H9252-catenin-GFP also varied significantly from cell to cell; however, we failed to observe any systematic influence on these ratios following expression of GSK-3 or GSK-3GR. Unexpectedly, we observed significantly higher levels of $\beta$H9252-catenin-GFP at the plasma membrane of cells expressing GSK-3GR than in cells expressing GSK-3 or control vector (Fig. 5C).

**DISCUSSION**

**GSK-3 Complexes**—The crystal structure of GSK-3$\beta$ shows that it forms an intimate head-to-tail dimer. The cross-linking data presented here show that GSK-3 dimers exist in solution but that the affinity of self-association is weak, possibly in the low micromolar range. Whether GSK-3 forms dimers in vivo is unclear. The local intracellular concentration of GSK-3 may be high enough to promote dimer formation, or dimers may be stabilized by additional cellular proteins. The intimate contacts observed in the crystal dimer preclude substrate access and predict that dimeric GSK-3 would be catalytically inactive. The regulatory Tyr216 residue is also buried within the GSK-3 dimer interface (37). Preliminary studies using phosphospecific antisera suggest that phospho-Tyr216 is under-represented within GSK-3 dimers in solution, raising the possibility that dimers can only form from unphosphorylated GSK-3.

4 E. Fraser, unpublished observation.
suggests that Axin and Frat binding sites may overlap the dimer interface or interfere with the orientation of residues important for dimer formation. Previous studies have shown that GSK-3 forms mutually exclusive complexes with Axin or Frat. Both Axin-GSK-3 and Frat-GSK-3 complexes are catalytically active (24, 25, 31, 38–40); thus, by interfering with GSK-3 dimer formation, Axin and Frat may enable substrate access to the catalytic site.

**GSK-3 Mutagenesis**—Based on the GSK-3 crystal structure, a library of mutations was generated to scan the surface of the molecule for differential binding to Axin and Frat. Four mutants were identified that clustered on the large lobe of the kinase at or close to the crystal dimer interface, further supporting the idea that Axin and Frat binding occurs to this face of the molecule. We conclude that the mutual exclusivity of GSK-3 homodimer and Axin and Frat heterodimer complexes is likely to arise by physical interference at a single surface on the GSK-3 protein. We can, however, exclude the possibility of competition for the same binding site, since we find mutations that specifically block interaction with Axin or Frat but still allow homodimerization.

Previous studies suggested that Axin binding may occur on the small lobe of GSK-3, based on the analysis of an L128A mutant that showed reduced kinase activity against Axin but not a prephosphorylated peptide substrate (12, 41). However, we note that the L128A change was not reported to directly interfere with Axin binding and that the residue is not exposed on the surface of GSK-3 (10).

**GSK-3-Axin Binding in Xenopus Embryos**—The GSK-3GR mutant was particularly useful, since it formed homodimers and heterodimers with Frat at close to wild type levels but had severely reduced binding to Axin. We therefore investigated the effects of this mutation in a number of organisms.

The ventral expression of kinase-dead GSK-3(KR) in Xenopus embryos causes axis duplication. This has been explained by the inactive GSK-3 displacing endogenous GSK-3 from the axin complex, allowing an increase in the concentration of β-catenin and induction of a second axis. We find that kinase-dead GSK-3 that is also unable to bind axin does not induce secondary axes. This observation is consistent with the hypothesis that endogenous Xenopus Axin is required for normal β-catenin turnover (25, 42, 43).

**The Axin-binding Motif Is Conserved in All GSK-3 Kinases**—The VE motif within GSK-3 is found in all identified GSK-3 homologues. Mammalian Axin interacted with a Saccharomyces pombe GSK-3 homologue, suggesting that the potential for interaction with Axin-like molecules may be evolutionarily conserved (44). In Dictyostelium, the VE to GR mutation interfered with the function of the GSK-3 homologue, GskA. The biology of GskAGR-expressing cells was different from both null gskA cells and GskA-overexpressing cells, arguing against simple dominant negative or overexpression phenotypes. The greater severity for GskA GR expression in the gskA mutant background suggests competition between wild type and GskA kinase molecules. The mechanism of deregulation is not presently understood, since an Axin-like protein has yet to be found in Dictyostelium.

In Dictyostelium, GskA lies genetically upstream of Aar, but in contrast to the GSK-3-β-catenin relationship in vertebrates and Drosophila, GskA positively regulates Aar function (35). A similar positive relationship has been observed between Caenorhabditis elegans GSK-3 and β-catenin homologues (45). The molecular mechanism underlying GskA-dependent activation of Aar is not understood. The data presented here argue that during growth and early Dictyostelium development, the GskA GR mutant has up-regulated its ability to activate Aar. At this stage of Dictyostelium development, misregulation of Aar activity has dramatic effects on cell shape and motility. In addition to their effect on gene expression, β-catenin proteins also interact with the actin cytoskeleton. This is also true for Aar in Dictyostelium (35). It is therefore unclear at present whether the effect of GskA GR on Aar is mediated by changes in gene expression or by a direct interaction with the actin cytoskeleton.

During multicellular development, expression of GskA GR has little effect under normal developmental conditions. A mutant that lacks the aar gene has a number of defects. These are both structural, such as the loss of adherens junctions, or signaling, such as the failure of extracellular cAMP to induce the prespore gene, psA, in isolated cells. Surprisingly, when GskA GR is expressed in a aar mutant, it restores expression of the psA. Nominally, this would place gskA genetically downstream of aar. Since this is in direct contradiction to all other interactions observed between gskA and aar, we favor an alternate explanation. We suggest that disruption of the VE motif in GskA leads to promiscuous behavior where GskA can target other substrates not normally phosphorylated in prespore cells. Alternative GskA substrates could be other β-catenin protein homologues, and there is evidence for a γ-catenin-like protein that is expressed during multicellular development (46). In addition, GskA targets the transcription factor D. discoideum STAT protein, which plays a regulatory role in later development (6).

In both early and late developmental effects, we observe a misregulation of GskA activity. This suggests that the role of the VE interaction is to restrict GSK-3-substrate interaction. In Dictyostelium this may occur via an Axin-like template protein that restricts GskA activity to Aar regulation. When the GskA interaction is lost, it may be able to phosphorylate other substrates to regulate the target genes. Although this is detected as a “rescue” of the response in our assays, we assume that this misregulation is detrimental in some developmental conditions.

**GSK-3 May Regulate β-Catenin through More than One Mechanism**—Our observations in mammalian culture also produced an unexpected result. Here GSK-3GR was expected to have a reduced ability to titrate Axin-dependent TCF transcription. However, no difference was observed between wild type GSK-3 and GSK-3GR over a range of GSK-3 levels. A trivial explanation may be that overexpression generated sufficient GSK-3 to mediate template-independent phosphorylation and degradation of β-catenin. However, in vitro studies suggest that template-independent phosphorylation of β-catenin is highly inefficient (40). Since all other evidence points to a failure of GSK-GR to bind Axin, this suggests that there may be an Axin-independent component to TCF-dependent transcription in the tissue culture system. One novel role for GSK-3GR suggested by our experiments may be to target β-catenin to the plasma membrane. An indirect consequence of localizing β-catenin at the membrane may be to inhibit nuclear β-catenin function and thereby repress TCF-dependent transcription. At present, it is unclear whether GSK-3GR regulation of β-catenin localization is a promiscuous gain of function or whether endogenous GSK-3 can regulate both β-catenin stability and localization.

The cell motility function that GskA GR regulates via Aar may depend on transcriptional functions of Aar; however, since Aar/β-catenin also functions at the cell membrane, we suggest that GskA GR may affect cell motility by deregulating Aar/β-catenin function in cytoskeleton-membrane interactions. A link between GSK-3 activity and β-catenin function at the plasma membrane is suggested by the observation that GSK-3-depend-
ent phosphorylation of APC enhances the affinity of APC for 
\(\beta\)-catenin (47, 48), whereas E-APC protein in Drosophila is 
required for \(\beta\)-catenin association with the plasma membrane 
(49). APC has also been suggested to inhibit colon cancer for-
mation and TCF-dependent transcription through a process that is Axin-independent, possibly by regulating \(\beta\)-catenin-de-
pendent nuclear export (50–52).

GSK-3GR could promote nuclear export of \(\beta\)-catenin by phos-
phorylation of APC. The removal of \(\beta\)-catenin from the nucleus 
thus would then block mammalian TCF-dependent transcription.
Thus, by inducing APC-dependent nuclear export of \(\beta\)-catenin, 
GSK-3 would control TCF by two processes. Only one of these, 
protein degradation, requires an Axin interaction. The separa-
tion of \(\beta\)-catenin localization from stability has been described 
in response to integrin-linked kinase, which is thought to act 
via GSK-3 (53). GSK-3 may also regulate nuclear localization of 
Aar in Dictyostelium where treatment with the GSK-3 inhibitor 
lithium causes redistribution of Aar from the cytoplasm to the 
nucleus.5

The studies described here may have a broader relevance to 
the family of GSK-3-related kinases including mitogen-activ-
ted protein kinases, since they are structurally highly con-
served in the equivalent region to that of GSK-3 that contains 
the VE/GR mutation. In the context of cancer studies, the 
details of the Axin and Frat interactions with GSK-3 should 
distinguish between the antioncogenic and oncogenic interac-
ations with GSK-3.

Acknowledgments—We thank Mike Jones for help in setting up the 
Xenopus axis duplication assays, Valerie Good for preparing batches 
of purified GSK-3, and Daruka Mahadevan for enthusiasm for the study 
of GSK-3 structure.

REFERENCES

1. Pyle, S. E., Hughes, K., Nikolakaki, E., Pulverer, B. J., and Woodgett, J. R. (1992) Biochim. Biophys. Acta 1114, 147–162
2. Kim, L., and Kimmel, A. R. (2000) Curr. Opin. Genet. Dev. 10, 508–514
3. Cohen, P., Parker, P. J., and Woodgett, J. R. (1985) Mol. Cell. Biol. 5, 3893–3902
4. Plyte, S. E., Olsnes, S., and Kimmel, A. R. (1997) Science 275, 893–895
5. Alt, J. R., Cleveland, J. L., Hannink, M., and Diehl, J. A. (2000) Genes Dev. 14, 3109–3114
6. Ginger, R. S., Dalton, E. C., Ryves, W. J., Fukuzawa, M., Williams, J. G., and Woodgett, J. R. (2000) EMBO J. 19, 5483–5491
7. Ginsburg, G. T., and Kimmel, A. R. (1997) Genes Dev. 11, 2112–2123
8. Pyle, S. E., Donovan, E., Woodgett, J. R., and Harwood, A. J. (1999) Development 126, 325–333
9. Cross, D. A. E., Alex, S. R., Cohen, P., Andjelkovich, M., and Olsnes, S., and Kimmel, A. R. (1995) Cell 83, 785–789
10. Darnani, A., Fraser, R. E., Roe, S. M., Young, N., Good, V., Dale, T. C., and Pearl, L. H. (2001) Cell 105, 721–732
11. ter Haar, E., Coll, T. J., Austen, D. A., Hsia, H. M., Swenson, L., and Jain, J. (2001) Nat. Struct. Biol. 8, 593–596
12. Frame, S., Cohen, P., and Biondi, R. M. (2001) Mol. Cell 7, 1321–1327
13. Rue1, S., Michel-Berry, V., Ali, M., Manoukian, A. S., and Woodgett, J. R. (1999) J. Biol. Chem. 274, 21790–21796
14. Kim, L., Liu, J., and Kimmel, A. R. (1999) Cell 99, 399–408
15. Ding, V. W., Chen, B. H., and McDermott, P. (2000) J. Biol. Chem. 275, 32475–32481
16. Stambollic, V., Ruel, L., and Woodgett, J. R. (1996) Curr. Biol. 6, 1664–1668

5 A. Harwood, unpublished observation.
Identification of the Axin and Frat Binding Region of Glycogen Synthase Kinase-3
Elizabeth Fraser, Neville Young, Rana Dajani, Jonathan Franca-Koh, Jonathan Ryves, Robin S. B. Williams, Margaret Yeo, Marie-Therese Webster, Chris Richardson, Matthew J. Smalley, Laurence H. Pearl, Adrian Harwood and Trevor C. Dale

J. Biol. Chem. 2002, 277:2176-2185.
doi: 10.1074/jbc.M109462200 originally published online November 13, 2001

Access the most updated version of this article at doi: 10.1074/jbc.M109462200

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 52 references, 24 of which can be accessed free at
http://www.jbc.org/content/277/3/2176.full.html#ref-list-1