Phosphorylation of the β-Galactoside-binding Protein Galectin-3 Modulates Binding to Its Ligands*

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The β-galactoside-binding protein galectin-3 has pleiotropic biological functions and has been implicated in cell growth, differentiation, adhesion, RNA processing, apoptosis, and malignant transformation. Galectin-3 may be phosphorylated at N-terminal Ser6, but the role of phosphorylation in determining interactions of this endogenous lectin with its ligands remains to be elucidated. We therefore studied the effect of phosphorylation on binding of galectin-3 to two of its reported ligands, laminin and purified colon cancer mucin. Human recombinant galectin-3 was phosphorylated in vitro by casein kinase I, and separated from the native species by isoelectric focusing for use in solid phase binding assays. Non-phosphorylated galectin-3 bound to laminin and asialomucin in a dose-dependent manner with half-maximal binding at 1.5 μg/ml. Phosphorylation reduced saturation binding to each ligand by >85%. Ligand binding could be fully restored by dephosphorylation with protein phosphatase type 1. Mutation of galectin-3 at Ser6 (Ser to Glu) did not alter galectin ligand binding. Metabolic labeling or separation by isoelectric focusing confirmed the presence of phosphorylated galectin-3 species in vivo in the cytosol of human colon cancer cells from which ligand mucin was purified. Phosphorylation significantly reduces the interaction of galectin-3 with its ligands. The process by which phosphorylation modulates protein-carbohydrate interactions has important implications for understanding the biological functions of this protein, and may serve as an "on/off" switch for its sugar binding capabilities.

Galectins (formerly known as S-type or S-Lac lectins) are a family of carbohydrate-binding proteins characterized by conserved amino acid sequences defined by structural similarities in their carbohydrate-binding domains and affinity for β-galactoside containing glycoconjugates (1–14). Galectin-3, a member of this galactose binding family, has pleiotropic biological functions and has been implicated in cell growth, differentiation, adhesion, RNA processing, apoptosis, and malignant transformation (1, 11, 15–28). The protein is found in the cytoplasm, on the cell surface, in the nucleus, and is secreted by tumor and inflammatory cells (22, 29–36). Potential ligands for galectin-3 include lysosomal-associated membrane proteins 1 and 2, IgE, laminin, and Mac-2-binding protein (37–39). Mucin derived from human colon cancer cells has been recently identified as an important ligand for galectin-3, and may represent a major circulating ligand in colon cancer patients (39).

Galectin-3 may be phosphorylated at N-terminal Ser6 (major) and Ser12 (minor) (40) and the major acidic residues on both sides of Ser6 make this a likely substrate for casein kinase I and/or for casein kinase II (26, 40, 41). Phosphorylated galectin-3 has been demonstrated to be present in the cytosolic and nuclear fractions of 3T3 fibroblasts (26, 41), and in cultured polarized canine epithelial (Madin-Darby canine kidney) cells (40). It has been suggested that phosphorylation may modulate the intracellular function and translocation of galectin-3, but the functional role of phosphorylation in determining interactions of this endogenous lectin with its ligands remains to be determined (40).

The present study employs large-scale separation of galectin-3 and its phosphorylated species to examine the effect of phosphorylation on the binding of galectin-3 to two of its reported ligands, laminin (38) and purified colon cancer mucin (39). Phosphorylation reduced saturation binding to each ligand by greater than 85%, and ligand binding could be fully restored by dephosphorylation with protein phosphatase type 1. Metabolic labeling confirmed the presence of phosphorylated galectin-3 in vivo in the cytosol of human colon cancer cells from which ligand mucin was purified.

EXPERIMENTAL PROCEDURES

Materials—Carrier-free [32P]orthophosphoric acid (5 mCi/ml in water, 9000 Ci/mmol) and [γ-32P]ATP (10 Ci/mmol) were purchased from PerkinElmer Life Sciences (Boston, MA). Rat recombinant casein kinase I (500,000 units/ml), and rabbit recombinant protein phosphatase 1 (1000 units/ml) were purchased from New England Biolabs (Beverly, MA). Rat hybridoma M3/38 producing monoclonal antibody (TIB–166, IgG2a) against galectin-3 was obtained from the American Type Culture Collection (Rockville, MD). Rabbit polyclonal anti-galectin-3 antibodies (R1) were generated as described previously (42). Both antibodies were purified with an Immunopure (G) kit (Pierce, Rockford, IL). Horseradish peroxidase–conjugated anti-rat and anti-rabbit antibodies and rat myeloma immunoglobulins were purchased from Zymed Laboratories Inc. (South San Francisco, CA). Biotinylated anti-rat and anti-rabbit Fab’s, fragments, protein G-agarose, blocking reagent, and protease inhibitor mixture were obtained from Roche Molecular Biochemicals (Indianapolis, IN).

Human recombinant galectin-3 and the Ser6 →Glu (SG-6) mutant were produced as described previously (36). Asialomucin from human colon cancer cell line LS LIM6 was prepared as described previously (39). Laminin from the basement membrane of the Engelbreth-Holm-Swarm sarcoma laminin, phosphatase inhibitors, and all other reagents, unless specified, were purchased from Sigma.

Cell Culture and Metabolic Labeling—Colon cancer cell lines
The separated proteins were concentrated on a Speed-Vac to 100
mM ammonium bicarbonate, pH 7.6, and 5 mM EDTA at 37 °C for 2 h.
nonphosphorylated galectin-3 bands were excised and eluted with 50
nonprotein phosphatase with activity toward phosphoserine/threonine resi-
Proteins were identified by either Coomassie Blue staining or immuno-
blots with R1 antibodies followed by drying and autoradiography.

Protein Analysis, SDS-PAGE, Isoelectric Focusing, and Immuno-
Protein concentration was determined using the Bio-Rad Protein
assay kit (Bio-Rad) and the Coomassie Plus protein assay (Pierce,
Rockford, IL). For protein separation the mini-protein unit (Bio-Rad) was
used under standard conditions. For isoelectric focusing the IEF
Ready Gel, pH 3–10, and IEF cathode and anode buffers (Bio-Rad) were
used according to the manufacturer’s instructions for non-denatured
protein samples. Western analysis was performed as described previ-
ously (22). After separation, protein was transferred to nitrocellulose
membranes in 0.7% acetic acid, blotted with anti-galectin-3 mAb
TIB166, and visualized using an enhanced chemiluminescent detection
system (Roche Molecular Biochemicals, Indianapolis, IN). Radiolabeled
proteins were detected by autoradiography. The relative amount of phos-
phorylated and nonphosphorylated galectin-3 was estimated by densi-
tometric scanning using a digital imaging system (Alpha Innotech,
Santa Clara, CA). The radioactivity incorporated into the phosphorylated
ganectin-3 excised protein band was determined by using a β-counter
(Packard, Downer Grove, IL).

Purification of Phosphorylated Galectin-3—For purification of phos-
phorylated galectin-3 (galectin-3-P), the casein kinase I phosphory-
lation reaction was scaled up. Affinity purified galectin-3 (280 µg) was
phosphorylated as above, except that 3500 units of casein kinase I and
34 mM [γ-32P]ATP were used in a final volume of 350 µL. The reaction
was carried out for 20 h at 30 °C, and terminated by addition of β-
mercaptoethanol (5 mM final) and glycerol (10% final). The reaction
mixture was immediately separated on an IEF gel pH 9–10.
The galectin-3-P protein band was visualized by autoradiography. The
non-phosphorylated galectin-3 was identified on a control lane by
staining with IEF staining solution (Bio-Rad). Both galectin-3-P and
nonphosphorylated galectin-3 bands were excised and eluted with 50
mM ammonium bicarbonate, pH 7.6, and 5 mM EDTA at 37 °C for 2 h.
The separated proteins were concentrated on a Speed Vac to 100 µL and
dialed against 50 mM Tris, pH 7.0, at 4 °C for 12 h.

Protein Phosphatase 1 (PP1) Assay—PP1 is a Mg2+-dependent pro-
tein phosphatase with activity toward phosphoserine/threonine resi-
dues.43 Galectin-3-P was digested with 1 unit of PP1 in 50 mM Tris,
ph 7.0, 1 mM MnCl2, 0.1 mM EDTA, 5 mM dithiothreitol, and 0.01% Brij
35 for 2 h at 30 °C.

Solid-phase Binding Assay—The individual wells of a 96-well poly-
styrene microtiter plate (Costar) were coated with either Engelbreth-
Holm-Swarm sarcoma laminin or asialomucin at 1 µg/100 µL of phos-
phate-buffered saline/well at 4 °C overnight. The nonspecific protein-
binding sites were saturated with 1% blocking buffer made in phosphate-buffered saline for 1 h at room temperature. Serial dilutions of
gel eluted galectin-3, galectin-3-P, SG-6 mutant galectin-3, and de-
phosphorylated galectin-3 samples in 0.5% blocking buffer were then
added to the coated wells and incubated for 1 h at 37 °C. Control

\[ \text{LS174T, LS1LM6, and HM7 were cultured as described previously (22).} \]
For 32P labeling in vivo, cells were grown in 162-mm² flasks (Costar,
Cambridge, MA) to confluence. Metabolic labeling and cell lysis was
performed according to the procedure described by Huflejt et al. (40).

Immuno precipitation—[32P]-Labeled galectin-3 was immunoprecipi-
tated from labeled cell lysates with TIB166 antibodies as described
previously (40). As controls, isotype matched normal rat myeloma
immunoglobulins were used (Zymed Laboratories Inc., South San
Francisco, CA). The immunoprecipitates were boiled for 5 min in
sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-
PAGE) sample buffer and separated on a 10% polyacrylamide gel (22).
Proteins were identified by either Coomassie Blue staining or immuno-
histochemistry or 0.5% blocking buffer for 30 min at 37 °C, followed by
incubation with biotinylated goat anti-rabbit IgG (Fab), fragment (1:4000)
for 30 min at 37 °C. In the last incubation step, ABC reagent (Vectastain
ABC Kit, Vector, Burlingame, CA) was added for 30 min at 24 °C. The protein-
coated wells were developed with the ABTS substrate and developed
color monitored at 405 nm using a plate reader (Bio-Rad).

RESULTS

Phosphorylation of Galectin-3 and Purification of Galectin-
3-P—Galectin-3 has been previously reported to be an in vitro
substrate for casein kinase I (40). Rat recombinant casein
kine I was therefore used to phosphorylate affinity purified
human galectin-3. Phosphorylated galectin-3 was characterized
on SDS-polyacrylamide gels, while retaining immunological rec-
ognition by both monoclonal and polyclonal antibodies to ga-
lectin-3 (Fig. 1). Upon isolectric focusing, two major bands
with distinct pI values resolved at pI 8.2 and pI 7.6 with 32P
radioactivity associated with the more acidic band (Fig. 2).
Immunoblot analysis confirmed that the two bands were
immunoreactive with antibody to galectin-3. This demonstrated
the feasibility of separating galectin-3 into its more acidic phos-
phorylated form and its nonphosphorylated form for further
experiments. Phosphogalactin was then purified using a “scaled up”
phosphorylation reaction described under “Experimental Proce-
dures.” After separation by isoelectric focusing, bands representing phosphorylated and nonphosphorylated ga-
lectin-3 species were excised and eluted by diffusion in ammo-
nium bicarbonate. Products were then analyzed by Western
analysis. Galectin-3 and galectin-3-P were purified to homoge-
nity from the reaction mixture (Fig. 3A) and all radioactivity
was found to be associated with galectin-3-P (Fig. 3B).

Phosphorylation Alters Binding of Galectin-3 to Its Li-
gands—A better understanding of the function of galectin-3 in normal
and neoplastic epithelium will require determination of the influx of
32P into human recombinant galectin-3 was carried out in vivo (32P)
with acute and chronic myeloid leukemia. Phosphogalactin was then purified using a

\[ 1 \text{ The abbreviations used are: PAGE, polyacrylamide gel electro-
phoresis; IEF, isoelectric focusing; galectin-3-P, phosphorylated galectin-
3; SG-6, galectin-3 mutated at serine 6 (Ser6 → Glu); ABTS, 2,2′-
azino-bis(3-ethylbenzthiazoline-6-sulfonic acid).} \]
This binding was sugar dependent as it was abrogated by 0.3 M lactose. Galectin-3-P exhibited markedly reduced binding, with saturation reaching only 15% at galectin-3 saturation binding levels for both ligands. Dephosphorylation of galectin-3-P fully restored galectin-3-ligand binding, confirming the role of phosphorylation in diminishing binding of galectin-3 to its ligands.

It has been previously demonstrated that galectin-3 is predominantly phosphorylated on Ser^6. Control phosphorylation reactions were performed with purified galectin-3 mutated at this site (Ser^6 → Glu) (36). Mutated galectin-3 purified from this source bound its ligands to a similar or increased degree compared with native galectin-3 (Fig. 5). These results confirm that the reduced ability of galectin-3-P to bind its ligands is not simply the result of the purification procedure or the phosphorylation reaction per se.

Galectin-3 Is Phosphorylated in Human Colon Cancer Cells—Colon cancer mucin is an important ligand for galectin-3, and interactions between the endogenous lectin and this ligand is altered by phosphorylation (Fig. 5). While phosphorylated galectin-3 has been demonstrated in cells such as 3T3...
Galectin-3 expression correlates with neoplastic transformation and tumor progression in the colon (22, 44, 46, 52), and colorectal cancer metastases express higher levels of galectin-3 than the primary tumors from which they arise (44, 52). Up-regulation of galectin-3 in colon cancer cells by stable transfection leads to an increase in spontaneous metastasis and liver colonization, while down-regulation by antisense methodology significantly reduces metastasis (22). This may be due in part to alterations in cellular adhesive interactions.

Phosphorylation of Galectin-3

Phosphorylation is important in modulating a variety of cell processes including cell growth and differentiation, and plays a major role in signal transduction pathways (48–50). It has recently become apparent that the phosphorylation or dephosphorylation of certain adhesion molecules may play a role in modulation of cell signaling (47, 48). E-selectin, for example, is constitutively phosphorylated in cytokine-activated human endothelial cells and undergoes enzymatically regulated dephosphorylation following leukocyte adhesion (47). Galectin-3 has been shown to be phosphorylated, predominantly at N-terminal Ser, and both phosphorylated and nonphosphorylated forms have been found in 3T3 fibroblasts and polarized canine kidney epithelial (Madin-Darby canine kidney) cells (26, 40, 41). Phosphorylated galectin-3 has been detected in both the cytoplasm and nucleus of 3T3 fibroblasts, and the ratio of phosphorylated to nonphosphorylated galectin-3 varies with the proliferative status of the cells. The precise functions of phosphorylated galectin-3 and the role of phosphorylation in determining the interactions of galectin-3 with its ligands remains unknown. Phosphorylation does not appear to play a role in regulating cellular localization of the lectin (36).

In the present study we confirmed that galectin-3 is a substrate for casein kinase I, and demonstrated that phosphorylation is associated with an alteration in molecular weight and electrophoretic mobility suggesting the possibility of a conformational shift induced by phosphorylation. We took advantage of the ability to separate phosphorylated galectin-3 from nonphosphorylated protein by isoelectric focusing to separate sufficient amounts of each species for comparative functional studies.

Phosphorylation reduced saturation binding of galectin-3 to laminin and mucin by greater than 85%. This was not a result of protein denaturation during in vitro phosphorylation since galectin-3 mutated at Ser bound in a manner similar to native galectin-3. A growing body of evidence suggests that protein phosphatases may play a role in modulating cell-cell or cell-matrix adhesion (48). We have demonstrated the ability of the protein serine-threonine phosphatase protein phosphatase 1 (PP1) (51) to dephosphorylate galectin-3-P in vitro. Dephosphorylation fully restored binding of galectin-3 to its ligands. It is possible that galectin-3 is constitutively phosphorylated in vivo by casein kinase-I, and that the ratio of phosphorylated to nonphosphorylated protein is regulated by phosphatase activity (40). A similar mechanism has been proposed for regulation of selectin phosphorylation and leukocyte adhesion to endothelia (50).

Galectin-3 expression correlates with neoplastic transformation and tumor progression in the colon (22, 44, 46, 52), and colorectal cancer metastases express higher levels of galectin-3 than the primary tumors from which they arise (44, 52). Up-regulation of galectin-3 in colon cancer cells by stable transfection leads to an increase in spontaneous metastasis and liver colonization, while down-regulation by antisense methodology significantly reduces metastasis (22). This may be due in part to alterations in cellular adhesive interactions.
Phosphorylated galectin-3 was detected in the cytosol of the colon cancer cells from which ligand mucin was purified, and was most abundant in cells with high metastatic potential. We have recently demonstrated that mucin produced by colon cancer cells is an important ligand for galectin-3 (39), and that galectin-3 may actually play a role in regulation of mucin synthesis (53). Our data suggest that phosphorylation of galectin-3 may play a role in regulation of these processes.

Phosphorylation of galectin-3 significantly alters the interaction of galectin-3 with its ligands. The process by which phosphorylation acts as an "on-off switch" for protein-carbohydrate interactions is unknown, but has important implications for understanding the biological functions of this protein. Galectin-3 is unique among galectins in that in addition to a typical carbohydrate recognition domain (located at the C-terminal), it has an unrelated non-carbohydrate-binding N-terminal domain including a 12-amino acid leader sequence containing a casein kinase I serine phosphorylation site (36). The x-ray crystal structure of the carbohydrate recognition domain of galectin-3 has been determined (54), and indicated structural differences between galectin-3 and other galectins which may impact carbohydrate binding specificity. The region corresponding to the dimer interface in galectin-1 and galectin-2 does not, for example, appear to serve a similar role in galectin-3, and oligomerization of galectin-3 may instead depend on interactions between the carbohydrate recognition domain and the N-terminal. While the three-dimensional structure of the N terminus of galectin-3 is not yet known, it appears that it is intact galectin-3, but not the carbohydrate recognition domain alone, shows avidity for multivalent glycoconjugates (55, 56), modulates cell adhesion (23), and induces intracellular signals (35). Phosphorylation of Ser6 at the N terminus of galectin-3 could lead to a conformational change in the protein, altering its ability to participate in multivalent interactions necessary for its biological functions.

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