Implication of Galectin-3 in Wnt Signaling

Tatsuo Shimura,[1,2] Yukinori Takenaka,[1,3] Tomoharu Fukumori,[1,4] Soichi Tsutsumi,[1,2] Kohji Okada,[2] Victor Hogan,[1] Akira Kikuchi,[5] Hiroyuki Kuwano,[2] and Avraham Raz[1]

[1]Department of Tumor Progression and Metastasis, Karmanos Cancer Institute, Wayne State University, Detroit, Michigan; [2]Department of General Surgical Science (Department of Surgery I), Graduate School of Medical Sciences, Gunma University, Gunma, Japan; [3]Department of Otolaryngology and Sensory Organs, Osaka University Graduate School of Medicine, Osaka, Japan; [4]Department of Urology, School of Medicine, University of Tokushima, Tokushima, Japan; and [5]Department of Biochemistry, Graduate School of Biomedical Sciences, Hiroshima University, Hiroshima, Japan

Abstract

Galectin-3 (gal-3), a member of the β-galactoside–binding proteins family, was identified as a binding partner of β-catenin. Analysis of the human gal-3 sequence reveled a structural similarity to β-catenin as it also contains the consensus sequence ($S_{92}XXXS_{96}$) for glycogen synthase kinase-3β (GSK-3β) phosphorylation and can serve as its substrate. In addition, Axin, a regulator protein of Wnt that complexes with β-catenin, also binds gal-3 using the same sequence motif identified here by a deletion mutant analysis. The data presented here give credence to the suggestion that gal-3 is a key regulator in the Wnt/β-catenin signaling pathway and highlight the functional similarities between gal-3 and β-catenin. (Cancer Res 2005; 65(9): 3535-7)

Introduction

Galectins are a family of carbohydrate-binding proteins characterized by conserved amino acid sequences of their carbohydrate-binding domains and affinity for β-galactoside–containing glycoconjugates (1). Galectin-3 (gal-3) exhibits pleiotropic biological functions and has been implicated in cell growth, differentiation, apoptosis, adhesion, malignant transformation, and RNA processing (1–4).

Previously, we have reported that gal-3 overexpression regulates the expression levels of cell cycle targets of Wnt pathway, like cyclin D_1 and c-myc (5–7), and found that gal-3 is a novel binding partner of β-catenin and is phosphorylated, like β-catenin, by casein kinase I (CKI; ref. 7–9). β-catenin is phosphorylated by a dual kinase system of CKIα and glycogen synthase kinase-3β (GSK-3β) in a complex containing adenomatous polyposis coli and axin, targeting β-catenin for ubiquitination and degradation (10–14). Gene mutations in APC, axin, or β-catenin augment phosphorylation, which, in turn, leads to its accumulation in the nucleus, resulting in activation of transcription of Wnt-target genes (11, 14). Because the nuclear import-export of gal-3, like that of β-catenin, is phosphorylation dependent (9), we questioned whether gal-3 may also be phosphorylated by a similar dual kinase system. A search of the human gal-3 protein amino acid sequence revealed that in addition to a CKI phosphorylation site (Ser^6; ref. 9), it also contains the GSK-3β phosphorylation consensus sequence ($S_{92}XXXS_{96}$). This prompted us to question whether gal-3 is phosphorylated by GSK-3β and whether gal-3 will bind Axin in a phosphorylation-dependent manner.

Requests for reprints: Avraham Raz, Tumor Progression and Metastasis Program, Karmanos Cancer Institute, Wayne State University, 110 East Warren Avenue, Detroit, MI 48201. Phone: 313-833-0715; Fax: 313-831-7518; E-mail: raza@kci.wayne.edu.

Materials and Methods

Cells and reagents. The origin and the culture conditions of the human breast cancer cell line BT549, the Gal-3–transfected cell clones (BT549-Gal), and the control transfectants (BT549-vCTR) were as described (15, 16). TIB166, monoclonal rat anti–gal-3, was purchased from American Type Culture Collection (Manassas, VA). hL31, anti–gal-3 antibody, was obtained as described (7). Anti-HA antibody was purchased from Cell Signaling Technology, Inc. (Beverly, MA) and anti-phospho-serine antibody from Sigma (St. Louis, MO). Recombinant GSK3β was purchased from New England BioLabs, Inc. (Beverly, MA).

Plasmid construction. pGEX-2T/rat Axin (rAxin; 298-832), pGEX-2T/rAxin (298-506), pGEX-2T/rAxin (1-529), pGEX-2T/rAxin (508-732), and pGEX-2T/rAxin (713-832) were described elsewhere (7). pcDNA3.1+/Zeo-HA-Axin was kindly provided by Dr. Shuichi Kusano (St. Marianna University School of Medicine, Kawasaki, Japan). pGEX-6P-2/gal-3 and gal-3 deletion mutants were as described (7). Production of recombinant glutathione *S*-transferase (GST) fusion proteins were produced and purified according to the manufacturer's instruction (Amersham Biosciences, Piscataway, NJ).

Immunoprecipitation. To determine whether gal-3 forms a complex with axin, BT549-Gal cells were transiently transfected in a 100-mm-diameter dish with pcDNA 3.1+/Zeo-HA-Axin using LipofectAMINE 2000 (Invitrogen, Carlsbad, CA). Twenty-four hours after transfection, cells were lysed in 800 μL ice-cold lysis buffer (procedural and technical detail as in ref. 7). The supernatant (200 μg protein) were immunoprecipitated with anti–gal-3 or anti-HA antibodies for 60 minutes at 4°C. Protein separations and identification were as described (7).

Mapping the Axin–galectin-3 binding region. Various deletion mutants of GST-β-rAxin (each at 250 nmol/L) were incubated with 250 nmol/L gal-3 (full length) for 1 hour at 4°C in 50 μL reaction mixture [20 mmol/L Tris-HCl (pH 7.5) and 1 mmol/L DTT]. GST-Axin deletion mutants were precipitated with glutathione-Sepharose 4B, and then the precipitates were probed with anti–gal-3 antibody. To examine the region of gal-3 that binds to rAxin, various deletion mutants of gal-3 (250 nmol/L each) were incubated with 250 nmol/L of GST-rAxin (298-832) for 1 hour at 4°C in 50 μL reaction mixture. GST-rAxin deletion mutants were precipitated with glutathione-Sepharose 4B and probed with the anti–gal-3 antibody.

***In vitro* kinase assay.** To examine whether gal-3 is phosphorylated by GSK-3β, *in vitro* kinase assay was done. Purified gal-3 (1 μg protein) was incubated in a kinase buffer containing 10 μCi [γ-^{32}P]ATP with indicated units of GSK-3β in the presence or absence of Axin (indicated amount). LiCl (30 mmol/L), a specific GSK-3β inhibitor, was used to establish specificity. The reaction was stopped by boiling, followed by SDS-PAGE and autoradiography (9).

Results and Discussions

Axin–galectin-3 interaction. Previously, it was reported that the β-catenin–Axin association promotes β-catenin phosphorylation by GSK3β (12, 13), whereas we found that gal-3 is a binding partner of β-catenin (7). Because human gal-3 contains the consensus sequence motif of GSK3β phosphorylation ($S_{92}XXXS_{96}$), we questioned whether gal-3 could bind Axin and be phosphorylated by GSK-3β. Thus, BT549-Gal cells were transiently transfected with HA-Axin cDNA, lysed, and immunoprecipitated with either anti-HA

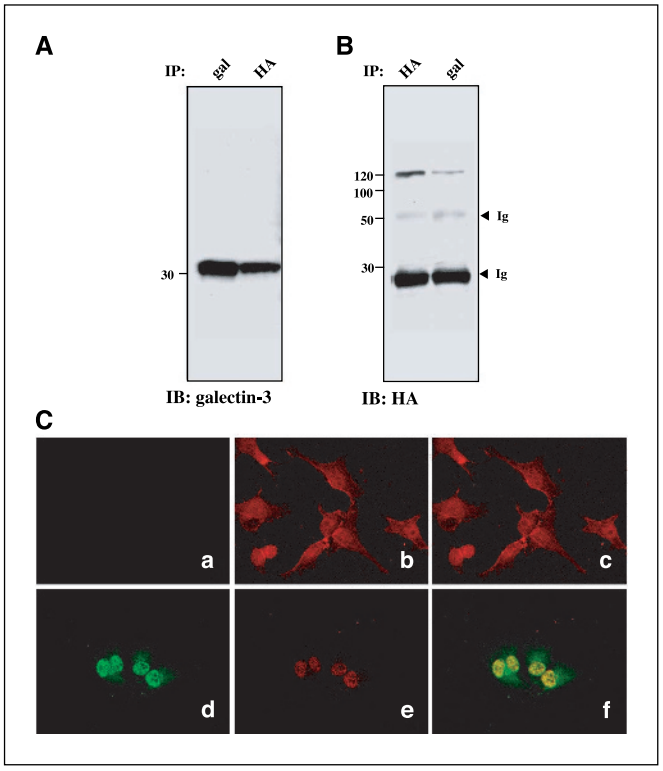

Figure 1. Interaction between gal-3 and HA-Axin. *A,* the lysates of BT549-Gal cells, transiently cotransfected with HA-Axin, were precipitated with anti–gal-3 antibody, hL31 (*left*), or with anti-HA antibody (*right*), and probed with TIB166. *B,* the same lysates were precipitated with anti-HA antibody (*left*) or with hL31 (*right*) and probed with anti-HA antibody. *C,* HA-Axin–transfected BT549 parent cells, which were gal-3–null cells (*a*, *b*, and *c*), and BT549-Gal cells transiently co-transfected with HA-Axin (*d*, *e*, and *f*) were cultivated on a cover glass for 48 hours. The cells were subjected to immunofluorescent analysis with anti-HA antibody (*a* and *d*) and hL31 (*b* and *c*). (*c*) and (*f*) are the merge images of (*a*) and (*b*), or (*d*) and (*e*), respectively.

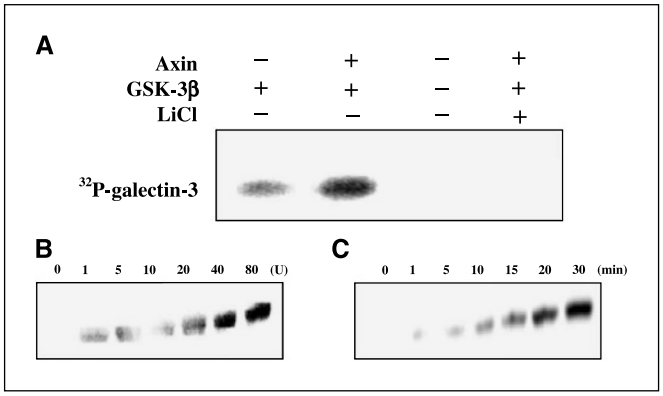

Figure 3. Phosphorylation of gal-3 by GSK-3β. *A,* gal-3 was subjected to *in vitro* kinase assay in the presence or absence of GSK3β, rAxin, and LiCl as described in Materials and Methods. The reaction mixtures containing 10 μCi [γ-^{32}P]ATP were subjected to SDS-PAGE. The effect of addition of 30 mmol/L LiCl, a specific inhibitor of GSK3β, was also examined. *B,* recombinant gal-3 was incubated for 30 minutes with the indicated amounts of GSK-3β in the kinase reaction mixture. The reaction mixtures were subjected to the same assay. *C,* recombinant gal-3 was incubated with 50 units GSK3β for the indicated periods. The reaction mixtures were subjected to SDS-PAGE. Data are representative of three independent sets of experiments.

(Fig. 1*A*, *right*; *B*, *left*) or anti–gal-3 antibodies (Fig. 1*A*, *left*; *B*, *right*) and Western blotted with the reciprocal antibodies (Fig. 1*A* and *B*, *bottom*). The results show that Axin coprecipitated with gal-3 and vice versa, suggesting that the two are complexed *in vivo*. Next, we confirmed that Axin–gal-3 are indeed colocalized *in vivo* by immunofluorescence (Fig. 1*C*) by using confocal microscopy analysis of BT549 (gal-3 null; Fig. 1*C a*, *b*, and *c*) parental and BT549-Gal (Fig. 1*C d*, *e*, and *f*) cells transiently transfected with HA-Axin (Fig. 1*C*). In cells expressing both Axin and gal-3, colocalization of the two proteins is readily observed (Fig. 1*C*, *f*). Subsequently, we

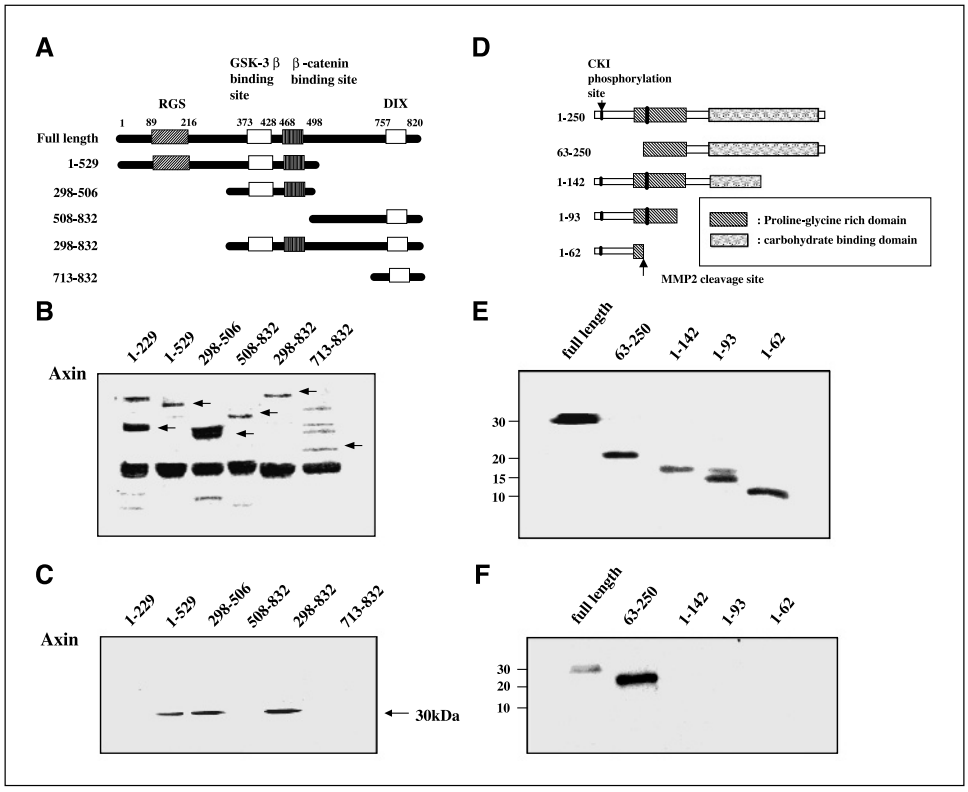

Figure 2. Binding region of gal-3 and Axin. *A,* the scheme of the deletion mutant of rAxin. *B,* GST-rAxin (1-229), GST-rAxin (1-529), GST-rAxin (298-506), GST-rAxin (508-832), GST-rAxin (298-832), and GST-rAxin (713-832) were subjected to SDS-PAGE followed by Coomassie Brilliant Blue staining. The common bands at ~25 kDa means separated GST. *C,* various deletion mutants of GST-β-rAxin (each at 250 nmol/L) were incubated with 250 nmol/L gal-3 (full length) for 1 hour at 4°C in 50 μL reaction mixture as described in Materials and Methods. GST-rAxin deletion mutants were precipitated with glutathione-Sepharose 4B. The precipitates were then subjected to SDS-PAGE and probed with TIB166. *D,* the scheme of the deletion mutants of gal-3. Ser^{6} is the phosphorylation site of casein kinase I. Matrix metalloproteinase has been reported to cleave gal-3 between residues 62 and 63. *E,* purified deletion mutants of gal-3 were subjected to SDS-PAGE followed by Coomassie brilliant blue staining. *F,* the deletion mutants of gal-3 was incubated with GST-rAxin (298-832) in the reaction mixture. The mixture was precipitated with glutathione-Sepharose 4B and then the precipitates were subjected to SDS-PAGE and probed with anti–gal-3 antibody, hL31.

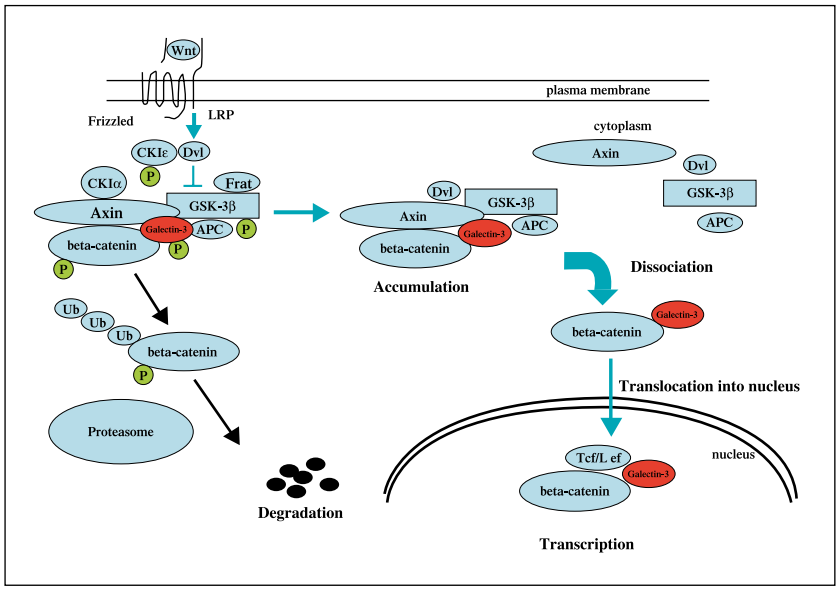

Figure 4. Gal-3–β-catenin interaction. Suggested model for gal-3–β-catenin interaction during Wnt-mediated signaling involving Axin and CKI and GSK-3β that regulates either protein degradation or transcription of genes.

have constructed and expressed deletion mutants of Axin (Fig. 2*A* and *B*) and gal-3 (Fig. 2*D* and *E*) to assist in determining the Axin–gal-3 interacting motifs necessary for their interaction. The Axin mutant peptides were purified as GST fusion proteins and gal-3 was precipitated with GST-Axin (1-528), GST-Axin (298-508), and GST-Axin (298-832) peptides but not with GST-Axin (1-229), GST-Axin (508-832), or GST-Axin (713-832) peptides (Fig. 2*C*). In the reciprocal experiments (Fig. 2*F*), only gal-3 (full length) and gal-3 (63-250) peptides were recognized by GST-Axin (298-508) peptide. Thus, we have concluded that the internal domain of Axin interacts with the COOH terminus of gal-3, encompassing amino acid residues 298-508 and 143-250, respectively.

GSK-3β phosphorylates galectin-3. Because the human gal-3 contains a GSK-3β phosphorylation consensus sequence ($S_{92}XXXS_{96}$), we first questioned its substrate suitability utilizing an *in vitro* kinase assay. Following incubation of gal-3 with or without GSK-3β in a reaction mixture containing [γ-^{32}P]ATP, we found that gal-3 was phosphorylated by GSK-3β and that the phosphorylation was specifically inhibited by a GSK-3β inhibitor, e.g., LiCl (Fig. 3*A*). Similar to β-catenin whereby Axin enhances its GSK-3β–dependent phosphorylation (10–13), Axin promoted the GSK-3β–dependent phosphorylation of gal-3 (Fig. 3*A*). Of note, the phosphorylation of gal-3 by GSK-3β was specific in a time- and dose-dependent manner (Fig. 3*B* and *C*). The above results and the previous data (7, 9) prompted the proposed model (Fig. 4) that revises the Wnt/β-catenin signaling pathway to include gal-3.

It was surprising to find that both gal-3 and β-catenin are substrates of CKI and GSK-3β. As for gal-3, phosphorylation of Ser^6 by CKI serves as a molecular switch for the sugar binding (17) and regulation of nuclear export (9); unlike the phosphorylation of β-catenin by CK1α and GSK3β that promotes its proteosomal degradation, the consequence of GSK-3β gal-3 phosphorylation mediated by Axin is yet to be determined. In addition, we will need to resolve whether the phosphorylation of gal-3 affects the status of phosphorylation of β-catenin or vice versa and to establish whether gal-3 engages/mediates/inhibits the ubiquitination/signaling of β-catenin.

Acknowledgments

Received 1/13/2005; revised 2/14/2005; accepted 3/1/2005.

Grant support: National Cancer Institute, NIH, grant CA46120 (A. Raz).

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.

References

1. Liu FT, Rabinovich GA. Galectin as modulators of tumour progression. Nat Rev Cancer 2005;5:29–41.
2. Inohara H, Raz A. Functional evidence that cell surface galectin-3 mediates homotypic cell adhesion. Cancer Res 1995;55:3267–71.
3. Ray S, Lukyanov P, Ocheing J. Extracellular functions of galectin-3. Glycoconj J 2004;19:527–35.
4. Dagher SF, Wang JL, Patterson RJ. Identification of galectin-3 as a factor in pre-mRNA splicing. Proc Natl Acad Sci U S A 1995;92:1213–7.
5. Kim HR, Lin HM, Biliran H, Raz A. Cell cycle arrest and inhibition of anoikis by galectin-3 in human breast epithelial cells. Cancer Res 1999;59:4148–54.
6. Lin H-M, Moon B-K, Yu F, Kim HR. Galectin-3 mediates genistein-induced G_2/M arrest and inhibits apoptosis. Carcinogenesis 2000;21:1941–5.
7. Shimura T, Takenaka Y, Tsutsumi S, Hogan V, Kikuchi A, Raz A. Galectin-3 a novel binding partner of catenin. Cancer Res 2004;64:6363–7.
8. Yoshii T, Fukumori T, Honjo Y, Inohara H, Kim HR, Raz A. Galectin-3 phosphorylation is required for its anti-apoptotic function and cell cycle arrest. J Biol Chem 2002;277:6852–7.
9. Takenaka Y, Fukumori T, Yoshii T, et al. Nuclear export of phosphorylated galectin-3 regulates its antiapoptotic activity in response to chemotherapeutic drugs. Mol Cell Biol 2004;24:4395–406.
10. Kikuchi A. Roles of Axin in the Wnt signaling pathway. Cell Signal 1999;11:777–88.
11. Ikeda S, Kishida S, Yamamoto H, Murai H, Koyama S, Kikuchi A. Axin, a negative regulator of the Wnt signaling pathway, forms a complex with GSK-3β and β-catenin and promotes GSK-3β-dependent phosphorylation of β-catenin. EMBO J 1998;17:1371–84.
12. Yamamoto H, Kishida S, Uochi T, et al. Axil, a member of the Axin family, interacts with both glycogen synthase kinase 3β and β-catenin and inhibits axis formation of *Xenopus* embryos. Mol Cell Biol 1998;18:2867–75.
13. Kishida S, Yamamoto H, Ikeda S, et al. Axin, a negative regulator of the wnt signaling pathway, directly interacts with adenomatous polyposis coli and regulates the stabilization of β-catenin. J Biol Chem 1998;273:10823–6.
14. Yost C, Torres M, Miller JR, Huang E, Kimelman D, Moon RT. The axis-inducing activity, stability, and subcellular distribution of β-catenin is regulated in *Xenopus* embryos by glycogen synthase kinase 3. Genes Dev 1996;10:1443–54.
15. Akahani S, Nangia-Makker P, Inohara H, Kim HR, Raz A. Galectin-3: a novel antiapoptotic molecule with a functional BH1 (NWGR) domain of Bcl-2 family. Cancer Res 1997;57:5272–6.
16. Nangia-Makker P, Thompson E, Hogan C, Ochieng J, Raz A. Induction of tumorigenicity by galectin-3 in a non-tumorigenic human breast carcinoma cell line. Int J Oncol 1995;7:1079–87.
17. Mazurek N, Conklin J, Byrd JC, Raz A, Bresalier RS. Phosphorylation of the β-galactoside-binding protein galectin-3 modulates binding to its ligands. J Biol Chem 2000;275:36311–5.