Biochimica et Biophysica Acta 1840 (2014) 1790–1797

Contents lists available at ScienceDirect

Biochimica et Biophysica Acta

journal homepage: www.elsevier.com/locate/bbagen

Galectin-3 binds to MUC1-N-terminal domain and triggers recruitment of β-catenin in MUC1-expressing mouse 3T3 cells

Shuhei Tanida, Yugo Mori, Akiko Ishida, Kaoru Akita, Hiroshi Nakada *

Department of Molecular Bioscience, Faculty of Life Science, Kyoto Sangyo University, Kamigamo-Motoyama, Kita-ku, Kyoto 603-8555, Japan

ARTICLE INFO

Article history:
Received 22 June 2013
Received in revised form 3 February 2014
Accepted 7 February 2014
Available online 18 February 2014

Keywords:
MUC1
Galectin-3
β-Catenin

ABSTRACT

Background: Galectin-3 is expressed in a variety of tumors and its expression level is related with tumor progression. Aberrant expression of MUC1 in various tumors is also associated with a poor prognosis. It has been reported that MUC1 is a natural ligand of galectin-3.
Methods: A stable MUC1 transfectant was produced by introducing MUC1 cDNA into mouse 3T3 fibroblasts (MUC1/3T3 cells). MUC1 was prepared from MUC1/3T3 cells; MUC1-N-terminal domain (MUC1-ND) and -C-terminal domain (MUC1-CD) were separated by CsCl ultracentrifugation, and then the galectin-3-binding domain was determined by co-immunoprecipitation assay. After ligation of galectin-3 to 3T3/MUC1 cells, MUC1-CD was immunoprecipitated from the cell lysate. The immunoprecipitate was subjected to SDS-PAGE and Western blotting, followed by detection of co-immunoprecipitated β-catenin.
Results: Galectin-3 binds to the N-terminal domain of MUC1 but not to the C-terminal one. Galectin-3 present on the cell surface increased with the expression of MUC1 and is colocalized with MUC1. It should be noted that β-catenin was detected in the immunoprecipitate with anti-MUC1-CD Ab from a lysate of galectin-3-treated 3T3/MUC1 cells.
Conclusions: Galectin-3 binds to MUC1-ND and triggers MUC1-mediated signaling in 3T3/MUC1 cells, leading to recruitment of β-catenin to MUC1-CD.
General significance: This signaling may be another MUC1-mediated pathway and function in parallel with a growth factor-dependent MUC1-mediated pathway.

1. Introduction

Galectin-3 is expressed in a variety of tumors and its expression level is related with tumor progression [1,2]. The effects of endogenous galectins on tumor progression seem to depend on the subcellular localization of the protein. Galectin-3 is widely distributed in different types of cells and tissues, and is found intracellularly in the nucleus and cytoplasm or is secreted via a non-classical pathway outside of cell, thus being found on the cell surface or in the extracellular space [3,4]. Extracellular galectins bind to a wide array of glycoproteins and glycolipids on the cell surface and in the extracellular matrix. By binding to these glycoconjugates, galectins can deliver signals intracellularly as well as mediate cell–cell and cell–matrix adhesion [2,5–7]. The effect of exogenously added galectin-3 on cell behavior such as proliferation differs among cell types, probably due to differences in the ligand glycoproteins to which the galectin binds.

MUC1 is a transmembrane mucin glycoprotein that is overexpressed and aberrantly glycosylated in many types of epithelial tumor cells, and it has been reported that an elevated level of MUC1 protein has been associated with a poor prognosis [8,9]. The MUC1 protein is translated as a single polypeptide, which is cleaved in the endoplasmic reticulum, yielding N- and C-terminal subunits that form a heterodimeric complex held together through non-covalent interactions [10,11]. The N-terminal ectodomain (MUC1-ND) consists of variable numbers of 20 amino acid tandem repeats that are extensively O-glycosylated [12]. The C-terminal domain (MUC1-CD) anchors MUC1-ND to the cell surface. MUC1-ND has an extended structure because it carries a large number of sialylated O-glycans. This extended structure is expected to act as a particularly effective scaffold for the presentation of oligosaccharide chains to lectins.

It has been reported that MUC1 is a natural ligand of endogenous galectin-3 [13–16]. Many studies have focused on the binding of galectin-3 to MUC1 and the resultant effect on cell behavior such as cell adhesion. Thus, it has been demonstrated that the galectin-3-MUC1 interaction induces the polarization of MUC1 on the cell surface and the exposure of cell adhesion molecules, facilitating homolytic aggregation of tumor cells and adhesion of cancer cells to endothelial cells [13,15,16]. With respect to galectin-3-binding domain, however, opposing data have been reported with respect to its binding domain. Yu et al. reported that recombinant galectin-3 interacts with the TF antigen expressed on MUC1-ND, facilitating the adhesion of epithelial

* Corresponding author. Tel./fax: +81 75 705 1888.
E-mail address: hnakada@cc.kyoto-su.ac.jp (H. Nakada).

http://dx.doi.org/10.1016/j.bbagen.2014.02.008
0304-4165/

cancer cells to the endothelium [13]. In contrast, Ramasamy et al. demonstrated that galectin-3 binds to N-glycans expressed on MUC1-CD, facilitating the interaction with receptors for growth factors [14]. In addition, many studies on MUC1-mediated signaling, which is initiated by growth factors, have been reported using epithelial tumor cells [17–20], and fibroblasts transfected with MUC1cDNA [8,21]. These studies have revealed that an FGF-triggered signal is mediated through MUC1 in MUC1cDNA-transfected fibroblasts like that initiated by EGF in epithelial tumor cells. Thus, we speculated that we could elucidate the function of MUC1 more clearly using 3T3/MUC1 cells lacking tumor associated genetic and epigenetic changes. These studies also demonstrated that MUC1-CD serves as a scaffold protein, enabling interactions between different regulators such as β-catenin. Dysregulation of β-catenin is known to be of great importance to the development of diverse human malignancies [17].

In the present study, we demonstrated that galectin-3 bound to MUC1-ND, but not to MUC1-CD, and that this direct binding of galectin-3 to MUC1 also triggered the recruitment of β-catenin to MUC1-CD in 3T3/MUC1 cells.

2. Materials and methods

2.1. Cells

Mouse NIH3T3 cells were obtained from the American Type Culture Collection. 3T3/MUC1 cells were prepared by introducing MUC1cDNA into mouse 3T3 fibroblasts as described previously [22], and 3T3 mock cells were prepared by transfection of the empty vector. 3T3/mock and 3T3/MUC1 cells were cultured in RPMI1640 containing 2 mM L-glutamine and 10% fetal calf serum.

2.2. Preparation of MUC1-ND and -CD

3T3/MUC1 cells (4.5×10^7 cells) were labeled with biotin according to the manufacturer's instructions. The cells were solubilized with a solubilizing solution (1% NP-40, 10 mM Tris–HCl; pH 8.0, 0.14 M NaCl, 1 mM EDTA, 1 mM PMSF and protease inhibitor cocktail (Nacalai tesque) and centrifuged at 14,000 ×*g* for 20 min. The supernatant was subjected to gel filtration on Sepharose™ CL-6B (2.8 × 91 cm; GE-Healthcare), and eluted with a gel filtration solution (25 mM Tris–HCl; pH 7.5, 0.15 M NaCl, 0.5% Triton® X-100), fractions of 8 ml being collected. Aliquots were dot-blotted onto a Zeta-Probe Blotting membrane (Bio-Rad) and biotin-labeled proteins were detected with HRP-streptavidin. The density was determined using Image J software (NIH) to reveal the elution pattern. The excluded fractions containing MUC1 were pooled and subjected to CsCl ultracentrifugation (36,000 rpm, 72 h; HITACHI, P40ST rotor) in the presence of 4 M guanidine–HCl. Aliquots (24 fractions) were dotted onto a Zeta-Probe Blotting membrane (BioRad), and MUC1-ND and -CD were detected with mouse anti-MUC1-ND Ab (BD Pharmingen™) and Armenian hamster anti-MUC1-CD Ab5 (Neo Marker), respectively.

2.3. Immunostaining

Immunostaining of the cell surface was performed as follows. After washing of 3T3/MUC1 and 3T3/mock cells (5×10^3 cell) with cold PBS, the cells were treated with PBS containing 10% goat serum, and then with mouse anti-MUC1-ND Ab, goat anti-galectin-3 Ab (Santa Cruz) or a control IgG in PBS containing 1% BSA at 4 °C for 2 h. After washing with cold PBS, the cells were stained with Alexa Fluor®-488 labeled rabbit anti-mouse IgG Ab or Alexa Fluor®-594 labeled rabbit anti-goat IgG Ab (Molecular Probe™) at 4 °C for 1 h. After treatment with PBS containing 4% paraformaldehyde, the nucleus was stained with diamidino-2-phenylindole (DAPI). Slides were mounted using ProLong® Gold Antifade Reagents (Invitrogen) and observed under a confocal fluorescence microscope (TCS SPE, Leica microsystems).

When the cells were immunostained intracellularly, they were fixed with PBS containing 4% paraformaldehyde, and then treated with PBS containing 10% goat serum and 0.1% Triton® X-100, followed by treatment with primary Abs as described above in PBS containing 1% BSA

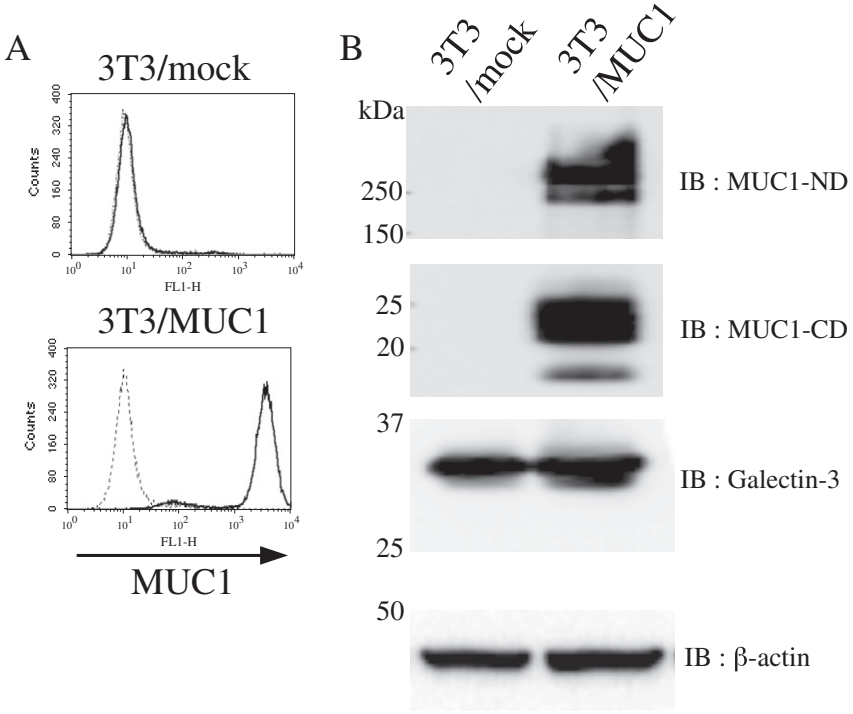

Fig. 1. Expression of MUC1 and galectin-3 in 3T3/MUC1 and 3T3/mock cells. A. 3T3/MUC1 and 3T3/mock cells were treated with anti-MUC1-ND Ab and then with FITC-labeled rabbit anti-mouse IgG Ab (——), and analyzed by flow cytometry. A control experiment was performed using a control mouse IgG Ab (– –). B. 3T3/MUC1 and 3T3/mock cells were solubilized and the lysates were subjected to SDS-PAGE and Western blotting, followed by detection with anti-MUC1-ND, anti-MUC1-CD, anti-galectin-3, and anti-β-actin Abs. Experiments were performed in duplicate and representative data are shown.

and 0.1% Triton® X-100 at room temperature for 2 h. After washing with PBS, the cells were stained with secondary Abs and DAPI, and the mounted slides were observed as described above.

2.4. Flow cytometry

3T3/MUC1 and 3T3/mock cells (1×10^6 cells) were treated with mouse anti-MUC1-ND Ab or a control mouse IgG in PBS containing 0.5% BSA at 4 °C for 1 h. After washing with PBS containing 0.5% BSA, the cells were incubated with FITC-labeled rabbit anti-mouse IgG Ab (Zymed) and analyzed with a FACS Calibur™ (Becton Dickinson).

2.5. Immunoprecipitation

3T3/MUC1 and 3T3/mock cells were solubilized with a solubilizing solution as described above. MUC1-CD and β-catenin were immunoprecipitated from the lysates (500 μg) by incubation with Armenian hamster anti-MUC1-CD Ab and mouse anti-β-catenin Ab, respectively, and then with Protein G-Sepharose™ 4 Fast Flow (GE Healthcare).

Immunoprecipitation of galectin-3 was also performed by incubation with goat anti-galectin-3 Ab, and then with PureProteome™ Protein G Magnetic Beads (MILLIPORE).

2.6. N-Glycanase, O-glycanase, and O-glycanase and neuraminidase treatment

Lysates (250 μg) of 3T3/MUC1 cells were treated with 10 mU of Glycopeptidase F (TaKaRa) at 37 °C for 17 h in 100 mM Tris–HCl; pH 8.6. The MUC1-ND fraction (10 μg) prepared as described above was treated with 2×10^5 U of O-glycanase (BioLabs inc.) or with 2×10^5 U of O-glycanase and 50 mU of neuraminidase (*Arthrobacter ureafaciens*, Nacalai tesque) at 37 °C for 17 h in 0.25% SDS, 1% NP-40, 20 mM DTT, and 50 mM sodium phosphate; pH 7.5.

2.7. Cell surface labeling with biotin

Cells were incubated in PBS containing 0.1 mg/ml EZ-Link® Sulfo-NHS-Biotin (Thermo) at 4 °C for 1 h. After washing with PBS containing 0.1 M glycine, the cells were dissolved with a solubilizing solution.

2.8. Western blot analyses

Lysates of 3T3/MUC1 and 3T3/mock cells, and immunoprecipitates were subjected to SDS-PAGE, followed by Western blotting using Immuno-Blot™ PVDF membranes (Bio-Rad), except for the detection

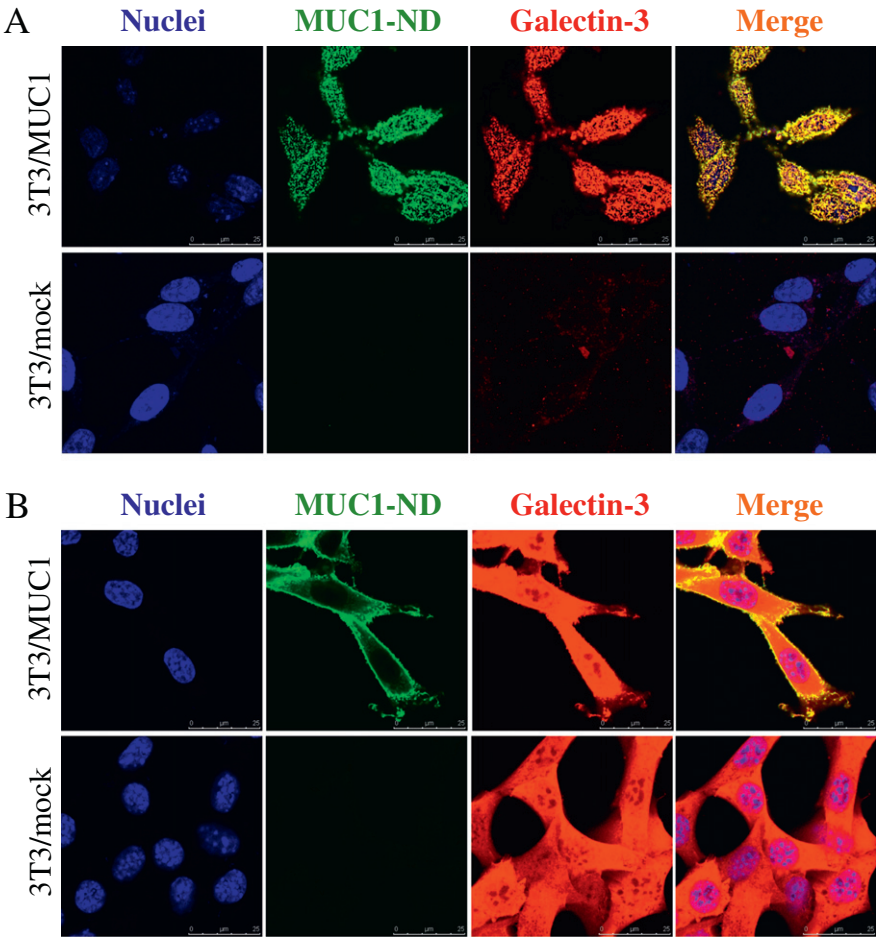

Fig. 2. Distribution of galectin-3 and MUC1 in 3T3/MUC1 and 3T3/mock cells. A. 3T3/MUC1 and 3T3/mock cells were stained immunochemically using the combinations of mouse anti-MUC1-ND and Alexa Fluor®-488 labeled rabbit anti-mouse IgG Ab, and goat anti-galectin-3 Ab and Alexa Fluor®-594 labeled rabbit anti-goat IgG Ab. B. 3T3/MUC1 and 3T3/mock cells were treated with PBS containing 10% goat serum and 0.1% Triton® X-100, and then stained immunochemically as described above.

of MUC1-ND. In the case of MUC1-ND, Zeta-Probe Blotting membranes were used.

Detection of MUC1-ND, β-catenin and β-actin was performed using mouse anti-MUC1-ND, anti-β-catenin and anti-β-actin Abs, and subsequently HRP-labeled rabbit anti-mouse IgG Ab (Zymed). The intensity of bands was determined using Image J software (NIH).

2.9. Detection of galectin-3 chemically cross-linked with MUC1 on the surface of 3T3/MUC1 cells

3T3/MUC1 cells (2×10^7 cells) were incubated with recombinant galectin-3 (1 μg/ml) at 4 °C for 30 min. After washing with PBS, the cells were treated at 4 °C for 1 h with a chemical cross-linking reagent, DTSSP [3,3′-dithiobis(sulfosuccinimidylpropionate)] (Thermo Fisher Scientific), which includes a disulfide bond. After washing with PBS containing 50 mM glycine, the cells were solubilized and subjected to CsCl ultracentrifugation, and then the MUC1-ND and -CD positive fractions were separated as described above. After dialysis of the pooled MUC1-ND and -CD positive fractions against a solubilizing solution, MUC1-ND and -CD were immunoprecipitated from the respective fractions and subjected to SDS-PAGE under reduced conditions, followed by detection of galectin-3.

2.10. Statistical analysis

Student's test was used to determine the significance of differences between sample means.

3. Results and discussion

3.1. Expression of MUC1 and galectin-3 in 3T3/MUC1 cells

MUC1cDNA was introduced into mouse 3T3 fibroblasts and stable transformants were obtained. Expression of MUC1 on the surface of 3T3/MUC1 cells was confirmed by flow cytometry. There was no detectable MUC1 expression on cells transfected with the empty vector (Fig. 1A). A cell lysate was subjected to SDS-PAGE, followed by Western blotting, and detection with anti-MUC1-ND and -CD Abs, and anti-galectin-3 Ab. For 3T3/MUC1 cells, a major band of MUC1-ND was detected at the position corresponding to about 250 kDa, and MUC1-CD gave several bands corresponding to molecular weights of 15 kDa to 25 kDa, which is considered to be due to different levels of N-glycosylation, as previously described [23]. It has been reported that MUC1 induces the expression of galectin-3, and galectin-3 also positively controls MUC1 expression in pancreatic cancer cells [24]. The level of galectin-3 in 3T3/MUC1 cells was slightly higher than that in 3T3/mock cells. One possibility may be due to the binding of galectin-3 to MUC1 on the surface of 3T3/MUC1 cells (Fig. 1B).

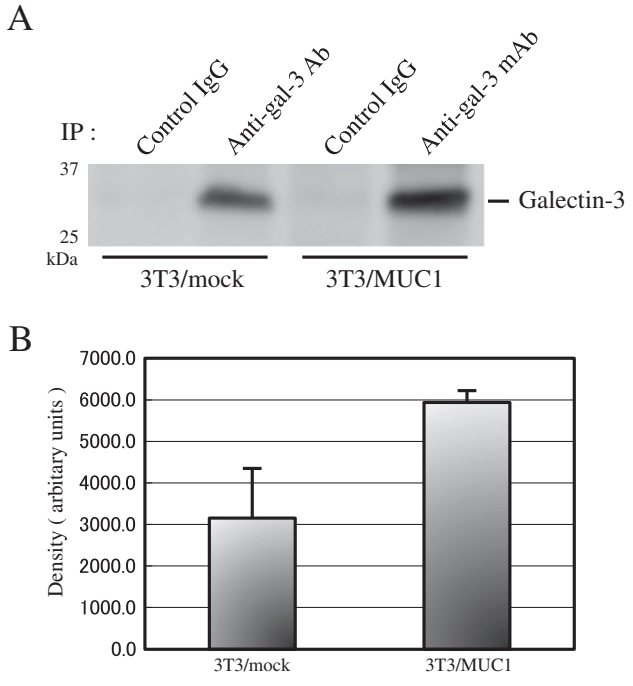

Fig. 3. Detection of cell surface galectin-3. A. 3T3/MUC1 and 3T3/mock cells were labeled with biotin as described under Materials and methods, and the immunoprecipitate obtained from the lysate with anti-galectin-3 Ab was subjected to SDS-PAGE and Western blotting, followed by detection with HRP-streptavidin. Experiments were performed in triplicate and representative data were shown. B. The intensity of the bands was determined using Image J software (NIH).

3.2. Distribution of galectin-3 and MUC1 on the surface of 3T3/MUC1 cells

Galectins are present both inside and outside the cells and function both intracellularly and extracellularly. It seems likely that extracellular galectins can bind to cell surface glycoconjugates bearing suitable galactose oligosaccharides. The distributions of MUC1 and galectin-3 on the surface of 3T3/MUC1 and 3T3/mock cells were observed using anti-MUC1-ND Ab and anti-galectin-3 Ab, and the corresponding fluorescein-labeled secondary Ab. MUC-1 was completely co-localized with galectin-3 (Fig. 2A). Therefore, it is speculated that ligation of

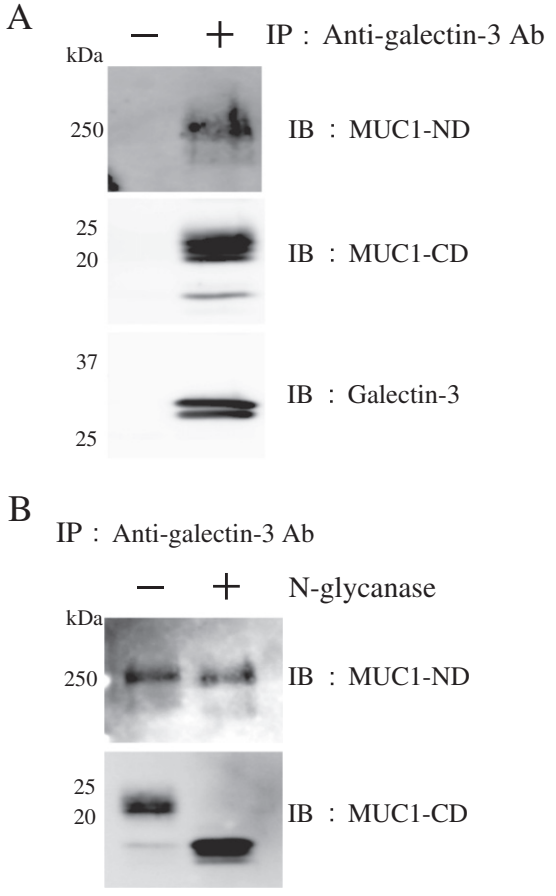

Fig. 4. Binding of galectin-3 to MUC1. A. Anti-galectin-3 Ab (1 μg/ml) was added to a lysate of 3T3/MUC1 cells and the immunoprecipitate was subjected to SDS-PAGE and Western blotting, followed by detection with anti-MUC1-ND, anti-MUC1-CD and anti-galectin-3 Abs. Experiments were performed in duplicates and representative data are shown. B. Lysates (250 μg) of 3T3/MUC1 cells were treated with N-glycanase as described under Materials and methods. MUC1-ND and MUC1-CD were detected as described in A. Experiments were performed in duplicates and representative data are shown.

galectin-3 may initiate the signaling through MUC1 like other cross-linked cell surface proteins. A low level of galectin-3 was detected on the surface of 3T3/mock cells compared with that on 3T3/MUC1 cells. It seems likely that N- and/or O-glycans borne on the MUC1 core proteins may be preferable ligands for galectin-3. Thus, galectin-3 seems to be retarded on the cell surface through its binding to MUC1. Cytoplasmic galectin-3 was also observed in 0.1% Triton-X 100-treated 3T3/MUC1 and 3T3/mock cells. Similar levels of galectin-3 were detected in the cytoplasm of both cells (Fig. 2B).

3.3. Level of cell surface galectin-3

To determine the level of galectin-3 present on the cell surface, 3T3/MUC1 and 3T3/mock cells were treated with biotin as described under Materials and methods. From the cell lysates, galectin-3 was immunoprecipitated and subjected to SDS-PAGE, followed by Western blotting and detection of galectin-3 with HRP-streptavidin. The level of galectin-3 on the surface of 3T3/MUC1 cells was about twice that on 3T3/mock cells, suggesting that galectin-3 was retained on the cell surface through binding to MUC1 (Fig. 3A,B).

3.4. Binding of galectin-3 to MUC1-ND

With respect to the binding domain of galectin-3, different results have been reported. Galectin-3 can interact either with O-glycans branched on MUC1-ND [13] or with N-glycans on MUC1-CD [14]. To determine which domain galectin-3 can bind to, galectin-3 was immunoprecipitated from lysates of 3T3/MUC1 cells and subjected to SDS-PAGE, followed by Western blotting and detection of co-immunoprecipitated MUC1. As shown in Fig. 4A, MUC1 was co-immunoprecipitated with galectin-3, and both MUC1-ND and -CD were detected, probably due to the tight association of both domains even if galectin-3 bound to either MUC1-ND or -CD. Ramasamy et al. reported that galectin-3 binds to N-glycans carried on MUC1-CD [14]. Thus, a similar experiment was performed after treatment of 3T3/MUC1 cell lysates with N-glycanase. The molecular weight of MUC1-ND was slightly decreased and with respect to MUC1-CD, 20–25 kDa molecular species disappeared and concomitantly a 17 kDa molecule increased, this being consistent with the fact that MUC1-CD contains multiple N-glycans. MUC1-ND and -CD were co-immunoprecipitated with galectin-3 even after N-glycanse treatment, suggesting that at least O-glycans expressed on MUC1-ND may be involved in the binding of galectin-3 (Fig. 4B). However, there is no direct evidence that galectin-3 can bind to MUC1-ND and/or MUC1-CD. To extend this analysis, it is necessary to separate MUC1-ND and -CD. The surface proteins of 3T3/MUC1 cells were labeled with biotin and the lysates were fractionated by gel filtration on Sepharose™ CL-6B. A part of the eluted proteins was blotted onto a membrane and cell surface proteins were detected with HRP-streptavidin (Fig. 5A) and an elution profile based on the density of dot blots is shown in Fig. 5B. The excluded fractions containing MUC1 were pooled, denatured with 4 M guanidine–HCl, and then subjected to CsCl centrifugation (Fig. 5C). MUC1-ND and -CD were detected in each fraction by dot blot analysis as shown in Fig. 5D. Fractions 3–5 (fraction a) and 21–23 (fraction b) containing MUC1-ND and -CD, respectively, were collected and subjected to SDS-PAGE, followed by Western blotting and detection of MUC1-ND and -CD. It was confirmed that fractions a and b exclusively contained MUC1-ND and -CD, respectively (Fig. 5E). MUC1-ND and -CD were incubated with recombinant galectin-3, and then anti-galectin-3 Ab was added. The immunoprecipitates were subjected to SDS-PAGE, followed by Western blotting. As shown in Fig. 6A,B, MUC1-ND but not MUC1-CD was co-precipitated with galectin-3. Furthermore, we analyzed the binding of galectin-3 to MUC1 under natural conditions. After incubation of 3T3/MUC1 cells with recombinant galectin-3 at 4 °C for 30 min and subsequent washing with PBS, cell surface proteins were cross-linked with DTSSP as described under Materials and methods. The cell lysates were subjected

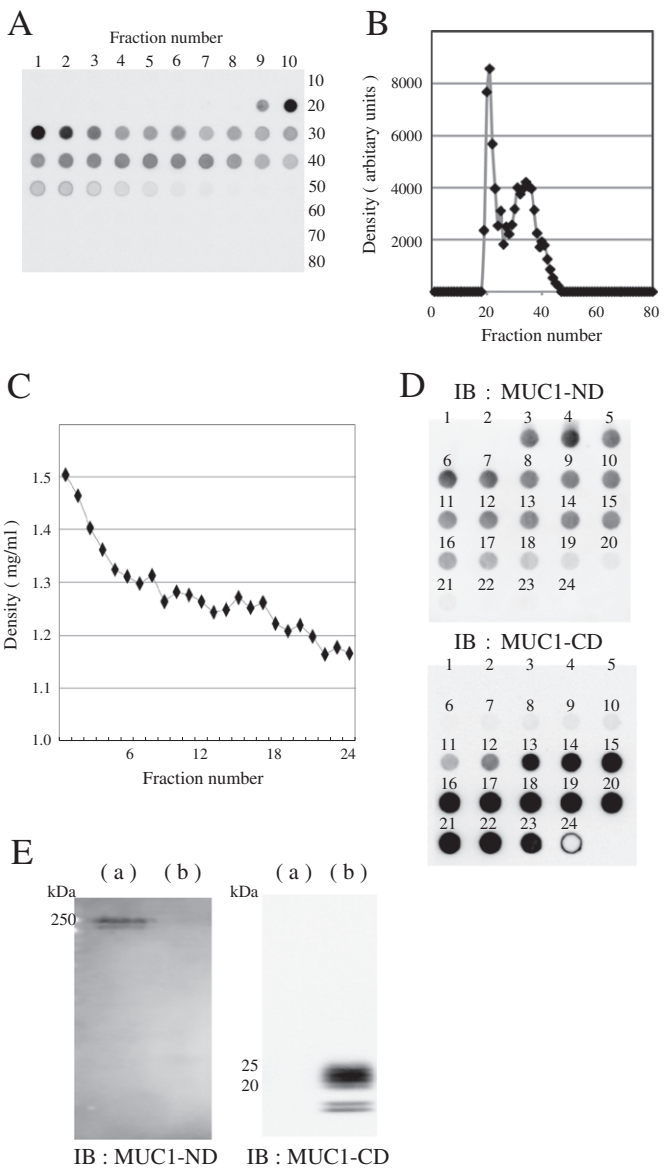

Fig. 5. Isolation of MUC1-ND and -CD. A. 3T3/MUC1 cells (4.5×10^7 cells) were labeled with biotin and solubilized as described under Materials and methods. The lysate was subjected to gel filtration on Sepharose™ CL-6B and aliquots were dot blotted, followed by detection of biotin-labeled proteins with HRP-streptavidin. B. Elution pattern of biotin-labeled proteins is shown according to the density of each dot. C. The excluded fractions (fractions 20 to 25) shown in B were pooled and fractionated according to buoyant density by CsCl ultracentrifugation as described under Materials and methods. The density of each fraction was determined gravimetrically. D. A part of each fraction (24 fractions) was dotted onto a membrane and MUC1-ND and -CD were detected as described under Materials and methods. E. The fractions exhibiting high buoyant density (fractions 3 to 5) and low buoyant density (fractions 21 to 23) were pooled and subjected to SDS-PAGE and Western blotting, followed by detection of MUC1-ND and -CD. (a) Fractions 3 to 5, (b) Fractions 21 to 23.

to CsCl ultracentrifugation, and MUC1-ND and -CD fractions were prepared as described above, and then dialyzed against a solubilizing solution. Separation of MUC1-ND and -CD was confirmed by SDS-PAGE and immunoblotting (Fig. 6C, lanes a, b). MUC1-ND and -CD were immunoprecipitated from the MUC1-ND and -CD positive fractions, respectively, and then subjected to SDS-PAGE, followed by Western blotting and detection of galectin-3. Galectin-3 was detected in the MUC1-ND fraction but not in the MUC1-CD one (Fig. 6C, lanes c, d), indicating that galectin-3 binds to MUC1-ND but not to MUC1-CD on the cell surface. Since MUC1-ND possesses a large number of O-glycans, we examined galectin-3 binding activity after O-glycanase treatment. O-

Glycanase treatment produced a small amount of MUC1-ND with a little lower molecular weight, most MUC1-ND hardly changing on SDS-PAGE (Fig. 6D, lane b). However, the major band of MUC1-ND was abolished and a lower molecular band appeared with neuraminidase and O-glycanase treatment (Fig. 6D, lane c). Binding of galectin-3 to MUC1-ND treated with these enzymes was examined as described above. Treatment of MUC1-ND with O-glycanase apparently had little effect on galectin-3 binding (Fig. 6D, lane e). However, galectin-3 binding activity was almost completely abolished by the treatment with neuraminidase and O-glycanase (Fig. 6D, lane f). Even after O-glycanase treatment, MUC1-ND still possessed galectin-3 binding activity, maybe due to incomplete cleavage by O-glycanase. After removal of sialic acid residues, the cleavage site may be more easily accessible to O-glycanase, resulting in almost complete cleavage of both TF antigens natively expressed on MUC1-ND, and produced from sialo- and disialo-TF antigens by neuraminidase treatment. These results suggest that TF antigen is responsible for the binding of galectin-3.

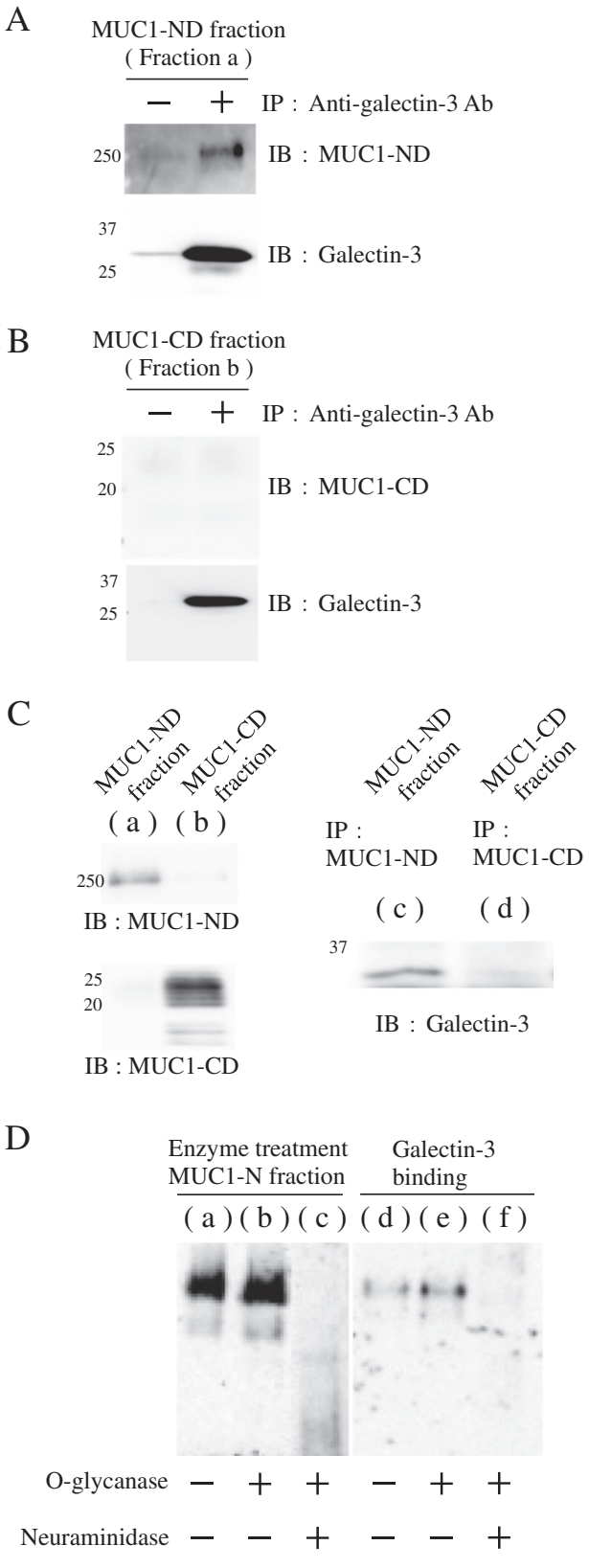

Fig. 6. Binding of galectin-3 to MUC1-ND. A, B. MUC1-ND and -CD (10 μg) separated by CsCl ultracentrifugation were mixed with recombinant galectin-3 (1 μg), and then anti-galectin-3 Ab was added. The proteins co-immunoprecipitated with galectin-3 were subjected to SDS-PAGE and Western blotting followed by detection of MUC1-ND (A) and -CD (B). C. The lysate of 3T3/MUC1 cells, which were treated with galectin-3 and then with DTSSP, was subjected to CsCl ultracentrifugation, and then MUC1-ND and -CD positive fractions were prepared as described under Materials and methods. Separation of MUC1-ND and -CD was confirmed by SDS-PAGE and immunoblotting (lanes a, b). MUC1-ND and -CD were immunoprecipitated from the respective fractions, and then subjected to SDS-PAGE, followed by Western blotting and detection of galectin-3 (lanes c, d). D. MUC1-ND was treated with O-glycanase (lane b), with neuraminidase and O-glycanase (lane c) or without these enzymes (lane a) as described under Materials and methods, and subjected to SDS-PAGE, followed by Western blotting and detection with anti-MUC1-ND Ab. Galectin-3 binding activity of MUC1-ND treated with O-glycanase (lane e), with neuraminidase and O-glycanase (lane f) or without these enzymes (lane d) was examined as described above. Experiments were performed in triplicate and representative data are shown.

3.5. Recruitment of β-catenin to MUC1-CD on treatment of 3T3/MUC1 cells with galectin-3

Extracellular galectins have been implicated in many biological processes such as cell–cell interaction/adhesion and lattice formation [25,26]. As galectin-3 can bind either bivalently or multivalently, it can cross-link cell surface glycoconjugates, which, like many other receptor–ligand systems, can trigger a cascade of transmembrane signaling events, but little is known about the signaling through MUC1 initiated by the direct binding of a ligand including a lectin such as galectin-3. In fact, studies on signaling through MUC1 following direct ligation of MUC1-ND with an endogenous ligand have been limited. Intracellular adhesion molecule (ICAM-1) serves as a ligand for MUC1, activating outside-in signaling via MUC1-CD [27,28]. *Pseudomonas aeruginosa* or its flagellin protein can serve as an activator of MUC1 signaling [29]. It has been reported that MUC1-CD serves as a scaffold protein, enabling interactions between different regulators such as β-catenin [8,17]. Dysregulation of β-catenin is known to be of great importance to the development of diverse human malignancies [17].

First, we examined the levels of β-catenin in 3T3/MUC1 and 3T3/mock cells. Cell lysates of both cells were subjected to SDS-PAGE, followed by Western blotting and detection of β-catenin. Similar levels of β-catenin were detected in these cells (Fig. 7A). To exclude the possible influences of growth factors included in serum and produced by the cells, 3T3/MUC1 cells were pre-cultured in the serum-free RPMI1640 medium for 1 h prior to analysis, and then extensively washed with the same medium containing 30 mM lactose and successively with serum-free RPMI1640 medium just before the treatment with galectin-3. 3T3/MUC1 cells (4.5×10^5 cells) were treated with galectin-3 (10 μg/ml) at 37 °C for 30 min, and MUC1-CD was immunoprecipitated from cell lysates (500 μg) and subjected to SDS-PAGE, followed by Western blotting and detection of co-immunoprecipitaed β-catenin. As shown in Fig. 7B, β-catenin was clearly co-immunoprecipitated with MUC1-CD. In a reciprocal experiment, MUC1-CD was detected in the immunoprecipitate with anti-β-catenin Ab after treatment with galectin-3. Next, we examined the time-dependency of recruitment of β-catenin to MUC1-CD in 3T3/

MUC1 cells. After treatment with galectin-3 for various times, MUC1-CD was immunoprecipitated from cell lysates, followed by SDS-PAGE and immunoblotting (Fig. 7C). The level of recruited β-catenin peaked at 30–40 min and then decreased to the control level at 50 min. c-Src is known to associate physically with MUC1 [8]. We also confirmed that c-Src was present in 3T3/MUC1 cells and a part of it was associated with MUC1-CD (data not shown). The conformational change of MUC1 with the ligation of galectin-3 may stimulate c-Src, maybe elevating the formation of the MUC1-CD-β-catenin complex. It has been reported that MUC1 associates with the EGF or FGF family of growth factor receptor kinases in some human carcinoma cells and mouse mammary gland, and treatment with these growth factors increases the binding of β-catenin to MUC1-CD [8,17,20,30–32]. Since 3T3/MUC1 cells express FGFR-3, a co-immunoprecipitation assay was performed. From a lysate of 3T3/MUC1 cells, MUC1-ND was immunoprecipitated and then subjected to SDS-PAGE, followed by Western blotting. FGFR-3 was not detected in the immunoprecipitate obtained with anti-MUC1-ND Ab (data not shown). Thus, we speculate that this signaling may be another MUC1-mediated pathway that promotes tumor progression even though growth factors such as EGF and FGF or their receptors are absent. The effect of exogenously added galectin-3 on cell growth differs among cell types, probably due to differences in the ligand glycoproteins to which galectin-3 binds [33–36]. Galectin-3 has been shown to bind to several cell surface glycoproteins including β1-integrin [37] and EGF receptor [38] to exert a variety of functions. In fact, Tadokoro et al. reported that ligation of galectin-3 to N-glycans borne on VCAM-1 down-modulates the phosphorylation of ERK1/2, leading to inhibition of proliferation of mouse 3T3 cells [39].

Oligomerization of galectin-3 is necessary for it to be functional. It is generally agreed that galectin-3 oligomerizes at higher concentration or when it binds to multivalent carbohydrates [40–42]. MUC1 seems to be one of the most preferable ligand glycoproteins for oligomerizing galectin-3, because O-glycans, which are the binding sites of galectins, may be expressed repeatedly on the tandem repeats of the MUC1 core protein. Thus, galectin-3 is expected to preferentially bind to MUC1 expressed on many epithelial tumor cells.

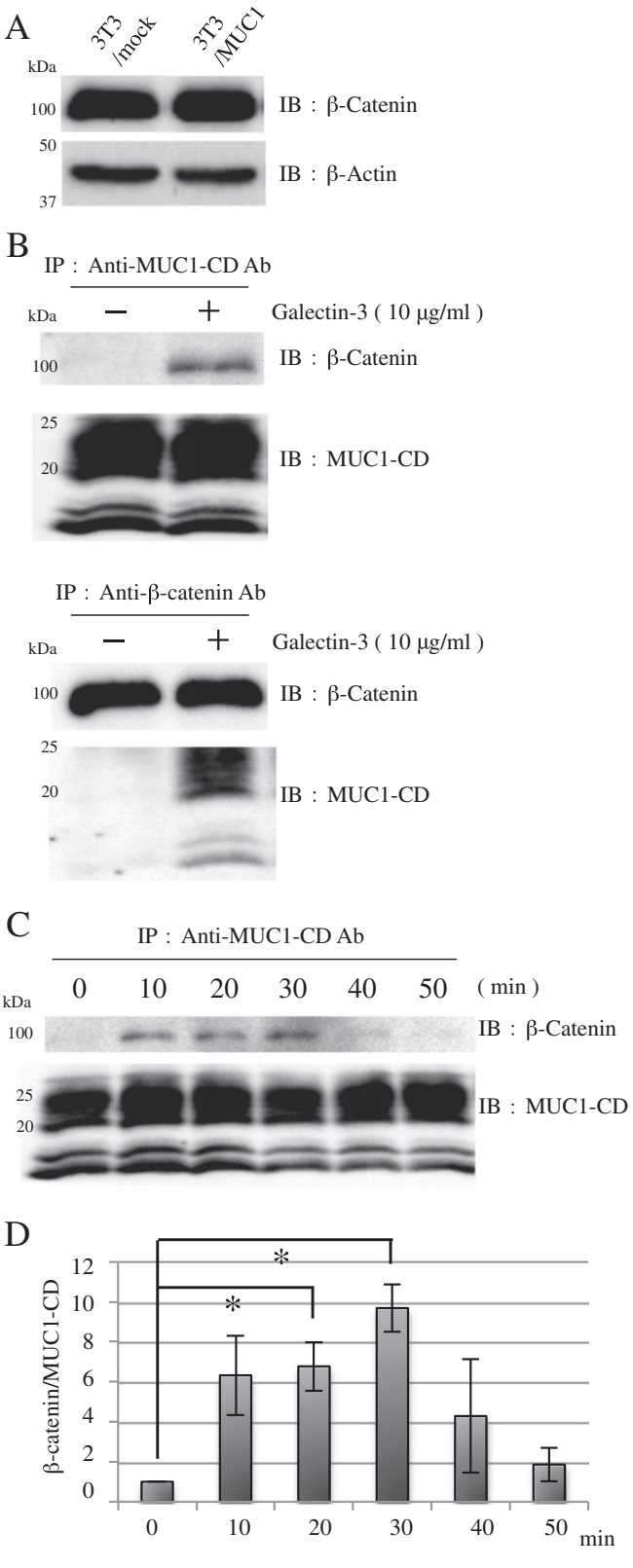

Fig. 7. Recruitment of β-catenin to MUC1-CD on ligation of galectin-3 with MUC1-ND in 3T3/MUC1 cells. A. Lysates of 3T3/MUC1 and 3T3/mock cells were subjected to SDS-PAGE and Western blotting, followed by detection of β-catenin. B. 3T3/MUC1 cells (4.5×10^5 cells) were treated with galectin-3 (10 μg/ml) for 30 min and then MUC1-CD or β-catenin was immunoprecipitated from the lysate with each antibody. The immunoprecipitates were subjected to SDS-PAGE and Western blotting, followed by detection with MUC1-CD and β-catenin. C. MUC1-CD was immunoprecipitated from lysates of 3T3/MUC1 cells treated with galectin-3 for 0–50 min. After SDS-PAGE and Western blotting, co-immunoprecipitated β-catenin was detected. Experiments were performed in triplicate and representative data are shown. D. The histogram shows the relative intensities of the recruited β-catenin in C in which each density was normalized as to MUC1-CD, and the value obtained in the experiment in which 3T3/MUC1 cells were not treated with galectin-3 is taken as 1 (means ± SD, $n = 3$, $^*p < 0.05$).

4. Conclusion

Galectin-3 was colocalized with MUC1 on the surface of mouse 3T3 cells stably transfected with human MUC1 cDNA, and galectin-3 bound to MUC1-ND but not to MUC1-CD. Binding of galectin-3 to MUC1-ND triggered recruitment of β-catenin to MUC1-CD. This galectin-3-triggered MUC1-mediated pathway may function co-operately with a growth factor-triggered pathway.

Acknowledgments

This study was supported by a Private University Strategic Research Foundation Support Program.

References

[1] A. Danguy, I. Camby, R. Kiss, Galectins and cancer, Biochim. Biophys. Acta 1572 (2002) 285–293.
[2] F.T. Liu, G.A. Rabinovich, Galectins as modulators of tumour progression, Nat. Rev. Cancer 5 (2005) 29–41.
[3] S. Sato, R.C. Hughes, Regulation of secretion and surface expression of Mac-2, a galactoside-binding protein of macrophages, J. Biol. Chem. 269 (1994) 4424–4430.
[4] I.K. Moutsatsos, M. Wade, M. Schindler, J.L. Wang, Endogenous lectins from cultured cells: nuclear localization of carbohydrate-binding protein 35 in proliferating 3T3 fibroblasts, Proc. Natl. Acad. Sci. U. S. A. 84 (1987) 6452–6456.
[5] A. Raz, D.G. Zhu, V. Hogan, N. Shah, T. Raz, R. Karkash, G. Pazerini, P. Carmi, Evidence for the role of 34-kDa galactoside-binding lectin in transformation and metastasis, Int. J. Cancer 46 (1990) 871–877.
[6] T. Fukumori, N. Oka, Y. Takenaka, P. Nangia-Makker, E. Elsamman, T. Kasai, M. Shono, H.O. Kanayama, J. Ellerhorst, R. Lotan, A. Raz, Galectin-3 regulates mitochondrial stability and antiapoptotic function in response to anticancer drug in prostate cancer, Cancer Res. 66 (2006) 3114–3119.
[7] J. Dumic, S. Dabelic, M. Flögel, Galectin-3: an open-ended story, Biochim. Biophys. Acta 1760 (2006) 616–635.
[8] Y. Li, D. Liu, D. Chen, S. Kharbanda, D. Kufe, Human DF3/MUC1 carcinoma-associated protein functions as an oncogene, Oncogene 22 (2003) 6107–6110.
[9] M.A. Hollingsworth, B.J. Swanson, Mucins in cancer: protection and control of the cell surface, Nat. Rev. Cancer 4 (2004) 45–60 (2004).
[10] M.J. Ligtenberg, L. Kruijshaar, F. Buijs, M. van Meijer, S.V. Litvinov, J. Hilkens, Cell-associated episialin is a complex containing two proteins derived from a common precursor, J. Biol. Chem. 267 (1992) 6171–6177.
[11] S. Parry, H.S. Silverman, K. McDermott, A. Willis, M.A. Hollingsworth, A. Harris, Identification of MUC1 proteolytic cleavage sites in vivo, Biochem. Biophys. Res. Commun. 283 (2001) 715–720.
[12] J. Siddiqui, M. Abe, D. Hayes, E. Shani, E. Yunis, D. Kufe, Isolation and sequencing of a cDNA coding for the human DF3 breast carcinoma-associated antigen, Proc. Natl. Acad. Sci. U. S. A. 85 (1988) 2320–2323.
[13] L.G. Yu, N. Andrews, Q. Zhao, D. McKean, J.F. Williams, L.J. Connor, O.V. Gerasimenko, J. Hilkens, J. Hirabayashi, K. Kasai, J.M. Rhodes, Galectin-3 interaction with Thomsen-Friedenreich disaccharide on cancer-associated MUC1 causes increased cancer cell endothelial adhesion, J. Biol. Chem. 282 (2007) 773–781.
[14] S. Ramasamy, S. Duraisamy, S. Barbashov, T. Kawano, S. Kharbanda, D. Kufe, The MUC1 and galectin-3 oncoproteins function in a microRNA-dependent regulatory loop, Mol. Cell 27 (2007) 992–1004.
[15] Q. Zhao, M. Barclay, J. Hilkens, X. Guo, H. Barrow, J.M. Rhodes, L.G. Yu, Interaction between circulating galectin-3 and cancer-associated MUC1 enhances tumour cell homotypic aggregation and prevents anoikis, Mol. Cancer 9 (2010) 154.
[16] Q. Zhao, X. Guo, G.B. Nash, P.C. Stone, J. Hilkens, J.M. Rhodes, L.G. Yu, Circulating galectin-3 promotes metastasis by modifying MUC1 localization on cancer cell surface, Cancer Res. 69 (2009) 6799–6806.
[17] L. Huang, D. Chen, D. Liu, L. Yin, S. Kharbanda, D. Kufe, MUC1 oncoprotein blocks glycogen synthase kinase 3beta-mediated phosphorylation and degradation of beta-catenin, Cancer Res. 65 (2005) 10413–10422.
[18] Y. Li, W.H. Yu, J. Ren, W. Chen, L. Huang, S. Kharbanda, M. Loda, D. Kufe, Heregulin targets gamma-catenin to the nucleolus by a mechanism dependent on the DF3/MUC1 oncoprotein, Mol. Cancer Res. 1 (2003) 765–775.
[19] J. Ren, A. Bharti, D. Raina, W. Chen, R. Ahmad, D. Kufe, MUC1 oncoprotein is targeted to mitochondria by heregulin-induced activation of c-Src and the molecular chaperone HSP90, Oncogene 25 (2006) 20–31.
[20] L. Huang, J. Ren, D. Chen, Y. Li, S. Kharbanda, D. Kufe, MUC1 cytoplasmic domain coactivates Wnt target gene transcription and confers transformation, Cancer Biol. Ther. 2 (2003) 702–706.
[21] D. Raina, S. Kharbanda, D. Kufe, The MUC1 oncoprotein activates the anti-apoptotic phosphoinositide 3-kinase/Akt and Bcl-xL pathways in rat 3Y1 fibroblasts, J. Biol. Chem. 279 (2004) 20607–20612.
[22] Y. Li, J. Ren, W. Yu, Q. Li, H. Kuwahara, L. Yin, K.L. Carraway, D. Kufe, The epidermal growth factor receptor regulates interaction of the human DF3/MUC1 carcinoma antigen with c-Src and beta-catenin, J. Biol. Chem. 276 (2001) 35239–35242.
[23] M.S. Lan, S.K. Batra, W.N. Qi, R.S. Metzgar, M.A. Hollingsworth, Cloning and sequencing of a human pancreatic tumor mucin cDNA, J. Biol. Chem. 265 (1990) 15294–15299.
[24] J. Merlin, L. Stechly, S. de Beaucé, D. Monté, E. Leteurtre, I. van Seuningen, G. Huet, P. Pigny, Galectin-3 regulates MUC1 and EGFR cellular distribution and EGFR downstream pathways in pancreatic cancer cells, Oncogene 30 (2011) 2514–2525.
[25] C.F. Roff, J.L. Wang, Endogenous lectins from cultured cells. Isolation and characterization of carbohydrate-binding proteins from 3T3 fibroblasts, J. Biol. Chem. 258 (1983) 10657–10663.
[26] J. Nieminen, A. Kuno, J. Hirabayashi, S. Sato, Visualization of galectin-3 oligomerization on the surface of neutrophils and endothelial cells using fluorescence resonance energy transfer, J. Biol. Chem. 282 (2007) 1374–1383.
[27] J.J. Rahn, Q. Shen, B.K. Mah, J.C. Hugh, MUC1 initiates a calcium signal after ligation by intercellular adhesion molecule-1, J. Biol. Chem. 279 (2004) 29386–29390.
[28] Q. Shen, J.J. Rahn, J. Zhang, N. Gunasekera, X. Sun, A.R. Shaw, M.J. Hendzel, P. Hoffman, A. Bernier, J.C. Hugh, MUC1 initiates Src-CrkL-Rac1/Cdc42-mediated actin cytoskeletal protrusive motility after ligating intercellular adhesion molecule-1, Mol. Cancer Res. 6 (2008) 555–567.
[29] E.P. Lillehoj, H. Kim, E.Y. Chun, K.C. Kim, Pseudomonas aeruginosa stimulates phosphorylation of the airway epithelial membrane glycoprotein Muc1 and activates MAP kinase, Am. J. Physiol. Lung Cell. Mol. Physiol. 287 (2004) L809–L815.
[30] Y. Li, H. Kuwahara, J. Ren, G. Wen, D. Kufe, The c-Src tyrosine kinase regulates signaling of the human DF3/MUC1 carcinoma-associated antigen with GSK3 beta and beta-catenin, J. Biol. Chem. 276 (2001) 6061–6064.
[31] J.A. Schroeder, M.C. Thompson, M.M. Gardner, S.J. Gendler, Transgenic MUC1 interacts with epidermal growth factor receptor and correlates with mitogen-activated protein kinase activation in the mouse mammary gland, J. Biol. Chem. 276 (2001) 13057–13064.
[32] M.R. Pochampalli, R.M. el Bejjani, J.A. Schroeder, MUC1 is a novel regulator of ErbB1 receptor trafficking, Oncogene 26 (2007) 1693–1701.
[33] T.M. Connolly, J.W. Jacobs, C. Condra, An inhibitor of collagen-stimulated platelet activation from the salivary glands of the Haementeria officinalis leech. I. Identification, isolation, and characterization, J. Biol. Chem. 267 (1992) 6893–6898.
[34] H. Inohara, S. Akahani, A. Raz, Galectin-3 stimulates cell proliferation, Exp. Cell Res. 245 (1998) 294–302.
[35] J. Kopitz, C. von Reitzenstein, S. André, H. Kaltner, J. Uhl, V. Ehemann, M. Cantz, H.J. Gabius, Negative regulation of neuroblastoma cell growth by carbohydrate-dependent surface binding of galectin-1 and functional divergence from galectin-3, J. Biol. Chem. 276 (2001) 35917–35923.
[36] N. Maeda, N. Kawada, S. Seki, T. Arakawa, K. Ikeda, H. Iwao, H. Okuyama, J. Hirabayashi, K. Kasai, K. Yoshizato, Stimulation of proliferation of rat hepatic stellate cells by galectin-1 and galectin-3 through different intracellular signaling pathways, J. Biol. Chem. 278 (2003) 18938–18944.
[37] V. Furtak, F. Hatcher, J. Ochieng, Galectin-3 mediates the endocytosis of beta-1 integrins by breast carcinoma cells, Biochem. Biophys. Res. Commun. 289 (2001) 845–850.
[38] E.A. Partridge, C. Le Roy, G.M. Di Guglielmo, J. Pawling, P. Cheung, M. Granovsky, I.R. Nabi, J.L. Wrana, J.W. Dennis, Regulation of cytokine receptors by Golgi N-glycan processing and endocytosis, Science 306 (2004) 120–124.
[39] T. Tadokoro, M. Ikekita, T. Toda, H. Ito, T. Sato, R. Nakatani, Y. Hamaguchi, K. Furukawa, Involvement of Galectin-3 with vascular cell adhesion molecule-1 in growth regulation of mouse BALB/3T3 cells, J. Biol. Chem. 284 (2009) 35556–35563.
[40] J. Ochieng, D. Platt, L. Tait, V. Hogan, T. Raz, P. Carmi, A. Raz, Structure-function relationship of a recombinant human galactoside-binding protein, Biochemistry 32 (1993) 4455–4460.
[41] S.H. Barondes, D.N. Cooper, M.A. Gitt, H. Leffler, Galectins. Structure and function of a large family of animal lectins, J. Biol. Chem. 269 (1994) 20807–20810.
[42] N. Ahmad, H.J. Gabius, S. André, H. Kaltner, S. Sabesan, R. Roy, B. Liu, F. Macaluso, C.F. Brewer, Galectin-3 precipitates as a pentamer with synthetic multivalent carbohydrates and forms heterogeneous cross-linked complexes, J. Biol. Chem. 279 (2004) 10841–10847.