Phosphorylation and externalization of galectin-4 is controlled by Src family kinases

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Galectin-4 is a cytosolic protein that lacks a signal sequence but is externalized and binds to 3-O-sulfated glycoconjugates extracellularly. The mechanism of subcellular localization and externalization of galectin-4 has not yet been determined. A preliminary experiment using pervanadate (PV) showed that galectin-4 is tyrosine-phosphorylated in cells and suggested that Src kinases are involved. Cell transfection with galectin-4 and active Src plasmids showed that galectin-4 can be tyrosine phosphorylated by members of the Src kinase family. The C-terminal peptide YYQI of galectin-4 was found to play an important role in its tyrosine phosphorylation, and the SH2 domains of Src and SHP2 were found to bind to this peptide. Immunofluorescence analysis showed that galectin-4 and phosphorylated proteins were intensely stained in the area of membrane protrusions of PV-treated or Src-activated cells. Furthermore, MUC1 derived from NUGC-4 cells was observed to bind to galectin-4, and externalization of the bound molecules from the cell to the medium increased in the hyperphosphorylated condition. Study of the transfection of the mutant galectin-4 which lacks the C-terminal peptide revealed that the phosphorylation status is important for externalization of galectin-4. These results suggest that externalization of galectin-4 can be regulated by signaling molecules and that it may function intracellularly as an adaptor protein serving to modulate the trafficking of glycoproteins.

Keywords: externalization / galectin-4 / phosphorylation / Src-family kinase

Introduction

Galectins are members of a family of lectins defined by their affinity for β-galactoside-containing saccharides and are involved in regulating diverse biological phenomena (Barondes et al. 1994; Hernandez and Baum 2002). Galectin-4, a member of the galectin family, is expressed abundantly in the epithelium of the alimentary tract. It has two tandemly repeated carbohydrate-recognition domains and binds with high affinity to an SO3 → 3Galβ1 → 3GalNAc pyranoside (Ideo et al. 2002). Previously, we found that galectin-4 binds strongly not only to glycosphingolipids carrying 3-O-sulfated galactose but also to GPI-anchored carcinoembryonic antigen (CEA) that is localized in the raft (Ideo et al. 2005). Galectin-4 has been reported as one of the major components of detergent-resistant membranes (DRMs) of the brush border of porcine intestine (Danielsen and van Deurs 1997; Hansen et al. 2001; Braccia et al. 2003) and of the HT-29 colon adenocarcinoma cell line (Delacour et al. 2005).

The brush border of the intestine is a specialized membrane designed to function both as a digestive/absorptive surface for dietary nutrients and as a permeability barrier to luminal pathogens. It contains greater quantities of glycolipids, enabling the formation of lipid rafts. The glycolipid rafts are stabilized by galectin-4, which forms cross-linked ligands and slows down the release of several brush border proteins into the gut lumen (Danielsen and Hansen 2008). As a glycolipid-binding lectin, galectin-4 may protect the epithelium from luminal pathogens that exploit lipid rafts as portals for entry into the organism (Ideo et al. 2009).

Galectins are cytosolic proteins that lack signal sequences for transport into the endoplasmic reticulum (ER) and are not glycosylated, indicating that they do not traverse the ER-Golgi network (Barondes et al. 1994; Hughes 1999). However, these proteins are also found in extracellular spaces (Barondes et al. 1994; Hughes 1999). There is abundant evidence for the extraacellular roles played by galectins as modulators of cell adhesion and signaling, although the secretion mechanism is not yet understood (Hughes 1999). Galectin-4 is also localized on the cell surface and binds to sulfated glycosphingolipids and CEA, thereby modulating the adhesion of the cells (Ideo et al. 2005). However, it is not clear how galectin-4 is externalized. It has been reported that galectin-4 recruits apical glycoproteins, including MUC1, sialyltransferase, DPP-IV, CEA, NCA and CD59, in DRMs, because these glycoproteins are depleted in DRMs isolated from galectin-4-knockdown HT-29 5M12 cells (Delacour et al. 2005; Stechly et al. 2009). This implies that...
galectin-4 plays an important role in trafficking by functioning as the carrier of these proteins. The interaction between oligosaccharides and galectin-4 seems to show only a limited aspect of its function. In a preliminary experiment, we found that galectin-4 is tyrosine phosphorylated in cells. We, therefore, investigated whether the activation of a signaling pathway affects the intracellular localization and externalization of galectin-4.

**Results**

**Tyrosine phosphorylation of galectin-4 in NUGC-4 cells**

Several reports indicate that addition of pervanadate (PV), a protein tyrosine phosphatase (PTP) inhibitor, enhances the phosphotyrosine levels of several proteins (Srivastava and St-Louis 1997; Fantus and Tsiani 1998). Staining of the galectin-4 immunoprecipitate by anti-tyrosine-phosphorylated (pY) antibody showed that galectin-4 was phosphorylated by PV in a time-dependent and dose-dependent manner (Figure 1A). PV treatment caused the protein band of phosphorylated galectin-4 to slightly shift to a higher molecular weight. The tyrosine-phosphorylated molecules of the NUGC-4 cells increase with PV treatment time and dose (Figure 1A).

Phosphorylation of galectin-4 was inhibited by pre-incubation with Src-family kinase (SFK) inhibitors, including herbimycin A (1 μM), PP2 (10 μM) and genistein (50 μM) (Figure 1B), whereas the phosphorylation level of total cell lysates was not altered by the inhibitors (data not shown). This suggests that certain members of SFK might be involved in the phosphorylation of galectin-4. Although PV was prepared from orthovanadate and H₂O₂, tyrosine phosphorylation of galectin-4 was not enhanced to the same extent as PV, when orthovanadate and H₂O₂ were added, respectively (Figure 1B). It has been reported that reactive oxygen species (ROS) generated by PV treatment increase the total levels of tyrosine-phosphorylated proteins (Natarajan et al. 1998). Tyrosine phosphorylation of galectin-4 was effectively inhibited by the ROS scavenger N-acetylcysteine (NAC) even at concentrations <20 μM (Figure 1B), although phosphorylation levels of the total cell lysates at 20 μM NAC were the same as those of cell lysates without NAC (data not shown). These results suggest that the process of tyrosine phosphorylation of galectin-4 is regulated by certain signaling pathways that include SFKs and ROS.

**Galectin-4 is phosphorylated by constitutively active Src kinase**

In preliminary experiments, involvement of SFKs was suggested (Figure 1B). To confirm this, plasmids expressing FLAG-tagged galectin-4 and constitutively active Src (Act-Src) were transfected to Chinese hamster ovary (CHO) cells. The tyrosine phosphorylation level of the CHO cell lysates increased with PV treatment (Figure 2A, lane 1) and constitutively Act-Src (Figure 2A, lane 2), but not by inactive Src (kinase-dead (KD)-Src) (Figure 2A, lane 3). Staining of the FLAG-tagged galectin-4 immunoprecipitate by anti-pY antibody was observed, showing the tyrosine phosphorylation of galectin-4 by Src kinase (Figure 2A, lane 4). Activated Src kinase was stained by antiphospho-Src (Tyr416) antibody in Act-Src-transfected CHO cell lysates (Figure 2B, lane 2), but not in PV-treated cell lysates (Figure 2B, lane 1). However, in the case of NUGC-4 cells, activated Src kinase was found only after PV treatment (Figure 2B, lanes 3 and 4).

When the mock, Act-Src and KD-Src plasmids were transfected to NUGC-4 cells, tyrosine phosphorylation levels of NUGC-4 cell lysates were increased by the presence of the Act-Src plasmid (Figure 3A, lane 2), but not by the mock (Figure 3A, lane 1) or the inactive Src (KD-Src) (Figure 3A, lane 3) plasmids. Staining of the galectin-4 immunoprecipitate by anti-pY antibody was observed, suggesting that galectin-4 was tyrosine phosphorylated by Src kinase (Figure 3B, lane 3). Activated Src kinase was stained by antiphospho-Src (Tyr416) antibody in Act-Src-transfected NUGC-4 cell lysates (Figure 3A, lane 2) but not by cells transfected with the mock plasmid (Figure 3A, lane 1) or the inactive Src plasmid (KD-Src) (Figure 3A, lane 3). It is interesting that nonphospho-Src (Tyr416) and nonphospho-Src (Tyr527) were observed in KD-Src-transfected cell lysates, suggesting that strong dephosphorylating activity is present in NUGC-4 cells.

After immunoprecipitation with anti-galectin-4 antibody from the Act-Src-transfected cell lysates, several bands, including galectin-4, were stained by pY antibody (Figure 3B, lane 3). This did not occur with lysates from cells transfected with the
mock plasmid (Figure 3B, lane 1). The band near 60 kDa stained by anti-pY antibody is possibly the active form of Src kinase, since it was stained by anti-pSrc (Tyr416) antibody (Figure 3B, lower panel, lane 3).

PTPs expressed in NUGC-4 cells
Since phosphorylation of galectin-4 was observed after the addition of PTP inhibitor, the activities of PTPs might be important in galectin-4 modification in NUGC-4 cells. We, therefore, studied PTPs expressed in NUGC-4 cells. The NUGC-4 cell lysates with or without PV treatment were immunoblotted with several kinds of PTPs. The NUGC-4 cells were found to express cytosolic PTPs, including PTP1B, SHP-1 and SHP-2 (Supplementary data, Figure S1), but not CD45, KAP, LAR, MKP2, RPTPβ, SRPc1 and VHR (data not shown). Among these, the protein band of SHP-2 was shifted to a higher molecular weight with PV treatment, suggesting that SHP-2 protein is phosphorylated and activated.

The N-terminal SH2 domain of SHP-2 and the SH2 domain of Src bind to galectin-4 C-terminal pY-peptide (DVTLS(p)YVQI)
Among the 14 tyrosine residues of the galectin-4 molecule, the Y-X-X-I/V/L sequence exists at the C-terminus of galectin-4 (320YVQI323), which is known as the SHP-1 and SHP-2 binding motif (Sweeney et al. 2005). Since SHP-1 and SHP-2 are expressed in NUGC-4 cells, we prepared a synthetic galectin-4 C-terminal peptide containing the tyrosine with or without phosphate to obtain kinetic data. Using the surface plasmon resonance (SPR) assay, the CDVTLSYPVQI-conjugated keyhole limpet hemocyanin (KLH) was found to bind to SHP-2 in a dose-dependent manner (data not shown). The KD values of galectin-4-C-terminal peptide (CDVTLSPVQI) and galectin-4-C-terminal-pY-peptide (CDVTLSPYVQI) to SHP-2 were
calculated as $1.0 \times 10^{-4}$ M and $1.3 \times 10^{-5}$ M, respectively. Various concentrations of bacterially expressed glutathione-S-transferase (GST) fusion domains of SHP-2 were incubated with galectin-4-pY-peptide-coated plates. After several washes, bound SHP-2 domains were detected by horseradish peroxidase (HRP)-conjugated anti-GST antibody (Figure 4). GST-N-terminal SH2 domain (SHP2-N) was found to bind to galectin-4-pY-peptide in a dose-dependent manner, whereas GST, GST-carboxyl-terminal SH2 (SHP2-C) and GST-PTP (SHP-2-PTP) domains did not bind. These results suggest that the N-terminal SH2 domain may bind to galectin-4-C-terminal pY-peptide.

Since involvement of Src kinase is suggested, we also studied the binding of Src-SH2 domain to galectin-4-C-terminal pY-peptide. The results show that the lower concentrations of the Src-SH2 domain bind to galectin-4-pY-peptide in a dose-dependent manner (Figure 4).

C-terminal peptide is important for galectin-4 phosphorylation

To study the phosphorylation of galectin-4, we constructed a galectin-4 mutant that has the following changes: Y9F, Y166F and Y320F. The three tyrosine residues in the native sequence are predicted by the NetPhos 2.0 Server to be likely phosphorylation targets (Blom et al. 1999). The mutated sequence was inserted into a FLAG-tagged expression plasmid for transfection into NUGC-4 cells. Significant decrease of tyrosine phosphorylation of galectin-4 was not observed (data not shown).

Next, we constructed a mutant of galectin-4 that lacks the C-terminal 320YVQI323 motif and inserted it into a FLAG-tagged expression plasmid for transfection into NUGC-4 cells (Figure 5A). The FLAG-tagged wild-type galectin-4 was immunoprecipitated by anti-FLAG or anti-galectin-4 and was eluted with FLAG peptide or sodium dodecyl sulphate (SDS) sample buffer and stained by anti-FLAG (Figure 5A, lanes 2 and 6) or anti-pY (Figure 5A, lanes 4, 8 and 10). The mutant galectin-4 immunoprecipitated by anti-FLAG or anti-galectin-4 was also stained by anti-FLAG (Figure 5A, lanes 1 and 5); however, after PV treatment, it was not stained by anti-pY (Figure 5A, lanes 3, 7 and 9). Two major bands were stained by anti-pY in the anti-FLAG immunoprecipitate (Figure 5A, lane 8). Bands a and b were identified as FLAG-tagged galectin-4 and endogenous galectin-4, respectively; because they were stained by anti-galectin-4 (data not shown). These results suggest that the C-terminal peptide is important for the tyrosine phosphorylation of galectin-4 and that a galectin-4–galectin-4 homophilic interaction occurs in the cells.

When Act-Src and the wild or the mutant FLAG-tagged galectin-4 were transfected to NUGC-4 cells, both wild and mutant FLAG-tagged galectin-4s were immunoprecipitated by anti-galectin-4 and stained by anti-FLAG antibody (Figure 5B, lanes 1 and 2). The wild FLAG-tagged galectin-4 was also stained by anti-pY (Figure 5B lane 3), but the tyrosine phosphorylation level of the mutant FLAG-tagged galectin-4 was significantly decreased (Figure 5B, lane 4).

Immunostaining of Galectin-4 in NUGC-4 cells

Next, we studied the localization of galectin-4 in NUGC-4 cells by immunostaining using confocal laser scanning microscopy. When the plasmid of FLAG-tagged galectin-4 was transfected into NUGC-4 cells, it was observed to be localized in the area of membrane protrusion after PV treatment (Figure 6B) in comparison with nontreated cells (Figure 6A). However, in the case of the mutant FLAG-tagged galectin-4 which lacks the C-terminal peptides, it was not observed to be localized in the area of membrane protrusion after PV treatment (Figure 6C), suggesting that phosphorylation status of galectin-4 may affect its localization. The pY-proteins and galectin-4 were also observed to be richly localized in membrane protruded area of the Act-Src-transfected NUGC-4 cells (Figure 6D).

By transfecting FLAG-tagged galectin-4s into galectin-4-negative CHO cells, PV treatment also increased the pY-proteins of CHO cells (Figure 2A, lane 1, Supplementary data, Figure S2A and B). However, not only the mutant FLAG-galectin-4, but also the wild-type was distributed throughout the cytosol (Supplementary data, Figure S2A and B). Because Act-Src kinase, detected by anti-pSrc (Tyr416) antibody, is present in PV-treated NUGC-4 cells (Figure 2B, lane 4) but not in PV-treated CHO cells (Figure 2B, lane 1), the involvement of Src kinase activity in galectin-4 localization also suggested. Immunofluorescence analysis showed that galectin-4 was intensely stained in the area of membrane protrusion of PV-treated NUGC-4 cells and Act-Src-transfected cells, indicating that Src kinase activity is important for subcellular localization of galectin-4.

PV treatment enhances galectin-4 and MUC1 externalization into the medium

To study whether Src kinase activity affects the externalization of galectin-4, we measured the amount externalized into the
medium from NUGC-4 cells. Since transfection efficiency of the Act-Src plasmid was not high to perform this study, we treated NUGC-4 cells with PV. The concentrations of galectin-4 in the medium increased in a dose-dependent manner upon treatment of NUGC-4 cells with PV (Figure 7A).

MUC1, a transmembrane mucin glycoprotein, is expressed in NUGC-4 cells and is also found in the medium. Galectin-4 binds dose-dependently to the MUC1, which is adsorbed by the anti-MUC1 antibody from the medium of NUGC-4 (Figure 7B). Since the regulation of galectin-4 in the apical delivery of glycoproteins has been reported (Stechly et al. 2009), we measured MUC1 in the medium with or without PV. MUC1 increased time-dependently, and PV treatment also enhanced the concentration of MUC1 in the medium (Figure 7C).

Activity of a cytosolic protein, lactate dehydrogenase (KDH), was not detected under these conditions (data not shown), suggesting that PV-dependent enhancement of MUC1 and galectin-4 externalization are not caused by simple breakage of the plasma membrane. Staining with propidium iodide, which is commonly used for identifying dead cells, also showed that the membrane integrity seemed to be maintained at the same level as untreated one within PV-treated time. Under permeabilizing conditions, confocal laser scanning microscopy of galectin-4 and MUC1 revealed that PV treatment seems to induce both proteins to move to the same sub-membrane area in NUGC-4 cells (Supplementary data, Figure S3).

Furthermore, we studied whether the phosphorylation status of galectin-4 affects its externalization from the cell under hyperphosphorylated conditions. When the wild-type FLAG-tagged galectin-4 plasmid was transfected into NUGC-4
cells, FLAG-galectin-4 were found in both cell lysate and culture medium after PV treatment. However, in the case of the mutant FLAG-tagged galectin-4 which lacks C-terminal domain, it was not detected in the immunoprecipitate from the culture medium, although it was detected in cell lysate of PV-treated NUGC-4 cells (Figure 7D). These results suggest that tyrosine-phosphorylation of galectin-4 is important for the externalization of galectin-4.

Discussion

We found that galectin-4 is phosphorylated by the Src kinase family. Previous reports of phosphorylation of galectins have been limited to reports of serine phosphorylation (Huflaut et al. 1993; Mazurek et al. 2000) and tyrosine phosphorylation of galectin-3 by c-Abl kinase (Balan et al. 2010). PV treatment was found to increase the levels of phosphorylated tyrosine of proteins, including galectin-4 and pY-proteins in NUGC-4 cells (Figure 1A). This work reveals that galectin-4 has peptide sequences that can be phosphorylated by members of the Src kinase family, shown by the fact that their inhibitors effectively block tyrosine phosphorylation of galectin-4 (Figure 1B). It was confirmed that transfected Act-Src kinase increased the phosphorylation of galectin-4 (Figures 2A and 3B).

Src is a nonreceptor protein tyrosine kinase that plays a multitude of roles in cell signaling, including cell adhesion, growth, movement, and differentiation. At the plasma membrane, Src can transduce signals from a variety of receptors to internal signaling pathways, associating many signaling molecules (Giannoni et al. 2005). Therefore, the association of galectin-4 with Src kinase may sequentially affect those molecules to which Src binds.

Immunostaining of the transfected galectin-4 visualized with confocal microscopy revealed that it becomes localized to a significant extent at the area of membrane protrusion of NUGC-4 cells (Figure 6B) after PV treatment. However, the mutant FLAG-tagged galectin-4 which lacks the C-terminal peptides was not observed to be localized in that area (Figure 6C). This difference is due to the phosphorylation status of galectin-4 and activation of the Src kinase family, because Act-Src, which is phosphorylated at Tyr416 (pY416), was found in PV-treated NUGC-4 (Figure 2B, lane 4), but not in PV-treated CHO cells (Figure 2B, lane 1). And it was also observed that galectin-4 and pY-proteins were intensely stained in the area of membrane protrusion in the NUGC-4 cells which were transfected by the Act-Src kinase (Figure 6D). In Act-Src-transfected cells, pY416 was found in both CHO (Figure 2B, lane 2) and (open circle). After medium change, 100 µM of PV was added to NUGC-4 cells, and the amounts of MUC1 in the medium were measured after 30 and 60 min. Pretreatment with PP2 (10 µM) preceding the PV (filled square) was also performed. (D) Staining of the FLAG-galectin-4 s in cell lysates of PV-treated NUGC-4 cells which were transfected with the wild-type FLAG-tagged galectin-4 (W) (lane 2) or the mutant (M) (lane 1). Staining of the FLAG-galectin-4 s in immunoprecipitates of medium PV-treated NUGC-4 cells which were transfected with the wild-type FLAG-tagged galectin-4 (W) (lane 4) or the mutant (M) (lane 3) transfected cells. FLAG-tagged galectin-4s
NUGC-4 cells (Figure 3A, lane 2). In KD-Src-transfected cells, pY416 was not detected, but some non-pSrc (Y416) and non-pSrc (Y527) were detected, suggesting that strong tyrosine phosphatase activity is present in NUGC-4 cells.

Among the cytosolic PTPs, including PTP1B, SHP-1 and SHP-2, which are expressed in NUGC-4 cells, the SHP-2 band was shifted to a higher molecular weight on SDS–polyacrylamide gel electrophoresis (PAGE), suggesting that SHP-2 is phosphorylated by PV treatment (Supplementary data, Figure S1). SHP-1 and SHP-2 bind to the Y-X-X-I/V/L motif located at the 320YVQI323 C-terminus of galectin-4 (Sweeney et al. 2005). Transfection of the wild or mutant galectin-4 gene into NUGC-4 cells followed by PV treatment or co-transfection with the Act-Src plasmid revealed that the C-terminal 320YVQI323 is important for the phosphorylation of galectin-4. Furthermore, the N-termini of SHP-2 and Src were found to bind to the phosphorylated C-terminal galectin-4 peptide through their SH2 domains (Figure 4). Since it was reported that the N-terminal SH2 domain regulates SHP-2 activity by masking the catalytic site (Guvenc et al. 2007), it is possible that phospho-tyrosinated galectin-4 modulates SHP-2 activity. It is interesting that the sialic acid-binding proteins known as siglec-7 and siglec-9 also have this motif and recruit SHP-2 in lymphocytes after PV treatment (Taylor et al. 1999; Avril et al. 2004).

To our surprise, the SH2 domain of c-Src bound to the phosphorylated C-terminal galectin-4 peptide at one-tenth the concentration of SHP-2, even though it is not the known preferred peptide sequence pYEEI for Src-SH2 (Songyang et al. 1993). It is known that the SH2 domain of Src interacts with phospho-Tyr-527 in its inactive form and that it binds to other phosphorylated proteins in its active form (Tatosyan and Mizzenina 2000). Dephosphorylation of the C-terminal tyrosine (Y527) by a PTP and binding of the SH2 domain to a competitive phospho-tyrosine residue, as seen in the case of c-Src binding to focal adhesion kinase, regulate its kinase activity (Tatosyan and Mizzenina 2000). Since we found anti-pSrc (Y416) antibody-reactive bands in galectin-4 immunoprecipitate and binding of Src-SH2 to the phosphorylated C-terminal peptide of galectin-4, galectin-4 may function as a modulator of Src kinase activity in epithelial cells through an association with Src and/or an Src kinase complex.

Inhibition of the phosphorylation of galectin-4 by N-acetylcyesteine, a ROS scavenger (Figure 1B), may also be explained in relation to Src activity, because ROS directly oxidize Src, promoting autophosphorylation at Tyr416, which in turn leads to enhanced Src kinase activity (Giannoni et al. 2005). Braccia et al. (2003) have demonstrated that galectin-4 can function as a core raft stabilizer in the microvillar membrane vesicles of intestinal brush border. On the cytoplasmic side of the raft, many signaling molecules respond to extracellular stimulation. Protein tyrosine phosphorylation is controlled by the balance of the tyrosine kinases and tyrosine phosphatases. Tyrosine kinase inactivation or tyrosine phosphatase activation may initiate intestinal epithelial cell death (Scheving et al. 1999). Many bacterial pathogens inject effector proteins into host cells that are substrates for host tyrosine kinases such as the Src and Abl kinase families. Phosphorylated effectors eventually subvert host cell signaling, aiding disease development (Mueller et al. 2012). Since our data suggest that Src kinase affects the localization of galectin-4, external stimuli that trigger the intracellular signaling pathway may subsequently affect the movement of galectin-4.

The localization of galectin-4 may subsequently affect the trafficking of apical proteins (Delacour et al. 2005; Stechly et al. 2009). Mehul and Hughes (1997) reported that galectin-3 underneath the plasma membrane is released by membrane blebbing, and movement of cytoplasmic galectin-3 to plasma membrane domains is a rate-limiting step in its secretion. In NUGC-4 cells, PV-induced externalization of galectin-4 in part seems to be related to its phosphorylation, because the level of the mutant FLAG-tagged galectin-4 in PV-treated cell medium was very low (Figure 7D) and it was not found in area of the cell membrane protrusions (Figure 6C). The increase in MUC1 externalization to the medium after PV treatment (Figure 7C) may in part also be related to the movement of galectin-4.

In the case of galectin-3, its N-terminal domains (Menon and Hughes 1999) and binding to fetuin (Zhu and Ochieng 2001) are reported to be involved in its secretion mechanism. However, the association between SFK and galectins and their effect on localization and externalization have not been reported. Externalized galectin-4 may follow an apical endocytic-recycling pathway (Stechly et al. 2009) by associating with its ligands such as glycoproteins and glycosphingolipids. Our results provide new information regarding the regulation of galectin externalization.

Cell surface expression and secretion of MUC1 is highly related to cancer malignancy by preventing access of chemotherapeutic drugs to the cancer cells and inhibiting the interaction of immune cells with receptors on the cancer cell surface (Hollingsworth and Swanson 2004). In fact, NUGC-4 cells derived from signet ring cell carcinoma of the stomach express MUC1 (Figure 7) and induce peritoneal metastasis (Nakashio et al. 1997). Hippo et al. (2001) studied the gene expression profiles of scirrhus gastric cancer cells and found that galectin-4 is upregulated in cells with a high potential for peritoneal dissemination. Accordingly, the expression of both galectin-4 and MUC1 may promote cell surface expression of MUC1, and might result in the acquisition of peritoneal metastatic ability of MUC1-expressing cancer cells.

Further study of galectin-4 is merited, not only for its properties as a carbohydrate binding protein, but also for its cellular role as an adaptor protein that associates with SH2 domains of SFKs and PTPs.

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**Materials and methods**

**Materials**

Asialofetuin, sodium orthovanadate (Na3VO4), NAC, p3XFLAG-CMV™-10 expression vector, anti-FLAG M2-agarose, TRITC-conjugated anti-rabbit IgG and FITC-conjugated anti-mouse IgG (Fab2) were purchased from Sigma Chemical Co. (St. Louis, MO). NaF, RPMI1640 medium and bovine liver catalase were obtained from Wako Pure Chemical Industries Ltd. (Osaka, Japan). Halt protease inhibitor cocktail and MagnaBind™ Carboxyl Derivatized Beads were obtained from Thermo Scientific, Inc. (Rockford, IL). The Src-family protein tyrosine
kinase inhibitor set including genistein, PP2 and herbimycin A was purchased from Calbiochem (Cambridge, MA).

The pGEX-6P-1 plasmid, Escherichia coli BL21 strain and CNBr-Glutathione-Sepharose were obtained from GE Healthcare (Buckinghamshire, UK).

Full-length His-tagged galectin-4 was prepared as described previously (Ideo et al. 2002). Antiserum against human galectin-4 was prepared as described previously (Ideo et al. 2005).

Peptides CDVTLSYQVI, CDVTVLSYP(V)QI and CDVTLSYP(V)QI-conjugated KLH were purchased from Medical & Biological Laboratories Co. Ltd. (Nagano, Japan). Monoclonal antibodies (mAbs) against PTP1B, SHP-1, SHP-2, CD45, KAP, LAR, MKP2, RPTPβ, SRPα1, VHR and β-catenin were purchased from BD Biosciences (San Jose, CA), The Src antibody sampler kit (including anti-Src rabbit monoclonal, antiphospho-Src-family (Tyr416) rabbit monoclonal, anti-nonphospho-Src (Tyr416) mouse monoclonal and anti-nonphospho-Src (Tyr527) rabbit polyclonal antibodies) was purchased from Cell Signaling Technology Japan, K.K. (Tokyo, Japan). Anti-MUC1 antibody was purchased from Mikuri Immunological Laboratories Co., Ltd, Kyoto, Japan.

**Cell lines**

NUGC-4 human gastric cancer cells and CHO cells were purchased from the RIKEN Cell Bank (Tsukuba, Japan) and Health Science Research Resources Bank (Osaka, Japan), respectively. The cells were maintained in RPMI 1640 medium supplemented with 10% fetal bovine serum (Thermo Scientific, Inc., Rockford, IL), penicillin and streptomycin (Invitrogen, Carlsbad, CA, USA).

**SPR assays**

Binding of phosphopeptides to SHP-2 was measured by an SPR assay using a Biacore 2000 instrument. GST-SHP-2 was immobilized on a CM5 sensor chip by the amine-coupling method according to the manufacturer’s instructions. Various concentrations of galectin-4-C-terminal peptide (CDVTLSYQVI), galectin-4-C-terminal-pY-peptide (CDVTLSYP(V)QI) and CDVTLSYP(V)QI-conjugated KLH were diluted in HBS-E buffer. The interaction was monitored at 25°C as the change in the SPR response. The dissociation constants were calculated using the BIAevaluation 3.0 software.

**PV treatment and inhibition study of Src kinase inhibitors and NAC**

PV solution was prepared by mixing Na3VO4 (6.6 mM) and H2O2 (0.1%) in phosphate buffered saline (PBS) for 5 min at room temperature. The solution was neutralized with dilute HCl, and excess H2O2 was destroyed by addition of bovine liver catalase (Sigma Chemical Co.). For PV treatment, the PV solution was added to the medium. PV concentration was taken as the final concentration of Na3VO4.

Each of the SFK inhibitors was dissolved in DMSO before the experiment. For the inhibition study, confluent 6-cm dish cultures of NUGC-4 cells were incubated with 2 μL of medium containing 2 μL of genistein (50 mM), PP2 (10 mM), herbimycin A (1 mM), solutions and DMSO for 1 h. NUGC-4 cells were subsequently incubated with 100 μM of PV.

For the NAC inhibition study, the confluent 6-cm dish cultures of NUGC-4 cells were incubated with 2 μL of medium containing various concentrations of NAC for 1 h, and subsequently incubated with 100 μM of PV.

**Immunoprecipitation and immunoblotting**

NUGC-4 and CHO cells were solubilized in lysis buffer (0.5% Triton X-100, 25 mM Tris–HCl [pH 7.4], 100 mM NaCl, 2 mM EDTA, 1/100 [v/v] Halt protease inhibitor mixture [Pierce], 10 mM NaF, and 1 mM Na3VO4) for 1 min at 4°C and harvested. The nuclear and insoluble components were removed by a 15,000 rpm centrifugation for 10 min at 4°C, and the supernatant was used for immunoprecipitation. Aliquots of the supernatants were incubated with anti-galectin-4-conjugated Sepharose 4B (5.5 mg of IgG/mL gel), control rabbit-IgG-conjugated Sepharose 4B (5 mg/mL) or anti-FLAG M2 agarose for 4–18 h at 4°C with constant rotation. The beads were washed five times with lysis buffer and boiled with Laemmlili sample buffer for 5 min. Samples were subjected to SDS-PAGE (12.5% acrylamide) followed by blotting onto a 0.2-μm nitrocellulose membrane and blocked in 1% bovine serum albumin (BSA) in PBS containing 0.05% Tween 20. The blots were probed with an anti-galectin-4 or an anti-FLAG M2 or anti-FLAG M2-HP conjugate (M2-HP; Sigma-Aldrich St. Louis, MO), and for phosphotyrosine detection, with an antiphosphotyrosine 4G10 (anti-pY) antibody (Upstate Biotechnology, Lake Placid, NY) followed by HRP-conjugated anti-mouse IgG (GE Healthcare). For detection of the blots of cell lysates, the membranes were probed with mAbs of phosphatases (PTP1B, SHP-1, SHP-2, CD45, KAP, LAR, MKP2, RPTPβ, SRPα1 and VHR), followed by anti-mouse IgG-HRP antibodies (GE Healthcare). Immunoblotted proteins were visualized using chemiluminescent detection (SuperSignal, Thermo Scientific, Inc.).

**Immunofluorescence analysis**

Human stomach cancer NUGC-4 and CHO cells grown on coverslips were washed with PBS and fixed in 4% paraformaldehyde for 15 min, followed by washing and incubation with 1% BSA for 1 h at room temperature. For permeabilization, cells were treated with 0.2% Triton X-100 in PBS for 5 min, followed by washing before the incubation with 1% BSA. For immunostaining, cells were incubated at 4°C overnight with anti-galectin-4 (rabbit IgG), anti-pY (mouse IgG), or anti-FLAG (mouse IgG) antibodies in 1% BSA. Cells were then rinsed several times and incubated for 1 h with TRITC-conjugated anti-rabbit IgG and/or FITC-conjugated anti-mouse IgG in PBS containing 0.1% BSA. After extensive washing, the coverslips were mounted on slides. Fluorescent images were obtained using a confocal laser scanning microscope (LSM 5 PASCAL; Carl Zeiss, Germany). Images were acquired using the multitrack mode to avoid signal crosstalk.
The specificity of labeling was assessed by incubation with control nonimmune primary antibodies.

**Site-directed mutagenesis of galectin-4**

Site-directed mutagenesis was performed using the QuikChange™ Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA), according to the manufacturer’s instructions. Nucleotide sequences were analyzed using an Applied Biosystems PRISM 310 Genetic Analyzer (PE Biosystems, Foster City, CA).

**Expression of wild and mutant FLAG-tagged galectin-4 in cells**

For the construction of FLAG-tagged galectin-4, galectin-4 cDNA was polymerase chain reaction (PCR) amplified using the following primers: Wild-type, 5′-TTTAAAGCTTatgggtagcaacaa-3′ and 5′-TTTGAAATTCTtagagtcgtgttaaggggc-3′; mutant, 5′-TTTAAAGCTTatgggtagcaacaa-3′ and 5′-TTTGAAATTCTtagagtcgtgttaaggggc-3′ and cloned into the HindIII and EcoRI sites of the pC3FLAG-CMV™-10 expression vector.

**Expression of constitutively Act-Src and KD-Src in cells**

For the construction of Act-Src harboring a tyrosine to phenylalanine substitution at codon 529, Src cDNA was PCR amplified using the following primers: Wild-type, 5′-TTTAAAGCTTatgggtagcaacaa-3′ and 5′-TTTGAAATTCTtagagtcgtgttaaggggc-3′; Act-Src, 5′-TTTGAAATCTatgggtagcaacaa-3′ and 5′-TTTGAAATTCTtagagtcgtgttaaggggc-3′ and cloned into the HindIII and EcoRI sites of the pcDNA3 expression vector. KD-Src (K298M mutant) was generated using the QuikChange™ Site-Directed Mutagenesis Kit (Stratagene, La Jolla, CA, USA) according to the manufacturer’s instructions. Transfection studies were performed using Lipofectamine™ 2000 reagent (Life Technologies, Carlsbad, CA, USA) according to the manufacturer’s instructions.

**Generation of GST-SHP-2 fusion proteins and the SH2 domain of Src kinase**

GST-fusion proteins containing various SHP-2 domains were generated. The plasmid containing full-length SHP-2 cDNA was amplified by PCR (30 cycles of 95°C for 0.5 min, 50°C for 1 min and 72°C for 2 min) using 5′- and 3′-primers containing the appropriate restriction enzyme sites as follows: The N-terminal SH2 domain fragment (amino acids 1–105, referred to as SHP2-N in Figure 4) was amplified using the primers 5′-TTTGGAATTCTagggctgaggag-3′ and 5′-TTTGCTGACGgcacacggtggtgaggc-3′; the C-terminal SH2 domain fragment (amino acids 109–220, referred to as SHP2-C in Figure 4) was produced with the primers 5′-TTTGGAATTCTatgggtagcaacaa-3′ and 5′-TTTGCTGACGgcacacggtggtgaggc-3′ and cloned into the HindIII and EcoRI sites of the pcDNA3 expression vector. GST-fusion proteins of the SH2 domain of Src kinase was generated in the same manner as SHP2-N, SHP2-C, SHP2-PTP and Src-SH2 in PBS-T were added to each well and the plate was kept at room temperature for 1.5 h. The plate was washed several times with PBS-T, and HRP-conjugated anti-GST antibody (GE Healthcare) diluted in PBS-T was added. After incubation for 1.5 h at room temperature, the plate was washed and incubated with 0.3% o-phenylenediamine (Nacalai Tesque, Inc., Kyoto, Japan) and 0.03% H2O2 in 10 mM acetate buffer (pH 5.0). The released chromogen was measured with a photospectrometer (microplate reader, Plate CHAMELEON V; Hitex, Turku, Finland).

**ELISA for the externalized galectin-4 and MUC-1 in the medium**

Galectin-4 antibody was chemically immobilized to MagnaBind™ Carboxyl Derivatized Beads according to the manufacturer’s instructions and labeled with HRP using the peroxidase labeling kit-NH2 (Dojindo Laboratories, Kumamoto, Japan). Standards and samples were pipetted into the wells, and galectin-4 present in the medium was bound by the immobilized antibody. After any unbound substances were washed away, HRP-labeled anti-galectin-4 was added to the wells. After a wash to remove any unbound antibody-enzyme reagent, o-phenylenediamine solution or SuperSignal ELISA Pico Chemiluminescent Substrate (Thermo Scientific, Inc.) were added and measured as described above. To quantify the amount of galectin-4 in the medium, NUGC-4 cells were grown on a 6-cm dish until confluent and transferred to fresh cell culture medium (2 mL total). After this incubation period, aliquots of the medium were removed and centrifuged at 12,000 × g for 5 min at 4°C to remove cells and cell debris to permit ELISA.

Galectin-4 and anti-MUC-1 antibody were also labeled with HRP using the peroxidase labeling kit-NH2. Each well was coated with 50 μL of anti-MUC1 antibody (5 μg/mL), after blocking with 1% BSA in PBS, aliquots of culture medium from the NUGC-4 cells were applied and the amounts of MUC1 were measured using HRP-conjugated anti-MUC1 antibody.
Variations of culture medium from the NUGC-4 cells were applied to each well which was coated with anti-MUC1 antibody, and the binding ability of galectin-4 to MUC1 was measured using HRP-conjugated galectin-4. The plate was washed and incubated with TMB Microwell Peroxidase Substrate system (2C) (KPL, Inc. Gaithersburg, MD).

LDH was assayed using the LDH cytotoxicity assay kit (Cayman Chemical Company, MI).

**Supplementary data**

Supplementary data for this article are available online at http://glycob.oxfordjournals.org/.

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**Conflict of interest**

None declared.

**Abbreviations**

Act-Src, active Src; BSA, bovine serum albumin; CEA, carcinoembryonic antigen; CHO, Chinese hamster ovary; DMSO, dimethyl sulfoxide; DRMs, detergent-resistant membranes; EDTA, ethylenediaminetetraacetic acid; ELISA, enzyme-linked immunosorbent assay; ER, endoplasmic reticulum; GST, glutathione-S-transferase; HRP, horseradish peroxidase; KD, kinase-dead; KLH, keyhole limpet hemocyanin; LDH, lactate dehydrogenase; mAbs, monoclonal antibodies; NAC, N-acetylcysteine; PAGE, polyacrylamide gel electrophoresis; PBS: phosphate buffered saline; PCR, polymerase chain reaction; PTP, protein tyrosine phosphatase; PV, pervanadate; pY, tyrosine-phosphorylated; ROS, reactive oxygen species; SDS, sodium dodecyl sulphate; SFR, Src-family kinase; SPR, surface plasmon resonance.

**References**


