Interactions between Galectin-3 and Mac-2-Binding Protein Mediate Cell-Cell Adhesion

Hidenori Inohara, Shiro Akahani, Kirston Koths, and Avraham Raz

Tumor Progression and Metastasis Program, Karmanos Cancer Institute [H. I., S. A., A. R.]; Departments of Pathology and Radiation Oncology, Wayne State University School of Medicine [A. R.], Detroit, Michigan 48201; and Chiron Technologies, Chiron Corporation, Emeryville, California 94608 [K. K.]

ABSTRACT

Galectin-3 is a \( \beta \)-galactoside-specific lectin implicated in diverse processes involved in cellular interactions. Recently, the Mac-2-binding protein, a heavily N-glycosylated secreted protein with a subunit \( M_r \) of 97,000, was identified as its ligand. The present study characterizes the interaction between galectin-3 and Mac-2-binding protein in whole cells and measures their relative expression levels. Incubation of A375 cells with affinity-purified Mac-2-binding protein resulted in its binding to galectin-3 on the cell surface in a specific carbohydrate-dependent manner. Mac-2-binding protein also induced homotypic cell aggregation, which was inhibited by lactose or Fab fragments of an anti-galectin-3 antibody. Northern blotting analysis revealed differences in the transcriptional regulation of galectin-3 and Mac-2-binding protein. These results provide the first direct evidence for a Mac-2-binding protein function and suggest that it may play a role in tumor cell embolization during metastasis through interaction with galectin-3.

INTRODUCTION

The galectins are a growing family of vertebrate carbohydrate-binding proteins that share two properties: affinity for \( \beta \)-galactosides and significant sequence homology of the carbohydrate-binding domain (1). Galectin-3 (also known as Mac-2, CBP-35, mL-29, L-31, and eBP; Refs. 2–6) is a \( M_r \sim 30,000 \) protein composed of two distinct structural motifs, an amino-terminal half containing Gly-X-Y tandem repeats characteristic of collagenas and a carboxy-terminal half containing the carbohydrate-binding site (2–5, 7, 8). Although galectin-3 is implicated in cell growth, differentiation, inflammation, transformation, and metastasis via interactions with a specific ligand(s), the mechanism of its diverse actions is only now beginning to be elucidated, in part by the identification of its native ligands, such as Mac-2-BP. Mac-2-BP, originally identified by its ability to bind to galectin-3 (6, 9, 10), is a heavily N-glycosylated secreted protein with a \( M_r \), 97,000 subunit that forms a native molecular complex with a molecular weight of several million (9, 10). Mac-2-BP is probably identical to two previously described proteins, the L3 lung tumor antigen (11) and a cytoplasmic melanoma-associated antigen (12). A molecular cloning study has revealed that Mac-2-BP is a member of the macrophage scavenger receptor cytoste-rich domain superfamily (10). Subsequently, Ulrich et al. (13) reported the cDNA sequence of a secreted tumor-associated antigen, designated 90k and found to be identical to the previously cloned Mac-2-BP (10). Although the serum level of L3/90k antigen has been found frequently elevated in patients with a diversity of malignant diseases (11, 14–16), its function has not yet been established. In addition, it has remained unproven that Mac-2-BP serves as a functional endogenous ligand for galectin-3. In this context, the present study was designed to examine the cellular localization, function, and expression of Mac-2-BP in relation to galectin-3. We show here that Mac-2-BP may interact with galectin-3 on tumor cell surfaces, resulting in the formation of multicell aggregates, and that the expression of Mac-2-BP is differentially regulated from that of galectin-3. The possibility that Mac-2-BP plays a role in the pathogenesis of metastasis through interaction with galectin-3 is discussed.

MATERIALS AND METHODS

Cells and Culture Conditions. The human melanoma cell line A375 was from Dr. W. A. Nelson-Rees (Naval Biosciences Laboratory, Naval Supply Center, Oakland, CA). The human breast cancer cell lines SK-BR-3, BT-549, T47-D, MDA-MB-231, and MDA-MB-435 were obtained from Dr. E. W. Thompson (Vincent T. Lombardi Cancer Research Center, Georgetown University Medical Center, Washington, D.C.). Breast cells were cultured on plastics in RPMI 1640, whereas A375 cells were cultured in DMEM, both supplemented with 10% heat-inactivated fetal bovine serum, 2 mM glutamine, nonessential amino acids, and antibiotics. The cells were maintained at 37°C in a humidified atmosphere of 7% CO2 and 95% air.

Indirect Immunofluorescence. A375 cells grown on glass coverslips were washed twice with PBS+, followed by fixation and permeabilization with prechilled (–70°C) 100% methanol incubated at –20°C for 30 min. The cells were then washed briefly with PBS+ and blocked with 2% NDS in PBS+ at 4°C for 1 h followed by incubation with 10 \( \mu \)g/ml of affinity purified anti-Mac-2-BP rabbit polyclonal antibody (10) and a rat anti-galectin-3 monoclonal antibody (American Type Culture Collection, Rockville, MD) in PBS+/NDS at 4°C for 1 h. After three washes with PBS+, the cells were labeled with a 1:20 dilution of FITC-conjugated donkey antirabbit IgG (Jackson Immunoresearch Laboratories, West Grove, PA) and a 1:50 dilution of B-phycoerythrin-conjugated Fab\( _2 \) fragment of donkey antirat IgG (Jackson Immunoresearch Laboratories, West Grove, PA) and a 1:50 dilution of B-phycoerythrin-conjugated Fab\( _2 \) fragment of donkey antirat IgG (Jackson Immunoresearch) in PBS+/NDS at 4°C for 1 h to localize Mac-2-BP and galectin-3, respectively, and finally washed three times as described above. The coverslips were mounted on slides in 90% glycerol and visualized using a Nikon Optiphot fluorescence microscope. Alternatively, the cells were processed as described above, except that they were fixed with 2% paraformaldehyde in PBS at 4°C for 30 min before labeling. The latter procedure was used to detect the cell surface antigens. No cross-reactivity between the rabbit anti-Mac-2-BP primary antibody and the donkey antirat IgG secondary antibody or between the rat anti-galectin-3 primary antibody and the donkey antirabbit IgG secondary antibody was observed. Controls receiving either no primary antibody, a nonspecific rabbit IgG or a nonspecific rat IgG exhibited no background labeling.

In a subset of assays, the cells were washed twice with PBS+, blocked with PBS+/NDS at 4°C for 1 h, and again washed twice with PBS+. Subsequently, the cells were incubated with 10 \( \mu \)g/ml of Mac-2-BP, which had been affinity purified from serum-free conditioned medium of A375 melanoma cells as described previously (9), in the presence or absence of 50 mM lactose at room temperature for 1 h, and gently washed twice with PBS+ followed by fixation with 2% paraformaldehyde in PBS at 4°C for 30 min. After washing three times for 5 min with PBS+, the cells were processed for immunofluorescent staining as described above.

Cross-Linking. A375 melanoma cell monolayers cultured on culture dishes 60 mm in diameter were washed twice with ice-cold PBS and exposed to a thiol-cleavable membrane-permeable cross-linking reagent DSP (Pierce Chemical Co., Rockford, IL), or an uncleavable, membrane-permeable cross-
linking reagent DSS (Pierce), freshly dissolved in DMSO, at final concentrations of 1 mM in 2 ml of PBS at 4°C for 1 h. The reaction was ended by the addition of 1 M Tris-HCl (pH 7.4) at a final concentration of 10 mM. After 15 min at 4°C, the reaction mixture was aspirated, and the cells were washed three times with ice-cold PBS. Then, 1 ml of lysis buffer (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1% Triton X-100, 0.1% SDS, 1% sodium deoxycholate, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 10 µg/ml leupeptin, 10 µg/ml aprotinin) with or without 50 mM lactose was added, and the cells were scraped off the dish with a rubber policeman. The suspensions were incubated at 4°C for 1 h, followed by centrifugation at 12,000 × g at 4°C for 30 min. The supernatants were saved and processed for immunoprecipitation and/or immunoblotting analyses as described below.

**Immunoprecipitation.** The cell lysates were precleared by overnight incubation at 4°C with 3 µg of rabbit IgG and 50 µl of protein A-Sepharose (Zymed, South San Francisco, CA). Immunoprecipitation was initiated by adding 3 µg of rabbit anti-Mac-2-BP to the precleared supernatant followed by 50 µl of protein A-Sepharose. The reaction mixture was incubated at 4°C for 2 h, followed by five washes with a lysis buffer with or without added 50 mM lactose.

**Immunoblotting.** The cell lysates and the immunoprecipitates were boiled in SDS-sample buffer (2% SDS, 62.5 mM Tris-HCl, pH 6.8, 10% glycerol, 5% β-mercaptoethanol) for 5 min, and aliquots of the supernatants obtained after centrifugation were subjected to SDS-PAGE on 4–20% gradient gels (Bio-Rad, Richmond, CA). After electrophoresis, the proteins were transferred to PVDF-Plus membranes (MSI, Westboro, MA) in the presence of 12.5 mM Tris and 120 mM glycine and quenched overnight at 4°C with 5% nonfat dry milk. Blots were probed with rat anti-galectin-3 at room temperature for 1 h, washed, and probed with 125I-sheep antirat IgG (ICN Biomedicals, Irvine, CA) at a concentration of 0.2 µCi/ml at room temperature for 1.5 h. After extensive washing, the blots were autoradiographed.

**Cell Aggregation.** A375 cells were harvested from monolayers with 0.02% EDTA in PBS, and single-cell suspensions (1 × 10⁶ cells/ml in PBS) were incubated with various concentrations of affinity-purified Mac-2-BP. Aliquots containing 0.5 ml of the cell suspension were placed in siliconized glass tubes and agitated at 80 rpm at 37°C for up to 1 h. The aggregation was then stopped by fixing the cells with 1% formaldehyde. The number of single cells in suspension was determined, and the extent of aggregation was calculated according to the following equation: (1-N/N₀) × 100, where N₀ (test) and N₀ (control) represent the number of single cells in the presence or absence of the tested compounds, respectively.

In a subset of assays, 100 µg/ml Fab' fragments of rabbit IgG antibody directed against a synthetic peptide of the carbohydrate-binding domain of galectin-3 (S), 100 µg/ml Fab' fragments of normal rabbit IgG, 50 mM lactose, or 50 mM sucrose were added in addition to 20 µg/ml of Mac-2-BP. Cell suspensions were agitated for 1 h as above and the extent of aggregation was determined. For the preparation of Fab' fragments, IgG fraction was purified from rabbit antiserum with the use of an IgG purification kit (Pierce). Oligonucleotide primers for PCRs were sense (5'-ATGCCCCATGTCAGGACCT-TGTC-3') and antisense (5'-ACCGATGCTACCATACCTAG-3') containing an added Sp6 restriction site, which correspond to nucleotides 784–808 and 1922–1937, respectively, in Mac-2-BP cDNA (10). The PCR reaction was run for 50 cycles at 94°C for 1 min, 60°C for 1 min, and 72°C for 2 min. The PCR product was cloned into pCR vector from TA cloning kit (Invitrogen, San Diego, CA). The identity of the PCR product was confirmed by restriction enzyme and sequencing analyses. The cDNA encoding human galectin-3 has been described previously (5).

**Northern Blotting.** Ten µg of total RNA was fractionated by electrophoresis through 1% agarose gels containing 17% formaldehyde and blotted onto a nitrocellulose membrane. The blots were prehybridized overnight at 42°C in 50% formamide, 5% dextran sulfate, 5× Denhardt’s solution, 0.05 µM sodium phosphate (pH 7.0), 5× SSC (1× SSC containing 0.15 M NaCl, 0.015 M sodium citrate), and 300 µg/ml denatured salmon sperm DNA. Hybridization was performed overnight at 42°C with random primed 32P-labeled cDNA probes prepared with the hybridization probe labeling system (DuPont, Boston, MA). The membrane was washed at 57°C with 2× SSC, 0.1% SDS for 30 min, 0.2× SSC, 0.1% SDS for 30 min, and 0.1% SDS for 15 min and autoradiographed.

**RESULTS**

**Cellular Localization of Galectin-3 and Mac-2-BP.** Mac-2-BP was originally identified by its ability to coprecipitate with galectin-3 using anti-galectin-3 antibodies (6). Our previous work further substantiated the idea that Mac-2-BP binds to galectin-3 through carbohydrate recognition (9, 10). Taken together, the results show that soluble Mac-2-BP can interact with galectin-3 and that Mac-2-BP may be designated as a ligand of galectin-3. The following experiments address whether Mac-2-BP is a functional ligand for the endogenous galectin-3.

Fig. 1A depicts a double indirect immunofluorescence staining of galectin-3 and Mac-2-BP in A375 melanoma cells. In permeabilized cells, galectin-3 molecules were diffusely distributed in the cytoplasm (Fig. 1A, a), and Mac-2-BP exhibited a similar distribution pattern in the cytoplasm (Fig. 1A, b), suggesting a possible colocalization of the two molecules. The possibility that anti-Mac-2-BP in the cytoplasm cross-reacted with some component in NDS, resulting in a false positive immunofluorescent labeling, can be ruled out since: (a) anti-Mac-2-BP did not react with any component of NDS when analyzed by immunoblotting; and (b) when NDS was replaced with BSA, the same cytoplasmic distribution pattern of Mac-2-BP in A375 cells was obtained. When the cell surface distribution of the two antigens was studied, an intense labeling of galectin-3 as microclusters was observed as previously described for other cells (17) with no obvious labeling of Mac-2-BP (Fig. 1A, c and d). Of note, the failure to detect the endogenous cell surface Mac-2-BP is due to the extensive washing of the cell cultures prior to the exposure to the antibodies, which has resulted from its removal from the cell surface. Labeling for Mac-2-BP could be observed in cells that did not undergo the washing cycles; however, the enormous fluorescence background resulting from such a procedure did not permit the submission of an adequate pictorial demonstration of the endogenous Mac-2-BP cell surface expression.

To ascertain the possible association of the two proteins in the cytoplasm, cross-linking experiments were done, followed by immunodetection analyses. In these studies, we considered that sensitivity of the anti-Mac-2-BP antibody is markedly augmented by reducing conditions and that Mac-2-BP has an apparent native molecular weight of several million (10). Thus, we have used membrane permeable, thiol-cleavable cross-linking DSP for chemical cross-linking and anti-Mac-2-BP and anti-galectin-3 antibodies for immunoprecipitation and Western analysis, respectively. In untreated cells, a galectin-3 was coimmunoprecipitated with Mac-2-BP by the anti-Mac-2-BP antibodies (Fig. 1B), whereas no coimmunoprecipitation was observed when lactose (the sugar competitor of carbohydrate binding by galectin-3) was added to the reaction mixture (Fig. 1B). When cells treated with DSP cross-linker were examined, no alteration in the M₈, detected and lactose again inhibited the coimmunoprecipitation of the two proteins (Fig. 1B). Thus, taken together, the immunolocalization and the cross-linking experiments suggest that the two molecules are not natively complexed together in the cytoplasm, although they probably reside in the same cellular compartment. Furthermore, when the cells were treated with a membrane permeable, uncleavable crosslinking DSP followed by immunoblotting analysis using anti-galectin-3 antibody, no molecules other than the species of M₈, 31,000 was detected (Fig. 1C).

**Galectin-3 and Mac-2-BP Interaction.** Next, we examined the possible interaction between galectin-3 and Mac-2-BP on the cell...
GALECTIN-3, MAC-2-BP, AND TUMOR CELL EMBOLIZATION

A

Cross-Link None DSP

Lactose - + - + - +

Fig. 1. A, double indirect immunofluorescence staining of galectin-3 and Mac-2-BP in A375 cells. Fixed and permeabilized (a and b) or fixed (c and d) cells were incubated with rat anti-galectin-3 (a and c) and rabbit anti-Mac-2-BP (b and d) followed by visualization with β-phycocerythrin-conjugated F(ab')2 fragment of donkey antirat IgG and FITC-conjugated donkey antirabbit IgG, respectively. B, chemical cross-linking analysis followed by immunoprecipitation and immunoblotting. A375 cells treated or untreated with 1 mM DSP, a thiol-cleavable permeating cross-linker, were lysed in the presence or absence of 50 mM lactose followed by immunoprecipitation with rabbit anti-Mac-2-BP. Immunoprecipitates were subjected to reducing SDS-PAGE on 4–20% gradient gels probed with rat anti-galectin-3 followed by treatment with 125I-labeled sheep antirat IgG and visualized by autoradiography. C, chemical cross-linking analysis followed by immunoblotting. A375 cells were treated with 1 mM DSS, an uncleavable permeating cross-linker, lysed, and subjected to SDS-PAGE on 4–20% gradient gels followed by immunoblotting with rat anti-galectin-3 as described above. Migration positions of molecular mass markers on the left.

B

C

Cross-Link None DSP

surface. A375 cells were incubated with affinity purified Mac-2-BP concentration of 10 µg/ml for 1 h, followed by double indirect immunofluorescence staining analysis of both galectin-3 and Mac-2-BP. The analysis revealed extensive colocalization of the two molecules on the cell surface (Fig. 2, a and b). Moreover, addition of lactose completely blocked Mac-2-BP from binding to the cell surface (Fig. 2d), but did not affect the surface distribution of galectin-3 (Fig. 2e). These results suggest that secreted Mac-2-BP molecules may bind to cell surface galectin-3 molecules through a carbohydrate recognition mechanism.

We have recently shown that cell surface galectin-3 is involved in a complementary serum glycoprotein-induced homotypic cell aggregation (18), whereas Mac-2-BP is a component of serum (10, 11, 14–16). Therefore, it was of interest to test whether Mac-2-BP can mediate homotypic cell aggregation by bridging surface galectin-3 molecules on adjacent cells. As shown in Fig. 3A, a, A375 cells formed aggregates in the presence of Mac-2-BP, whereas without it, most of the cells remained as single cells in suspension (Fig. 3A, b), and Mac-2-BP induction of cell-cell aggregation process was both time- and dose-dependent (Fig. 3, B and C). Lactose, the competitive disaccharide, markedly inhibited the cellular interactions, whereas sucrose, a control disaccharide of similar size, was ineffective (Fig. 3D). Moreover, monovalent Fab' fragments of rabbit IgG antibody directed against a synthetic peptide of the carbohydrate-binding domain of galectin-3 significantly inhibited cell aggregation, whereas Fab' fragments of nonspecific control rabbit IgG were without effect (Fig. 3D). These results suggest that secreted Mac-2-BP may interact and bind with galectin-3 molecules on the surface of adjacent cells, leading to melanoma cell homotypic aggregation.

Expression of Galectin-3 and Mac-2-BP. The above observations raised the question of whether the expression of galectin-3 is correlated to that of Mac-2-BP. To study this we took advantage of a panel of human breast cancer cell lines, including galectin-3 null expressors (17). As shown in the Northern analysis of Fig. 4, galectin-3 mRNA was not transcribed in SK-BR-3 and BT-549, whereas all of the human breast cancer cell lines, including the two null galectin-3 lines, expressed varying amounts of Mac-2-BP mRNA. The expression of β-actin, which was used as a control for the amount and integrity of loaded RNA, exhibited no significant difference in (data not shown). These results imply that the expression of galectin-3 and Mac-2-BP is independently regulated.

DISCUSSION

The results presented have established (a) that galectin-3 and Mac-2-BP are colocalized in tumor cell cytoplasm, although they are apparently not complexed; and (b) that Mac-2-BP mediates cell-cell adhesion via bridging galectin-3 molecules on adjacent cells in a carbohydrate-dependent manner and that transcription of galectin-3 and Mac-2-BP mRNAs is independently regulated.

Gaalctin-3 is presumed to be involved in multiple biological processes, such as cell growth, differentiation, inflammation, transformation, and metastasis (1–5, 7, 8, 19), which may reflect its ability to interact with a diversity of complementary glycoconjugates. To date,
examined the Mac-2-BP glycoprotein as the serum candidate for galectin-3 and is normally present in serum (6, 9-13). Mac-2-BP, also a galectin-3 ligand in mediating tumor cell embolization in vivo. The functional relevance of these glycoforms as native ligands of galectin-3 awaits further analysis; in this context, the Mac-2-BP-galectin-3 relationship was analyzed here.

Recently, we have provided functional evidence that cell surface galectin-3 mediates homotypic cell adhesion (18). This and other studies are usually involved allo-glycoprotein-like asialofetuin or Mac-2-BP-galectin-3 relationship was analyzed here.

Fig. 3. Mac-2-BP-induced homotypic aggregation of A375 cells. A, phase-contrast photographs of homotypic cell aggregation in the presence (a) and absence (b) of 20 μg/ml of Mac-2-BP. ×200. B, concentration dependence of Mac-2-BP-induced homotypic cell aggregation. The cells were agitated for 1 h in the presence of varying concentrations of Mac-2-BP, and the extent of aggregation was determined as described in "Materials and Methods." C, time course of Mac-2-BP-induced homotypic cell aggregation. A375 cells were allowed to aggregate for the indicated times with agitation in the presence or absence of Mac-2-BP. D, inhibition of Mac-2-BP-induced homotypic cell aggregation by lactose and anti-galectin-3 antibody. The cells were agitated for 1 h in the presence of 20 μg/ml of Mac-2-BP with or without added Fab' fragments of rabbit anti-galectin-3 (100 μg/ml), Fab' fragments of normal rabbit IgG (100 μg/ml), lactose (50 mM), or sucrose (50 μM). Columns, mean of three different experiments; bars, SD. *, statistically significant differences from control; P < 0.05; t-test.

a spectrum of glycosylated molecules, including laminin (20, 21), lysosome-associated membrane proteins (9, 22), Mac-2-BP (6, 9, 10), carcinoembryonic antigen (8), IgE (23), and certain lipopolysaccharides (24), have been suggested as putative ligands of galectin-3. However, the functional relevance of these glycoforms as native ligands of galectin-3 awaits further analysis; in this context, the Mac-2-BP-galectin-3 relationship was analyzed here.

Recently, we have provided functional evidence that cell surface galectin-3 mediates homotypic cell adhesion (18). This and other studies are usually involved allo-glycoprotein-like asialofetuin or whole syngeneic serum (17, 19, 25), and the serum component(s) involved in the aggregation process has not been identified. Here, we examined the Mac-2-BP glycoprotein as the serum candidate for galectin-3 ligand in mediating tumor cell embolization in vivo.

Mac-2-BP has been recently identified as a secreted ligand for galectin-3 and is normally present in serum (6, 9-13). Mac-2-BP, also known as tumor-associated antigen 90k, was shown to be elevated in the serum of some cancer patients and to reflect the progression of various malignant disease (11, 14-16). Similarly, in human colon cancer, a correlation was found between the level of galectin-3 and the stage of tumor progression (26, 27). The tissue levels of galectin-3 are higher in certain primary gastric cancers compared with adjacent normal mucosa and to correlate with metastasis (28). More recently, it was found that galectin-3 is a marker for the human anaplastic large-cell lymphoma disease (29). The introduction of recombinant galectin-3 into null-expressing, nontumorigenic BT-549 cells resulted in the acquisition of anchorage-independent growth properties and tumorigenicity in nude mice (19). Thus, we question whether the expressions of the two proteins are coordinately regulated. To address this, we took advantage of the recent finding that some human breast carcinoma cells express galectin-3 whereas others do not (17). The results showed that the transcriptions of the two genes are differentially regulated, since BT-549 and SK-BR-3 cells that do not express galectin-3 do express significant levels of Mac-2-BP. The finding that galectin-3 and Mac-2-BP are not complexed in the cytoplasm implies that their transport to and through the cell membranes is also independently regulated.

Fig. 4. Galectin-3 and Mac-2-BP transcripts in five human breast cancer cell lines. Ten μg of total cellular RNA was fractionated by electrophoresis on 1% denaturing formaldehyde agarose gels, transferred onto nitrocellulose membranes, and hybridized with galectin-3 or Mac-2-BP cDNA probes randomly labeled with 32P. The 1.2-kb band for galectin-3 and the 2.2-kb band for Mac-2-BP mRNA were detected.
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Hidenori Inohara, Shiro Akahani, Kirston Koths, et al.


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