Galectin-3 Phosphorylation Is Required for Its Anti-apoptotic Function and Cell Cycle Arrest

Tadashi Yoshiii, Tomoharu Fukumorii, Yuichiro Honjo$$, Hidenori Inohara$$, Hyeong-Reh Choi Kimr, and Avraham Raz$$

From the ¶Tumor Progression and Metastasis Program, Karmanos Cancer Institute, Wayne State University, Detroit, Michigan 48201, the ¶¶Department of Otolaryngology and Sensory Organ Surgery, Osaka University Graduate School of Medicine, Suita, Osaka 565-0871, Japan, and the ¶¶¶Department of Pathology, Wayne State University School of Medicine, Detroit, Michigan 48201

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Galectin-3, a β-galactoside-binding protein, is implicated in cell growth, adhesion, differentiation, and tumor progression by interactions with its ligands. Recent studies have revealed that galectin-3 suppresses apoptosis and anoikis that contribute to cell survival during metastatic cascades. Previously, it has been shown that human galectin-3 undergoes post-translational signaling modification of Ser6 phosphorylation that acts as an “on/off” switch for its sugar-binding capability. We questioned whether galectin-3 phosphorylation is required for its anti-apoptotic function. Serine to alanine (S6A) and serine to glutamic acid (S6E) mutations were produced at the casein kinase I phosphorylation site in galectin-3. The cDNAs were transfected into a breast carcinoma cell line BT-549 that innately expresses no galectin-3. The cDNAs were transfected into the breast carcinoma cell line BT-549 that innately expresses no galectin-3. Metabolic labeling revealed that only wild type galectin-3 undergoes phosphorylation in vivo. Expression of Ser6 mutants of galectin-3 failed to protect cells from cisplatin-induced cell death and poly(ADP-ribose) polymerase from degradation when compared with wild type galectin-3. The non-phosphorylated galectin-3 mutants failed to protect cells from anoikis with Galectin-3 at Ser6 serves as an “on/off” switch for its sugar-binding activity. In 3T3 fibroblasts, quiescent cells express phosphorylated Gal-3 both in the cytoplasm and in the nucleus, whereas proliferating cells show an increased level of phosphorylated Gal-3 ratio in the cytoplasm (24). It was also shown that canine Gal-3 is phosphorylated at the NH2-terminal Ser9 (major) and Ser12 (minor) (25), and the major acidic residues on both sides of Ser6 make it a substrate for casein kinase I and/or for casein kinase II (24, 25). The human Gal-3 undergoes phosphorylation by casein kinase I only at Ser6, and the phosphorylation significantly reduces its binding ability to its ligands, e.g. laminin and asialomucin, whereas dephosphorylation fully restores the sugar binding activity (26), implying that phosphorylation of Gal-3 at Ser6 serves as an “on/off” switch for its sugar-binding capabilities. The function of Gal-3 phosphorylation to its other biological activities, however, remained unknown. We investigate the cellular function of Gal-3 phosphorylation and its impact on apoptosis. Gal-3 mutants generated by site-directed mutagenesis were transfected into the breast carcinoma BT-549 cells that are Gal-3 null. Ser6 mutation resulted in loss of Gal-3 anti-apoptotic activity and its involvement in acids that controls its cellular targeting; a repetitive collagen-like sequence rich in glycine, tyrosine, and proline, which serves as a substrate for matrix metalloproteinases; and a COOH-terminal domain of galectin-1 with a globular structure encompassing the carbohydrate-binding site (1–3). Gal-3 was shown to be involved in cell growth, cell adhesion, differentiation, and tumor progression through binding to complementary glycoconjugates (1, 4–8).

Recent studies (9–12) have revealed that Gal-3 inhibits apoptosis induced by anti-Fas antibody, staurosporine, chemotherapeutic reagent, tumor necrosis factor, radiation, and nitric oxide. Gal-3 also prevents cells from undergoing anoikis, a specific form of apoptosis induced by loss of cell-substrate interactions, by inducing G1 arrest (13). Although Gal-3 does not belong to the Bcl-2 family, it contains the Asp-Trp-Gly-Arg amino acid anti-death sequence, which is a highly conserved sequence within the BHL1 domain of the Bcl-2 family (9, 10, 14, 15). Recent reports (16–21) have shown that phosphorylation of Bcl-2 at Ser70 appears to be critical for its anti-apoptotic function. However, the role of this phosphorylation is debatable due to conflicting reports demonstrating that such a phosphorylation may activate or inactivate Bcl-2 anti-apoptotic function (17–21). It was argued that these contradictory conclusions stemmed from results obtained from using different cell types and death signaling molecules (16–23). Due to the functional anti-death mimicking of Bcl-2 and Gal-3 and the fact that both undergo post-translational modification of serine phosphorylation, we questioned the role of Gal-3 phosphorylation in its anti-apoptotic activity.

In 3T3 fibroblasts, quiescent cells express phosphorylated Gal-3 both in the cytoplasm and in the nucleus, whereas proliferating cells show an increased level of phosphorylated Gal-3 ratio in the cytoplasm (24). It was also shown that canine Gal-3 is phosphorylated at the NH2-terminal Ser9 (major) and Ser12 (minor) (25), and the major acidic residues on both sides of Ser6 make it a substrate for casein kinase I and/or for casein kinase II (24, 25). The human Gal-3 undergoes phosphorylation by casein kinase I only at Ser6, and the phosphorylation significantly reduces its binding ability to its ligands, e.g. laminin and asialomucin, whereas dephosphorylation fully restores the sugar binding activity (26), implying that phosphorylation of Gal-3 at Ser6 serves as an “on/off” switch for its sugar-binding capabilities. The function of Gal-3 phosphorylation to its other biological activities, however, remained unknown. We investigate the cellular function of Gal-3 phosphorylation and its impact on apoptosis. Gal-3 mutants generated by site-directed mutagenesis were transfected into the breast carcinoma BT-549 cells that are Gal-3 null. Ser6 mutation resulted in loss of Gal-3 anti-apoptotic activity and its involvement in

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† To whom correspondence should be addressed: Karmanos Cancer Institute, 110 E. Warren Ave., Detroit, MI 48201. Tel.: 313-833-7518; E-mail: raza@karmanos.org.

‡ From the Department of Pathology, Wayne State University School of Medicine, Detroit, Michigan 48201, the Department of Otolaryngology and Sensory Organ Surgery, Osaka University Graduate School of Medicine, Suita, Osaka 565-0871, Japan, and the Department of Pathology, Wayne State University School of Medicine, Detroit, Michigan 48201.

§ The abbreviations used are: Gal-3, galectin-3; CDDP, cis-diaminedichloroplatinum (cisplatin); CDK, cyclin-dependent kinase; CMF-PBS, calcium-magnesium-free phosphate-buffered saline; DMEM, Dulbecco’s modified Eagle’s medium; FBS, fetal bovine serum; PARP, poly(ADP-ribose) polymerase; poly-HEMA, poly-hydroxyethylmethacrylate.

Glycoconjugates (1, 4–8).
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cell cycle arrest in response to loss of cell-substrate interactions as compared with the wild type species.

EXPERIMENTAL PROCEDURES

Cells and Monolayer Culture Conditions—The breast carcinoma cell line BT-549 was obtained from Dr. E. W. Thompson (Vincent T. Lombardi Cancer Research Center, Georgetown University Medical Center, Washington, D. C.). Cells on tissue culture dishes were grown in DMEM (Invitrogen) supplemented with 10% heat-inactivated FBS, 2 mm glutamine, nonessential amino acids, and antibiotics (Invitrogen) and maintained at 37 °C in a humidified atmosphere of 95% air and 5% CO2.

Site-directed Mutagenesis—To generate Ser6 to Ala (S6A) and to Glu (S6E) point mutations on Gal-3 cDNA, a Quick Change Site-directed Mutagenesis Kit (Stratagene, La Jolla, CA) was employed with appropriate primers as described previously (2). Briefly, pGEM (7+) vector containing human wild type Gal-3 cDNA was used as a template for PCR to generate S6A and S6E point mutations. After amplification, the template DNA was cleaved with DpnI restriction enzyme, and the DpnI-treated DNA was transferred into Escherichia coli XL1-Blue supercompetent cells. Recombinant plasmid pGEM (7+)Gal-3 mutant S6A and S6E were purified and sequenced. The sequences were confirmed by the Macromolecular Core Facility of Wayne State University.

Stable Transfection of Gal-3 cDNAs—Wild type and Ser6 mutant Gal-3 cDNAs were excised from pGEM (7+) containing them with EcoRI and inserted into a mammalian expression vector pBK-CMV (Stratagene) at the EcoRI site in the sense direction. The proper orientation of the cDNA insert was confirmed by restriction enzyme analysis. Then each purified plasmid DNA was transfected into BT-549 using LipofectAMINE (Invitrogen). After 48 h, 800 μg/ml G418 (Invitrogen) was added to the culture for 14 days to obtain stable transfected clones. The resultant clones were named BT-549/V for the control vector-transfected cells, BT-549/Gal-3WT1 and BT-549/Gal-3WT2 for the wild type Gal-3-transfected cells, BT-549/Gal-3S6A1 and BT-549/Gal-3S6A2 for the S6A mutant Gal-3-transfected cells, and BT-549/Gal-3S6E1 and BT-549/Gal-3S6E2 for the S6E mutant Gal-3-transfected cells, respectively. Transfected cells were maintained in complete DMEM containing 400 μg/ml G418 sulfate.

Gal-3 Expression in Parental BT549 and Transfected Cells—Subconfluent cells were harvested and washed with CMF-PBS. Two million cells were then lysed with 500 μl of SDS-PAGE sample buffer (1% SDS, 62.5 mM Tris-HCl, pH 6.8, 10% glycerol). Each protein concentration was measured using BCA protein assay reagent (Pierce). Fifteen micrograms of each sample, immunoprecipitation was initiated by adding 2 μl of protein A-Sepharose 6MB (Amersham Biosciences) in ice-cold lysis buffer. After adjusting radioactivity and volume (800 μl) of each sample, immunoprecipitation was initiated by adding 2 μg of polyclonal rabbit anti-Gal-3 antibody (2) to the precleared supernatant. The reaction mixture was incubated at 4 °C for 2 h and then 30 μl of 1:2 slurry of protein A-Sepharose 6MB in ice-cold lysis buffer was added to the mixture. After incubation at 4 °C for 1 h, the immunoprecipitates were washed five times with ice-cold lysis buffer. The washed precipitates were boiled for 5 min in SDS-PAGE sample buffer containing 5% β-mercaptoethanol and then separated by 12.5% SDS-PAGE. Gal-3 phosphorylation state was analyzed by PhosphorImager (Molecular Dynamics, Sunnyvale, CA), and immunoprecipitated Gal-3 was detected by immunoblotting using TIB166. Equal loading amount was confirmed by checking protein expression in the same membrane.

Cell Viability and CDDP Treatment—Cell viability was assessed by a trypsin blue dye exclusion test. Briefly, 8 × 106 cells were cultured in a 60-mm dish with 25 μM CDDP (Sigma) for the indicated times. Both attached and detached cells were then thoroughly collected, and cell viability was determined by trypsin blue exclusion.

Apoptosis Assays—Apoptosis was assessed by the PARP degradation and the accumulation of cells at sub-G1 fraction. Briefly, cells incubated with or without 25 μM CDDP for 72 h were completely harvested. For the former analysis, the cells were lysed with SDS lysis buffer as described above, and 20-μg aliquots of the cell lysates were separated by 6% SDS-PAGE, transferred to a polyvinylidene difluoride membrane, and probed with mouse anti-PARP antibody (C-2; 10; Biomol Research Laboratories, Plymouth Meeting, PA). For the latter analysis, the cells were fixed with 80% ethanol at −20 °C for at least 4 h. After a wash with CMF-PBS, the cells were resuspended at a concentration of 1 × 106 cells/ml with staining solution that consisted of 50 μg/ml propidium iodide (Sigma), 0.1% Triton X-100, 0.1 mM EDTA, pH 8.0, and 50 μg/ml RNase A in CMF-PBS. The samples were then incubated at room temperature for 20 min in the dark and analyzed by flow cytometry (FACSCalibur, Becton Dickinson, San Jose, CA). CellQuest (Becton Dickinson) and ModFit LT (Verity Software House, Topsham, ME) were used as software for the analysis.

Cell Suspension—Poly-HEMA (Sigma) was solubilized in methanol (50 mg/ml) and diluted in ethanol to a final concentration of 10 mg/ml. To prepare poly-HEMA-coated dishes, 4 ml of poly-HEMA solution were placed onto 100-mm dishes and dried in a tissue culture hood. The poly-HEMA coating was repeated twice, followed by three washes with CMF-PBS. One million five hundred thousand cells were plated onto poly-HEMA-coated dishes for 24 h.

Anoikis and Cell Cycle Distribution—Anoikis was assessed by the PARP degradation and the sub-G1 fraction of propidium iodide-labeled cells, and cell cycle distribution was assessed by each cell cycle phase fraction of propidium iodide-labeled cells. Briefly, cells incubated as monolayer or in suspension culture for 24 h were collected and used for PARP immunoblot and flow cytometric analyses as described above. Cells grown for 24 h as monolayer or in suspension were harvested and lysed with SDS lysis buffer. Equal amounts of the cell lysates were separated by SDS-PAGE and transferred to a polyvinylidene difluoride membrane. Expression levels of cyclins were measured using the following antibodies: anti-cyclin A (SC-239; Santa Cruz Biotechnology, Santa Cruz, CA), anti-cyclin D1 (DCS-6; Sigma), anti-cyclin E (HE-12; PharMingen, San Diego, CA), anti-p21WAF1/CIP1 (SX118; PharMingen), anti-p27KIP1 (M-197; Santa Cruz Biotechnology).

RESULTS

Gal-3 Expression and Phosphorylation in Stable Transfectants of BT-549 Cells—To investigate whether human Gal-3 phosphorylation at Ser6 affects its biological functions, wild type and Ser6 mutant Gal-3 cDNAs as well as control vectors were transfected into the human breast carcinoma cell line BT-549 which expresses no Gal-3. Single G418-resistant colonies were selected and expanded in growth medium under continuous drug selection. Several clones of the control, wild type, and Ser6 mutant Gal-3 transfectants were obtained. A control clone (BT-549/V), two wild type Gal-3 (BT-549/WT1 and BT-549/WT2), two clones of the Ser6 mutant (Ser6 to Ala) (BT-549/S6A1 and BT-549/S6A2), and two clones of the Ser6 mutant (Ser6 to Glu) Gal-3 (BT-549/S6E1 and BT-549/S6E2) were used for further analyses. Gal-3 expression in stable transfectants was determined by Western blot analysis. All the transfected cell clones showed similar expression levels, and cell viability was assessed by a trypsin blue dye exclusion test. Briefly, 8 × 106 cells were cultured in a 60-mm dish with 25 μM CDDP (Sigma) for the indicated times. Both attached and detached cells were then thoroughly collected, and cell viability was determined by trypsin blue exclusion.

Next, we examined whether wild type and Ser6 mutant Gal-3 were phosphorylated in vivo. BT-549/V, /WT1, and /S6A1 cells were metabolically labeled with 32P as described under “Experimental Procedures,” and cell extracts were immunoprecipitated with anti-Gal-3 polyclonal antibody. Western blot anal-
Ser<sup>6</sup> Mutant Gal-3 Fails to Inhibit Anoikis and to Induce Cell Cycle Arrest—A recent study revealed that Gal-3 inhibits anoikis-inducing G<sub>1</sub> arrest (13). We therefore examined the effect of Gal-3 phosphorylation on its regulation of anoikis and cell cycle arrest. Anoikis was induced by culturing cells in suspension for 24 h on a non-adhesive substrate of poly-HEMA. Subsequently, we presented the data of BT-549 WT1, /S6A1, and /S6E1 as their representative to avoid redundancy. We monitored the PARP degradation as a marker for anoikis. No PARP degradation was detected in all clones when cells were cultured as a monolayer (Fig. 3I); however, when cells were cultured in suspension, proteolytic cleavage of PARP was readily detected in BT-549/V, /S6A, and /S6E cells, whereas it was significantly inhibited in BT-549/WT cells (Fig. 3I).

Flow cytometric DNA analysis to determine the cell cycle phase distribution of cells grown as monolayer or in suspension for 24 h was performed next, considering that cell cycle regulation is associated with a loss of cell-matrix interactions (13, 28). In monolayer cultures, the cell cycle phase distribution of both BT-549/V, /WT, and /S6A cells showed a similar pattern (Fig. 3II, A–C). In suspension cultures, however, there were two prominent differences in the DNA histogram pattern among these three clones (Fig. 3II, D–F). First, the percentages of BT-549/V and /S6A cells in sub-G<sub>1</sub> phase were 10.6 and 10.7%, respectively, whereas only 0.1% of BT-549/WT cells were in sub-G<sub>1</sub> phase. Second, a dramatic decrease in S phase (from 47.8 to 8.8%) and a dramatic increase in G<sub>1</sub> phase (from 38.1–75.1%) were detected in BT-549/WT cells in response to a loss of cell-substrate interactions, whereas no such remarkable change was observed in BT-549/V and /S6A cells. These results of the PARP degradation and flow cytometric DNA analysis suggest that Gal-3 protects BT-549 cells from anoikis by cell cycle arrest at G<sub>1</sub> phase in response to a loss of cell-matrix contact and that a mutation at Ser<sup>6</sup> abrogates such a function of Gal-3.

Wild Type Gal-3 Alters Expression Levels of Cell Cycle Regulators—To have a further understanding of the differences in cell cycle responses between BT-549/WT and /S6 mutant cells following loss of cell-substrate interactions, we examined the Gal-3 effect on the expression levels of cell cycle regulators. It was shown that expression of cyclin D<sub>1</sub>, an early G<sub>1</sub> cyclin that promotes cell cycle into late G<sub>1</sub> phase, is dependent upon cell adhesion (28, 29). As shown in Fig. 4, cyclin D<sub>1</sub> expression was up-regulated in BT-549/WT cells when cultured as monolayer. Moreover, cyclin D<sub>1</sub> expression in BT-549/WT cells was further up-regulated in suspension culture, showing that wild type Gal-3 induces cyclin D<sub>1</sub> overexpression independent of cell-matrix interactions. Cyclin E is a nuclear protein essential for the G<sub>1</sub> to S phase transition (28, 29). Expression of cyclin E was down-regulated in BT-549/WT cells, and the expression level was not significantly altered by the culture conditions (Fig. 4).

Expression of cyclin A, a late G<sub>1</sub> to S cyclin, is coincident with and necessary for the onset of S phase (28). In a monolayer culture, cyclin A expression was up-regulated in BT-549/WT cells and was significantly down-regulated in BT-549/WT in response to a detachment of cells from the substrate (Fig. 4). p21<sup>WAF1/CIP1</sup> and p27<sup>KIP1</sup> are CDK inhibitors that mainly act as negative regulators of G<sub>1</sub> to S phase transition (30, 31). As
shown in Fig. 4, the expression of \( p21^{\text{WAF1/CIP1}} \) and \( p27^{\text{KIP1}} \) was up-regulated in BT-549/WT cells when cultured as monolayer, and their expression was further up-regulated in suspension culture. Taken altogether, these results suggest that in response to loss of cell-substrate interactions, wild type Gal-3 up-regulates cyclin D1 expression resulting in passing the apoptosis-sensitive point in early G1 and up-regulates expression of CDK inhibitors (\( p21^{\text{WAF1/CIP1}} \) and \( p27^{\text{KIP1}} \)) and down-regulates cyclin A expression resulting in late G1 arrest. In contrast, such alteration in expression levels of cyclins and CDK inhibitors was observed in neither BT-549/V nor /S6 mutant cells in response to loss of cell-substrate interactions (Fig. 4). The clear difference in the expression levels of cell cycle regulators between BT-549/WT and /S6 mutant cells in response to loss of cell-substrate interactions demonstrates that G1 arrest induced by Gal-3 to avoid anoikis depends on its ability to undergo phosphorylation.

**DISCUSSION**

The \( \beta \)-galactoside-binding lectin Gal-3 plays a central role on diverse biological functions including regulation of apoptosis. The post-translational modification of Gal-3 by phosphoryla-
tion regulates its carbohydrate recognition (24). Studies using site-directed mutagenesis have shed new light on diverse protein functions (2, 10, 15–18, 20, 21); thus we employed this method to gain an insight into the relationship between Gal-3 phosphorylation and its biological function. From the data, we have concluded that Gal-3 phosphorylation is required for its anti-apoptotic activity and anti-anoikis activity with G1 cell cycle arrest.

It is now well established that Gal-3 overexpression correlates with increased metastatic potential in some cancers (1, 6, 8). Gal-3 may render this property to tumor cells by its anti-apoptotic and anti-anoikis activities, which are thought to be critical for anchorage-independent cell survival in the circulation during dissemination. We show that the anti-death activity of Gal-3 is regulated by its phosphorylation. Moreover, the enhanced metastatic potential related to Gal-3 overexpression seems to be promoted by the property that cell surface Gal-3 mediates homotypic cell aggregation and tumor cell adhesion to endothelial cells and to extracellular matrix through binding with its complementary glycoconjugates, which is also regulated by its post-translational modification, i.e. Ser6 phosphorylation of human Gal-3 significantly reduces the saturation binding to its complementary glycoconjugates and dephosphorylation fully restores the binding (5, 7, 26). Taken together, Gal-3 phosphorylation may play a pivotal role in the biological function of Gal-3.

Although Gal-3 does not belong to the Bcl-2 family, it con-
tains sequence and functional similarities to Bcl-2; Gal-3 has the four amino acid anti-death motif (Asp-Trp-Gly-Arg) that is highly conserved within the Bcl-2 family (9, 10, 14, 15), and both molecules undergo post-translational modification of serine phosphorylation. Recently, it has been shown (16–21) that phosphorylation of Bcl-2 at Ser70 appears to be critical for its anti-apoptotic function; however, the role of this phosphorylation is uncertain due to conflicting reports (17–21) showing that such a phosphorylation may activate or inactivate Bcl-2 anti-apoptotic function. These contradictory conclusions may result from the fact that these studies were performed using different types of tumor cells and apoptotic signals or from the fact that multiple kinases have been implicated in the phosphorylation of Bcl-2, including Raf-1 kinase (22), protein kinase C (17), protein kinase A (23), Jun NH2-terminal kinase/stress-activated protein kinase (20), and v-cyclin-CDK6 (21). On the other hand, the human Gal-3 is phosphorylated at Ser6 by casein kinase I (25, 26). Casein kinase I was shown to phosphorylate tumor necrosis factor receptor, which negatively regulates receptor-mediated tumor necrosis factor signaling for apoptosis (33). Furthermore, casein kinase I was suggested to be a conserved component of the Wnt pathway (34) and to be a positive regulator of this pathway and a link between upstream signals and the complexes that regulate ß-catenin (35). Casein kinase I might also act in the ß-catenin-independent pathway (36). Recently, it was found that Wnt signaling can inhibit drug-induced apoptosis suggesting that it may exhibit its oncogenic potential through a mechanism of anti-apoptosis (37). Thus, it appears that casein kinase I plays a role in regulation of apoptotic function of diverse molecules and signaling pathways.

We demonstrated here that Gal-3 also modulates its anti-apoptotic activity and cell cycle arrest in response to a loss of cell-substrate interactions via its phosphorylation. Thus, the finding of a new similarity in post-translational modification between Gal-3 and Bcl-2 should lead to further understanding of regulation of Gal-3 signaling. Taken together, these finding indicate that the regulation of Gal-3 phosphorylation transduces signal(s) for carbohydrate binding and anti-apoptotic function.

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