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Recent reports have shown that only nuclear forms of Notch1-IC (20) and Notch2-IC (N2-IC) (21) can be phosphorylated and that ligand binding induces both Notch2 hyperphosphorylation and nuclear translocation (22). Furthermore, both phosphorylation and nuclear translocation appear to be necessary for oncogenic transformation mediated by Notch (23). By

Received for publication, April 16, 2003, and in revised form, June 6, 2003
Published, JBC Papers in Press, June 6, 2003, DOI 10.1074/jbc.M304001200

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This work was supported by Comisión Interministerial de Ciencia y Tecnología, Plan Nacional de Salud Grant SAF2001-1191. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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The abbreviations used are: Notch-IC, intracellular Notch fragment; N2-IC, intracellular Notch2; N2-ICΔOPA, N2-IC deletion of OPA/PEST region; MT, Myc tag; FL-N2, full-length Notch2; RBP-Jκ, recombination binding protein-Jκ; GSK-3β, glycogen synthase kinase-3β; CBP, CREB-response element-binding protein binding protein; STR, serine/threonine-rich; Hes, hairy/Enhancer of Split; ANK, ankyrin; PMSF, phenylmethylsulfonyl fluoride; HEK-293T, human embryonic kidney 293T (cell); GST, glutathione S-transferase; HA, hemagglutinin A; LiCl, lithium chloride; PBS, phosphate buffered saline; NCR, Notch cytokine response.
contrast, we have shown recently that specific phosphorylation of N2-IC in the Ser/Thr rich (STR) domain renders the molecule inactive and permits cytokine-induced cell differentiation in a myeloid cell line (21). We now report that GSK-3β is able to induce specific phosphorylation in the previously identified STR domain of Notch2. We show that GSK-3β directly binds at C-terminal of the Notch2 ankyrin repeats and phosphorylates Thr2076 and Thr2081. The Thr2081 phosphorylation is known to affect transcriptional activation of different Notch target genes. Phosphorylation of Notch2 by GSK-3β is reversed in the presence of Wnt-1, resulting in the up-regulation of Notch-dependent transcription of the Hes-1 promoter.

**EXPERIMENTAL PROCEDURES**

**Plasmids and Notch Constructs**—Full-length Notch2 (Fl-N2) (amino acids 1–2472), N2-IC (amino acids 1699–2472), and N2-IC<sup>ΔN</sup> (amino acids 1699–2155) constructs cloned in the pcDNA3-MT have been described (21, 24). GSK-3β and Wnt1-hFc expression vectors have been reported previously (25, 26). Hes1-luc, 4×RBWt-luc (23A), and 4×RB-Pmut-luc (25A), have been described previously (27, 28). Notch2 constructs were generated by PCR using murine Fl-N2 cDNA as a template and then cloned into the pEGX3 vector. The primers used were as follows: 5′-CAAGCTGCATGTTCCGAGGCCGAATCTCC-3′ and antisense 5′-GAAGCTCTAGCTCCATGCAAAGAGCAGGCGGACACTTCC-3′; 5′-AGAATTCAGCAGGAGGTTACAGGT-3′ and antisense 5′-CAAGCTCTAGCTCCATGCAAAGAGCAGGCGGACACTTCC-3′; 5′-AGAATTCAGCAGGAGGTTACAGGT-3′ and antisense 5′-CAAGCTCTAGCTCCATGCAAAGAGCAGGCGGACACTTCC-3′. All amino acid numbers correspond to peptide 2020 as a template (21). All amino acid numbers correspond to the rat Notch2 sequence (29). Constructs were all verified by automatic sequencing.

**Pull-down Assay**—GST fusion proteins were purified from the *Escherichia coli* BL-21 strain in a buffer containing 20 mM Tris-HCl, pH 7.4, 1 mM NaCl, 0.2 mM EDTA, 1 mM dithiothreitol, 1 mM lysozyme, 1 mM PMSF, and protease inhibitor complex (Roche Applied Science) and bound to glutathione-Sepharose (Amersham Biosciences). Sepharose beads with GST fusion proteins were first blocked with untransfected HEK-293T cell lysates by 1 h of incubation at 4 °C and then incubated with 400 μg of HEK-293T-transfected cell lysate in a buffer containing 50 mM Tris-HCl, pH 7.5, 150 mM NaCl, 1% Nonidet P-40, 5 mM EDTA, 5 mM EDTA, 20 mM NaF, and protease inhibitor complex (Roche Applied Science). The reaction was incubated for 2 h at 4 °C and then washed six times with the same buffer. Bound proteins were analyzed by Western blotting.

**Western Blotting**—Cells were lysed in 1% Nonidet P-40, 10 mM Tris-HCl, pH 7.5, 140 mM NaCl, 5 mM EDTA, 50 mM NaF, and 0.4 mM sodium orthovanadate, 1 mM PMSF, and 10 μg/ml leupeptin and aprotinin. Lysates were boiled for 5 min and normalized for protein. The lysates were fractionated by electrophoresis, transferred onto Hybond-N<sup>+</sup> membranes, and immunostained with antibodies against Notch2, Hes1, and RBP-J<sub>k</sub> (Santa Cruz Biotechnology; data not shown). The membranes were incubated with peroxidase-conjugated secondary antibodies and subsequently exposed to film. The bands were quantitated densitometrically with Adobe Photoshop.

**RESULTS**

**Phosphorylation of the Notch2 Molecule by GSK-3β**—Our previous work showed that specific Notch2 phosphorylation can modify Notch function in myeloid differentiation (21). In those studies we showed that cytokine-dependent phosphorylation of Notch2 in the STR region resulted in the inactivation of the Notch2 molecule, thus permitting cell differentiation in the 32D myeloid cell system. In other systems, Notch phosphorylation has been correlated with Notch activation (22) or enhanced RBP-Je binding (31), although nothing is known about the kinases involved. To answer this question, we analyzed the sequence of the Notch2 protein and tested the ability of different candidate kinases to phosphorylate Notch2 in vitro (data not shown). In this work we present evidence that GSK-3β...
notch2 physically interacts with gsk-3β. a, lysates from hek-293t cells transfected with ha-gsk-3β were incubated with gsk or gnt2-n2-icop fusion proteins. complexes were precipitated using glutathione-agarose, electrophoresed, and analyzed by western blot with α-ha antibody to detect gsk3β. one-fifth of the input protein is shown. the bottom section shows coomassie staining of the gel. b, whole-cell lysates from hek-293t cells cotransfected with mt-n2-ic alone or with ha-gsk-3β were immunoprecipitated with α-ha-conjugated sepharose beads followed by immunoblotting with α-myc. one-fifth of the input protein is shown. bottom sections show the same membrane stripped and reprobed with anti-ha to detect precipitated gsk3β. c, lysates from hek-293t cells transfected with ha-gsk-3β were incubated with the indicated gnt-notch2 fragments. immunoblot (wb) with α-ha is shown in the top section. one-fifth of the input protein is shown. bottom section shows the coomassie staining of the same gel. schematic representation of gnt-notch2 constructs used is shown above.

antibody. consistent with the results from pull-down experiments, n2-ic was specifically detected in the gsk3β immunoprecipitates (fig. 2b). together, these results indicate that notch2 interacts with gsk3-β in vitro and that the interacting domain is within the n2-ic-op fragment.

the gsk-3β binding domain of notch2 is within ankyrin repeat number six—because we showed that the n2-ic-op molecule binds to gsk3β (fig. 2a), we next characterized the region of notch2 responsible for this binding. we generated different gnt2-n2-ic-op deletion mutants, which are graphically represented above fig. 2c, and performed pull-down assays with extracts from ha-gsk-3β-transfected hek-293t cells. we observed that notch2 molecules containing the n-terminal ankyrin repeats (ank, amino acids 1824–2064) could only weakly bind gsk-3β, similar to the peptide containing ank1–3 (amino acids 1824–1885). however, peptides exclusively containing the c-terminal ankyrin repeat number six (ank6, amino acids 2020–2064) showed considerably more gsk-3β binding activity relative to other mutants (fig. 2c). this suggests that, although gsk-3β can interact with different ankyrin repeats on the notch2 protein, the higher affinity interaction domain involves ankyrin number 6.

gsk-3β phosphorylates three specific residues in the str domain of notch2—to determine which notch2 residues are targets for gsk-3β phosphorylation, we first tested whether the notch2 fragments used for binding experiments were phosphorylated in vitro. as a positive control we used n2-ic-op construct, and, surprisingly, we observed that neither the fragment containing ankyrin 6, which showed high binding affinity
suggested that additional GSK-3β phosphorylation sites are present within the amino acid fragment 2065–2088.

To identify the specific phosphorylated residues, we generated several point mutations and deletion mutants in peptides 2020–2105 and 2020–2088. Fig. 3C shows that mutation of S2093A in peptide 2020–2105 resulted in a notable decrease in phosphorylation compared with that obtained with peptide 2020–2088, which lacks Ser-2093. This result suggests that Ser-2093 of Notch2 is one of the main targets for GSK-3β phosphorylation. Next, we characterized the residues responsible for phosphorylation of peptide 2020–2088. Deletion of amino acids 2077–2081 containing three Ser/Thr residues (TSALS) in the 2020–2088 fragment did not show any change in the phosphorylation. However, a double mutant (T2068A/S2070A) in the 2020–2088 fragment or an additional point mutation (S2074A) in the ΔTSALS fragment showed reduced phosphorylation compared with the wild type fragments (Fig. 3D). Altogether, these results demonstrate that residue Ser-2093 is efficiently phosphorylated by GSK-3β and, to a minor extent, residues Thr-2068 and/or Ser-2070 and Thr-2074 of Notch2 are also targets for GSK-3β-dependent phosphorylation.

Subcellular Localization of GSK-3β Is Modulated by Activated Notch2—GSK-3β is localized in either the nucleus or the cytosol. This different localization is dependent on cell type and, in some cases, correlates with levels of the Frat GBP protein, which is involved in its nuclear export (32). As shown in Fig. 4A, GSK-3β expressed in HEK-293T cells is primarily cytoplasmic (Fig. 4A, section 1), whereas activated Notch (N2-IC) is exclusively nuclear (Fig. 4A, section 8). Because our results demonstrated that GSK-3β phosphophates activated Notch in vivo in this cell line, we investigated the possibility that subcellular localization of N2-IC or GSK-3β may change when both molecules are coexpressed. We transfected HEK-293T cells with GSK-3β alone or in the presence of N2-IC and determined their subcellular localization by immunofluorescence. In most of the cells coexpressing both proteins (75%), GSK-3β was localized in the nucleus (Fig. 4A, sections 7–9). Because inactive full-length Notch2 is mainly localized in the membrane and cytoplasm of the cells (Fig. 4A, section 5) we hypothesized that the coexpression of Fl-N2 would not have such a great effect on the cytoplasmic localization of GSK-3β. To test this, we coexpressed GSK-3β with Fl-N2 and observed that both molecules colocalized in the cytoplasm in 50% of cells (Fig. 4A, sections 4–6). Of note, the coexpression of Fl-N2 leads to a moderate nuclear accumulation of GSK-3β in ~45% of the cells (Fig. 4A, section 4), which correlates with increased nuclear Notch staining. This may correspond to intracellular Notch generated by cellular processing of the overexpressed full-length Notch-2 molecule. Taking all this together, we find that, expressing either N2-IC or Fl-N2, GSK-3β colocalized with α-N2 staining in the majority of the cells. Moreover, because GSK-3β was mainly cytoplasmic in the absence of N2-IC, these results suggest that GSK-3β may translocate to the nucleus bound to the activated Notch2.

GSK-3β Interacts with but Does Not Phosphorylate Full-length Notch2—Because immunofluorescence staining patterns suggested that GSK-3β colocalized with both full-length and N2-IC proteins, we further studied the interaction between GSK-3β and both N2-IC and full-length molecules. Coimmunoprecipitation assays with α-N2 antibody from HEK-293T-transfected cell lysates showed that GSK-3β bound more efficiently to full-length Notch2 compared with the N2-IC molecule (Fig. 5A). Because we also detected GSK-3β in precipitates from cells transfected with GSK-3β plasmid alone (Fig. 5A), we speculated that GSK-3β may precipitate bound to the endoge-
GSK-3β Phosphorylates and Inhibits Notch2 Activity

Figure 4. Subcellular localization of GSK-3β is modulated by activated Notch2. A, HEK-293T cells were plated on slides and transfected with HA-GSK-3β plasmid alone (sections 1–3) or in the presence of Fl-N2 (panels 4–6) or N2-IC plasmids (panels 7–9). After 24 h, cells were fixed, permeabilized, and stained with α-HA and α-N2 antibodies. Proteins were detected with goat anti-rabbit Cy3 secondary antibodies. The nucleus was detected by 4,6-diamidino-2-phenylindole staining. Cells were visualized and counted in an Olympus BX-60 microscope at 400×. B, graph represents the percentage of cells showing predominantly nuclear (N) or predominantly cytoplasmic (C) staining. One of two representative experiments is shown.

nous Notch2 protein. To confirm the specificity of this interaction, we performed an additional control eliminating protein–protein interactions by boiling the samples in 1% SDS, followed by a 1% TritonX-100 treatment to allow the antibody binding (33). As expected, GSK-3β was not detected in these control precipitates, whereas the interaction of Notch2 protein with the α-N2 antibody was not affected (Fig. 5A, boiled).

As GSK-3β binds to full-length Notch2, we next investigated whether this molecule was also a substrate for GSK-3β activity. We transfected HEK-293T cells with GSK-3β and Fl-N2 expression vectors or N2-IC vector as a positive control and performed in vivo phosphorylation assays by incubating the cells with [32P]orthophosphate. Cells were lysed, and Notch2 proteins were precipitated with the α-N2 antibody. Precipitates were then electrophoresed in a SDS-PAGE and exposed in a PhosphorImager. Autoradiography of the gel showed that Fl-N2 was not phosphorylated, whereas N2–IC was efficiently phosphorylated under the same conditions (Fig. 5B). These results indicate that GSK-3β efficiently binds to the inactive Fl-N2 even though only the processed protein is a substrate for this kinase.

GSK-3β Induces Down-regulation of Notch-dependent Gene Transcription—Phosphorylation of Notch molecules has been associated with ligand-dependent Notch activation, cell transformation, and increased transcriptional activity. Because our results showed that GSK-3β binds and modifies the non-active Fl-N2 and the processed N2-IC differently, we asked whether GSK-3β phosphorylation had any effect on Notch activity. To answer this, we cotransfected NIH-3T3 cells with N2-IC and increasing amounts of GSK-3β expression vectors and measured the transcriptional activity of the Notch-dependent Hes1-luc promoter. This promoter contains a binding site for RBP-Jκ, the intracellular partner of Notch, and activates transcription in the presence of Notch-IC (27). We observed a GSK-3β dose-dependent inhibition of the Notch2-dependent transcriptional activity of this promoter (Fig. 6A). To further confirm that GSK-3β activity was responsible for the inhibition of the promoter, we performed the same experiment in the presence of different concentrations of the GSK-3β inhibitor LiCl. As expected, the addition of LiCl reversed the effect of GSK-3β on the Hes-1 reporter in a dose-dependent manner without affecting N2-IC protein levels (see bottom sections of Fig. 6, A and B).

To test whether this mechanism could be playing a role in vivo, we also examined the effect of GSK-3β inhibition on different endogenous Notch target genes. We obtained RNA from HEK-293T (Fig. 6C) and compared, using Northern blot, the expression of Hes-1, Hes-5m and Herp-2 genes in the presence or absence of LiCl. All of these genes contain RBP-Jκ binding sites in their promoters, and their transcription is dependent on Notch activity (27, 34, 35). Consistent with the results obtained in the Hes1 reporter assay, we observed an increased expression of the endogenous Hes1 and Herp-2 genes in the cells exposed to LiCl (Fig. 6C), whereas the Hes-5 signal was not detected (data not shown). These experiments support the hypothesis that GSK-3β-dependent phosphorylation may exert a negative regulation of Notch2 activity, because inhibition of GSK-3β activity by LiCl leads to increased expression of different Notch targets both in vitro and in vivo.

Wnt-1 Signaling Up-regulates Notch2 Transcriptional Activity by Inhibiting GSK-3β-dependent Phosphorylation—Cross-talk between Notch and Wnt signaling pathways has been proposed previously (36, 37). To date, it remains unclear
whether this cross-talk results in a synergistic or an antagonistic effect (38). Because Wnt glycoproteins signal through Frizzled receptors resulting in the inhibition of GSK-3β, we hypothesized that Wnt signaling could up-regulate Notch transcriptional activity in our system. To test this, we transfected NIH-3T3 with N2-IC in the presence of increasing amounts of Wnt1-hFc (26) along with the Hes1-luc reporter. Consistent with our hypothesis, coexpression of Wnt1 with N2-IC resulted in up-regulation of the Hes1 promoter in a dose-dependent manner (Fig. 7A), whereas no effect was observed when Wnt1 was expressed in the absence of N2-IC (Fig 7A). To further confirm that the effect of Wnt-1 on the Hes1 promoter was dependent on RBP-jk/Notch2 signaling, we assayed another reporter construct exclusively containing four repeats of the RBP-jk binding sites (4×RBPwt-luc) and compared it with a construct in which these binding sites had been mutated (4×RBPmut-luc) (28). In Fig. 7B we show that Wnt-1 also has a positive effect on the N2-IC-mediated transcriptional activation of the wild type 4×RBP-jk promoter, whereas neither N2-IC nor Wnt-1 had any effect on the mutated promoter. To investigate whether the up-regulation of Hes-1 transcription by Wnt-1 correlates with the inhibition of GSK-3β activity Notch2 phosphorylation, we transfected HEK-293T cells with different combinations of N2-IC, GSK-3β, and Wnt-1 expression vectors and incubated the cells with [32P]orthophosphate. When we analyzed the Notch2 immunoprecipitates, we observed that overexpression of Wnt-1 with GSK-3β results in a 35% reduction in Notch phosphorylation compared with GSK-3β alone (Fig. 7C, right panel). Coexpression of Notch-IC with Wnt-1 in the absence of transfected GSK-3β also leads to decreased phosphorylation compared with basal conditions (Fig 7C, left panel). These results suggest that the effect of Wnt-1 in Hes-1 activity is the result of a decrease in GSK-3β-dependent Notch2 phosphorylation.

**DISCUSSION**

The results presented in this study reveal a novel mechanism for Wnt and Notch pathways cross-talk involving GSK-3β phosphorylation. We demonstrate that GSK-3β is able to associate with and phosphorylate Notch2 in vivo. This phosphorylation occurs in the previously described STR domain, and, more specifically, in residues Thr-2068 and/or Ser-2070. The results presented in this study also show that overexpression of both Fl-Notch2 and N2-IC induces the accumulation of GSK-3β into the nucleus, suggesting that this kinase may be modulating active Notch. In this sense,
we have confirmed that GSK-3\(\beta\)/H9252 is able to inhibit Notch-mediated transcription of the Hes-1 promoter, and this is in agreement with the up-regulation of Notch target genes by the GSK-3\(\beta\)/H9252 inhibitor LiCl. Moreover, Wnt-1 inhibits GSK-3\(\beta\)/H9252-dependent phosphorylation of Notch2 and leads to the up-regulation of the Hes-1 promoter.

Phosphorylation of the Notch Molecule—There is now substantial evidence that Notch molecules can be phosphorylated. In previous reports, Notch phosphorylation has been correlated with Notch activation, nuclear translocation, and cellular transformation (8, 22) as well as inhibition of Notch activity (21). Here we describe a different aspect of regulation by specific phosphorylation. We present evidence that GSK-3\(\beta\)/H9252 induces phosphorylation of the N2-IC, thus negatively regulating its transcriptional activity. While this work was in progress, Foltz et al. demonstrated that GSK-3\(\beta\)/H9252 was able to phosphorylate Notch1 in vitro (39). Moreover, they showed that activation of the Hes-1 promoter was decreased in the GSK3-deficient fibroblasts compared with the wild type cells and proposed that these results were due to differences in Notch stability. Although we have analyzed the effect of GSK-3\(\beta\)/H9252 phosphorylation in the Notch2 homologue, we should consider the possibility that GSK-3\(\beta\)/H9252 was also affecting the stability of the Notch2 protein. Nevertheless, when we determined the N2-IC half-life in the presence or absence of GSK-3\(\beta\)/H9252 or LiCl, we did not observe any remarkable difference (data not shown).

Together, our results indicate that GSK-3\(\beta\)/H9252 activity is important for regulating Notch-dependent gene transcription. Because the Notch pathway is crucial for controlling many cell fate decisions, specific combinations of active/inactive GSK-3\(\beta\)/H9252 and Notch may result in completely different outcomes.

Importance of the STR Domain of Notch Molecules in Different Systems—The STR region, located at C-terminal of the ankyrin repeats, has been reported to play an active role in different Notch-dependent events. We originally identified this region because of its role in regulating Notch function in a cytokine-specific context. We described how Notch1 would inhibit granulocyte colony stimulating factor (G-CSF)-induced differentiation, whereas Notch2 would inhibit granulocyte macrophage colony stimulating factor (GM-CSF)-induced dif-
ferentiation, and this cytokine response specificity resided in the STR region, also called the Notch cytokine response (NCR) domain (24). In subsequent studies we observed that deletion of the STR domain in the N2-IC molecule would result in a cytokine-independent activated molecule that inhibits differentiation in the presence of either the granulocyte colony stimulating factor or the granulocyte macrophage colony stimulating factor. We also mapped some phosphorylation events taking place in STR region that were involved in cytokine-specific response (21). Now we have identified three new phosphorylation sites within this region that are GSK-3β-dependent and have shown that phosphorylation of these residues is important for regulating Notch-dependent transcription. Thus, the role of GSK-3β-dependent phosphorylation of the STR region may be important in regulating Notch1 and Notch2 specificity. In addition to its role in myeloid differentiation, the importance of this region in the neoplastic transformation capacity of activated Notch has also been reported (40). More precisely, the minimal region of Notch1 that is necessary to induce transformation in EIA-immortalized baby rat kidney (RKE) cells was defined within amino acids 2105–2114 (equivalent to 2053–2062 of Notch2), and deletion of this domain is sufficient to abolish Notch-IC transactivation and transformation capacities. Because we have shown that inactivation of Notch2 by GSK-3β implies phosphorylation of several residues adjacent to the minimal transformation domain, we would like to speculate that GSK-3β-dependent phosphorylation may be affecting the accessibility of Notch coactivators to this region. Using coimmunoprecipitation assays, we have analyzed whether overexpression of GSK-3β or incubation with the GSK-3β inhibitor LiCl would modify the binding efficiency of Notch and RBP-Jκ, and we have not observed any difference (data not shown). However we speculate that GSK-3β may be modulating the interactions between Notch and other proteins such as CBP/p300 (41) or Mastermind (42). The former is particularly interesting, because the CBP binding domain in the Notch molecule has been mapped next to the phosphorylation sites described here (43). The effect of GSK-3β phosphorylation on the binding of Notch to other proteins is currently under investigation.

Regulation of Notch Activity following Ligand Activation and Nuclear Translocation—Notch proteins are transmembrane receptors that localize on the cell surface when they are not activated. Interaction of Notch with its ligands induces proteolytic cleavages that liberate the intracellular domain, which translocates to the nucleus to activate transcription. Once activated, the mechanisms involved in the termination of the Notch signal are not well known. One of these mechanisms could be related to the interaction between Notch-IC and the F-box protein SEL-10 that targets the protein for proteasomal degradation. Although phosphorylated forms of Notch interact with SEL-10 (44), our results show that phosphorylation by GSK-3β is not targeting the Notch molecule for degradation. We observed that decreased or increased transcriptional activation of the Hes-1 promoter, either by GSK-3β or Wnt-1, respectively, is not associated with changes in Notch2 protein levels. This indicates that GSK-3β-dependent phosphorylation is down-regulating Notch2 activity through a different mechanism. Moreover, we showed that GSK-3β and Notch2 colocalize in the nucleus in the majority of the Notch-IC-expressing cells. When we transfected Fl-N2, 50% of the cells showed cytoplasmic GSK-3β, whereas the other half showed nuclear staining. Intriguingly, cells showing nuclear GSK-3β also presented higher levels of nuclear Notch2, which is probably due to cellular processing of the transfected full-length. From these results, we hypothesized that GSK-3β may be bound to the inactive Notch complex that resides in the cell membrane and may be translocated to the nucleus when Notch gets activated to module or participate in the termination of the Notch pathway signaling.

Connection of Wnt and Notch Pathways—Wnt and Notch pathways cross-talk in many different systems. The outcome of this cross-talk is somehow controversial and may involve distinct elements in the different experimental systems. Although most of the published work seems to favor an opposing interaction between the Wnt and Notch pathways (18, 36), there are also examples of both pathways synergistically activating gene transcription. For example, in the pre-patterning of the margin in Drosophila wing development, both Wingless (wg) and Serrate (ser) collaborate to induce the expression of vestigial (vg), and a functional Notch receptor is necessary for this induction (19). Moreover, incubation with Wingless of full-length Notch-expressing S2 cells also up-regulates transcription of patched (ptc) and Shaggy/GSK-3β (sigg) compared with wild type S2 cells (17). These observations have been classically explained by Wingless binding directly to Notch and activating an alternative Notch-dependent pathway (45). However, an alternative explanation would be that inhibition of GSK-3β by Wingless results in higher Notch activity or may render Notch competent to interact with other proteins. In this situation, alternative Notch-dependent genes could also be activated. It should be interesting to test whether expression of Wnt family members are affecting Notch-target gene expression in different systems; in this sense, we did find overexpression of the hes1 and herp2 genes in response to GSK-3β inhibition by LiCl in different cell types. Our results do not provide an explanation for all the genetic interactions that have been described in Drosophila between Wnt and Notch signaling components; however, it may be worth reconsidering that GSK-3β-mediated Notch phosphorylation is playing a role in some of the described effects.

Acknowledgments—We acknowledge J. R. Woodgett for HA-GSK-3β, S.D. Hayward for 23A and 25A for the RBP-Jκ reporters, A. Israel for Hes1-luc, and S. Aaronson for Wnt1-hFc plasmids. We are thankful to H. Evans for helping with language correction.

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J. Biol. Chem. 2003, 278:32227-32235.
doi: 10.1074/jbc.M304001200 originally published online June 6, 2003

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