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Intracellular sorting of galectin-8 based on carbohydrate fine specificity

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Galectin-8 has two carbohydrate recognition domains (CRDs), both of which bind β-galactosides, but have different fine specificity for larger saccharides. Previously we found that both CRDs were needed for efficient cell surface binding and signaling by soluble galectin-8, but unexpectedly binding of the N-CRD to its best ligands, α2-3-sialylated galactosides, was not needed. In search for another role for this fine specificity, we now compared endocytosis of galectin-8 in Chinese hamster ovary (CHO) cells and in a mutant (Lec2) lacking sialylated glycans, by fluorescence microscopy. Galectin-8 was endocytosed in both cells by a non-clathrin and non-cholesterol dependent pathway, but surprisingly, the pathway after endocytosis differed dramatically. In wild type (wt) cells, galectin-8 was found along the plasma membrane, near the nucleus, and in small vesicles. In the Lec2 cells, galectin-8 was found in larger vesicles evenly spread in the cell, but not along the plasma membrane or near the nucleus. A galectin-8 mutant with an N-CRD having reduced affinity to sialylated glycans and increased affinity for other glycans, gave a Lec2 like pattern in the wt CHO cells, but a wt pattern in the Lec2 cells. Moreover, the pattern of galectin-3 after endocytosis differed from that of both the wt and mutant galactin-8. These data clearly demonstrate a role of galectin fine specificity for intracellular targeting.

Keywords: endocytosis/galectin/intracellular/sialic acid/sorting

Introduction

Galectin-8 belongs to a β-galactoside binding protein family (Leffler et al. 2004) and is one of the most widely expressed members in human tissues (Lahm et al. 2004). Accumulating evidence pose galectin-8 as a modulator of cell functions including cell adhesion, spread, growth and apoptosis (Lahm et al. 2004; Zick et al. 2004; Arbel-Goren et al. 2005; Carcamo et al. 2006), but no precise mechanism or general physiological role has yet emerged. Galectin-8 is composed of two carbohydrate recognition domains (CRDs) with different fine specificity for ligands, and connected by a linker of varying length, giving rise to different isoforms (Bidon-Wagner et al. 2004; Zick et al. 2004). The N-terminal CRD has a particularly strong affinity for 3-sialylated and sulphated galactosides not shared by the C-CRD (Ideo et al. 2003; Carlsson et al. 2007). Despite this N-CRD fine specificity, sialylation was unexpectedly not required for cell surface binding of intact galectin-8 or its activation of cells (Patnaik et al. 2006; Carlsson et al. 2007). Presence of both CRDs, apparently binding to lower affinity ligands, was required and sufficient.

To search for an intracellular role of galectin-8 fine specificity, we studied endocytosis of galectin-8, reported for other galectins in a few cases (Furtak et al. 2001; Baptiste et al. 2007) and proposed to have functional effects (Nagaoka et al. 2002; Ochieng et al. 2002; Boura-Halfon et al. 2003; Partridge et al. 2004; Baptiste et al. 2007; Lau et al. 2007). Now, surprisingly, the binding of galectin-8 to sialylated galactosides had a dramatic effect, determining its intracellular targeting.

Results and discussion

Sialylation determines the cellular distribution of endocytosed galectin-8 in Chinese hamster ovary (CHO) cells

The endocytosis of galectin-8 was compared in wild type (wt) CHO cells and the Lec2 mutant devoid of glycan sialylations (Patnaik and Stanley 2006); the cells were incubated with Alexa Fluor488 labeled thioredoxin-fused human galectin-8 (G8S) for 30 min at 37°C. In wt cells this resulted in staining along the plasma membrane, in intracellular vesicles and in a larger perinuclear structure (Figure 1A). In Lec2 cells, the staining was completely different, found in larger evenly distributed intracellular vesicles but not along the plasma membrane or in a perinuclear structure.

The different staining in Lec2 cells could be either directly due to lack of sialylated ligands for galectin-8, or indirectly due to a general sorting defect in these cells. To distinguish these possibilities, a G8S Q47A mutant, which has decreased affinity for sialylated galactosides was examined (Table I (Carlsson et al. 2007)). In wt CHO cells, the mutant gave a pattern after endocytosis similar to what was found with wt galectin in Lec2 cells (Figure 1A). Moreover, in Lec2 cells, the mutant galectin gave a pattern similar to wt galectin in wt cells. This shows that there is no general sorting defect in Lec2 cells, and gives strong evidence that the wt sorting depends on the interaction of the galectin-8 N-CRD with sialylated saccharides.

Another CHO cell mutant, Lec1, devoid of galactose containing *N*-glycans (Patnaik and Stanley 2006), previously showed low binding of wt galectin-8 (Patnaik et al. 2006) and now, as expected, low but significant endocytosis in vesicles (Figure 1A). This is possibly due to binding to residual sialylated galactosides in glycolipids or *O*-glycans, as the mutant G8S Q47A gave no endocytosis in the Lec1 cells.

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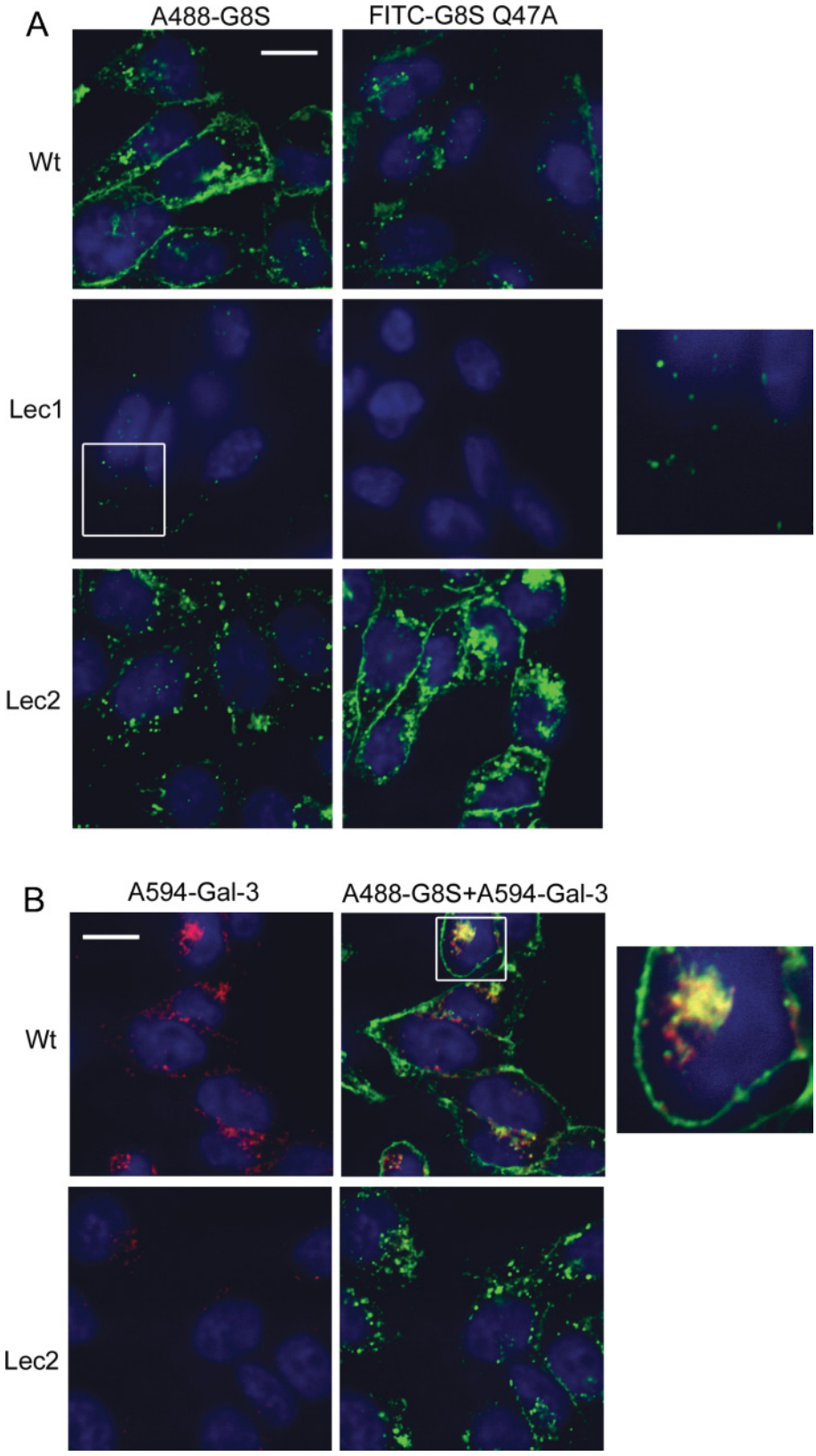

Fig. 1. Sialic acid dependent intracellular targeting of galectin-8 in CHO cells. (**A**) Wt CHO cells and CHO cell mutants Lec1 (lacking *N*-glycans) and Lec2 (lacking sialic acids) were incubated with 0.5 μM AlexaFluor488-labelled galectin-8 (A488-G8S) or FITC-labelled mutant G8S Q47A, with decreased affinity for sialic acid, for 30 min at 37°C. Cells were fixed in formaldehyde and examined by confocal microscopy. Boxed section in Lec1/A488-G8S is magnified to the right. (**B**) Endocytosis of Alexa Fluor594-galectin-3 (performed as described for G8S above) in wt and Lec2 cells. Second panels show overlay with A488-G8S and boxed section is magnified to the right. Bars represent 10 μm.

Table I. Relative affinities of G8S and G8S Q47A for various saccharides measured by FP inhibition assay, with an SA-Lac containing fluorescent probe. The K_d value for lactose is given within parenthesis

Saccharide	Structure	G8S	G8S Q47A
Lactose	Galβ1-4Glc	1 (195)	1 (145)
3′SA-Lac	Neu5Acα2-3Galβ1-4Glc	140	7.3
3′SA-Lacto-N-biose	Neu5Acα2-3Galβ1-3GlcNAc	120	10
3′SA-Tag	Neu5Acα2-3Galβ1-3GalNAc	85	12
3′Su-LacNAc	3OSO-3′LacNAc	5.0	1,8
3′Su-Lacto-N-biose	3OSO-3′Galβ1-3GlcNAc	31	7.7
Lacto-N-Triose	GlcNAcβ1-3Galβ1-4Glc	0.4	7.3
LNnT	Galβ1-4GlcNAcβ1-3Galβ1-4Glc	4.8	87

Targeting after endocytosis of galectin-3 with different carbohydrate fine specificity (Hirabayashi et al. 2002) only partially overlapped with endocytosed G8S in wt CHO cells (Figure 1B). Galectin-3 was found less along the plasma membrane and mainly in intracellular structures different from those of G8S, although there was some co-localisation mainly in the perinuclear structure in some cells. Galectin-3 endocytosis was clearly diminished in Lec2, and any changes in intracellular distribution were difficult to estimate.

In conclusion, the data above clearly show that a galectin's fine specificity can determine its intracellular targeting after endocytosis. The pattern for wt G8S in wt CHO cells resembles the one seen for recycling endosomes with subsequent targeting to the plasma membrane and Golgi seen for other molecules in CHO cells (Iglesias-Bartolome et al. 2006), although this remains to be proven. Apparently sialic acid can be a determinant of such targeting, which agrees with the sorting found for some sialylated glycoconjugates (Slimane et al. 2000; Potter et al. 2006b).

Gain of function and gain of carbohydrate affinity of G8S Q47A

G8S Q47A with decreased affinity for sialylated glycans had not only lost the wt targeting in wt CHO cells, but also, apparently, gained a wt targeting pattern in Lec2 cells (Figure 1A). To see if it had also gained in affinity for any saccharides, its specificity was examined in further detail (Table I). The results confirmed decreased affinity for 3-sialylated and 3-sulphated β-galactosides (Ideo et al. 2003; Carlsson et al. 2007), but also showed markedly increased affinity for galactosides extended with GlcNAc instead, such as GlcNAcβ1-3Galβ1-4GlcNAc (Lacto-N-biose) and LNnT. Although the connection between the two gains of function remains unproven, one may speculate that the lack of sialic acid in the CHO cells have made galactosides available to other glycosyltransferases (e.g. a GlcNAcβ1-3 transferase) resulting in glycans with higher affinity for the mutant galectin-8; this interaction could in turn have taken the place of the lost interaction of wt galectin-8 with sialylated galactosides.

Galectin-8 endocytosis in different human cells

Next we investigated galectin-8 endocytosis in other cell types, as shown for Jurkat T-cells in Figure 2A. After 10 min at 37°C, G8S could be seen inside the cells and it persisted during the length of the experiment (30 min). As seen for CHO cells, the plasma membrane was evenly stained in addition to intracellular vesicles. However, the precise intracellular distribution was hard

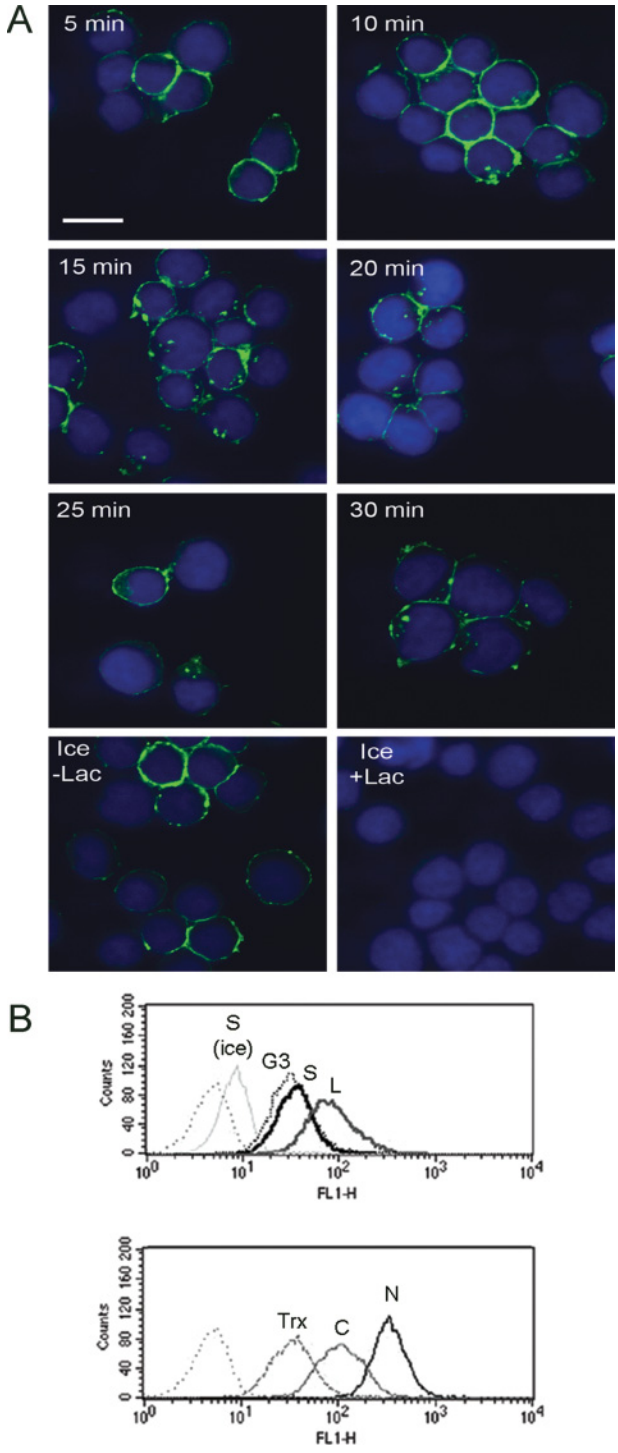

Fig. 2. Time and temperature dependent endocytosis of galectin-8 in Jurkat cells. (**A**) Jurkat cells were incubated with 0.5 μM A488-G8S at 37°C for 5–30 min and each time point stopped by addition of ice cold PBS. Cells incubated on ice for 30 min (ice) were either washed in 150 mM lactose to remove non-endocytosed galectin (+Lac) or not (−Lac). After incubation cells were treated and examined as for Fig. 1. Bar represents 10 μm. (**B**) FACS analysis of Jurkat cells incubated with FITC-labelled galectin for 30 min at 37°C followed by wash with 150 mM lactose on ice. (*Top panel*) FITC-G8S (0.5 μM, thick black line), -G8L (0.5 μM, thick dark grey line), -Gal-3 (0.5 μM, dashed grey line). The result with G8S on ice followed by lactose wash is marked S (ice) (thin grey line). (*Bottom panel*) FITC-G8N (5 μM, thin black line), -G8C (5 μM, dark grey thin line) or -Trx (5 μM dashed grey line). Unlabelled cells are a sparsely dotted grey line in each panel.

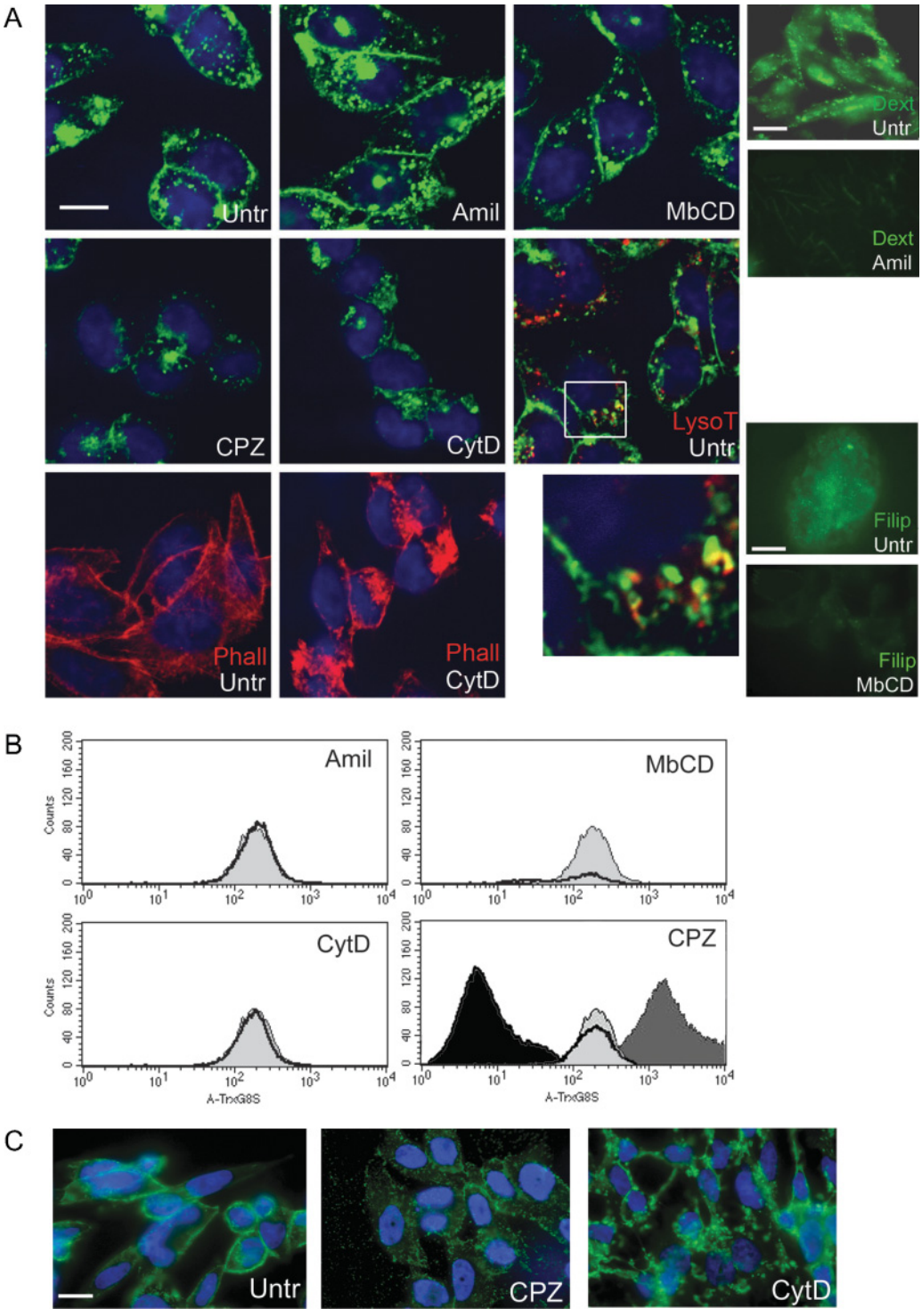

Fig. 3. Galectin-8 endocytosis is not inhibited by amiloride, methyl-β-cyclodextrin, chlorpromazine or cytochalasin D and does not co-localise with acidic vesicles. (**A**) Confocal imaging of wt CHO cells pre-incubated for 30 min in serum-free medium with endocytosis inhibitors amiloride (Amil, 100 μM), methyl-β-cyclodextrin (MbCD, 5 mM), chlorpromazine (CPZ, 14 μM) or cytochalasin D (CytD, 4 μM) or without (Untr) before incubation with 0.5 μM A488-G8S for 30 min at 37°C in continued presence of inhibitor. In co-localisation experiments, LysoTracker Red DND99 (LysoT, 300 nM) was added in addition to A488-G8S, and a magnification is shown below the LysoT panel (boxed area). F-actin was visualised with Alexa Fluor594-phalloidin (Phall). The effect of amiloride and methyl-β-cyclodextrin was visualised by epifluorescence microscopy using fluorescently labeled dextran (0.2 mg/mL, Dext) and filipin-III (300 μM, Filip) respectively (small panels to the right). Bar represents 10 μm. (**B**) Pre-incubation of Jurkat cells with amiloride (Amil, 1 mM), methyl-β-cyclodextrin (MbCD, 10 mM), cytochalsin C (CytD, 40 μM) or chlorpromazine (CPZ, 50 μM) before incubation with 0.5 mM A488-G8S for 30 min at 37°C. Cells were washed with 150 mM lactose before flow cytometric analysis. Closed histogram (light grey) represents untreated cells and open histogram inhibitor treated cells. Transferrin uptake in non-treated cells (closed histogram, dark grey) and in cells treated with 14 μM chlorpromazine (closed histogram, black) are also shown in bottom right panel. (**C**) Epifluorescence microscopy of endocytosis of galectin-3 (0.5 μM) in CHO-cells under conditions as described above (part A) with panels showing untreated cells (Untr), and chlorpromazine (CPZ, 14 μM) and cytochalasin D (CytD, 4 μM) treated cells. Bar represents 10 μm.

to compare between the two cell lines because most of the Jurkat cell volume is taken up by the nucleus, leaving only a thin space for the cytosol. The mutant G8S Q47A was not found along the plasma membrane but in intracellular vesicles (not shown), in general agreement with the finding in CHO cells.

Relative endocytosis was estimated by flow cytometry (Figure 2B) of cells that had been chilled after the incubation at 37°C thus stopping endocytosis, and had the surface bound galectin removed by washing with lactose (Figure 2A, bottom panels). This showed that both G8S and long linker galectin-8 (G8L) were internalised to a similar extent in Jurkat cells. The N-CRD (G8N) and the weak cell surface binding C-CRD (Patnaik et al. 2006; Carlsson et al. 2007) by themselves were likewise internalised when the concentration added to the cells was 10 times higher. In addition, galectin-3 also behaved in a similar manner. Thus, taking the specific activity into account, the rate of uptake correlates roughly with cell surface affinity (Carlsson et al. 2007). There is also some uptake independent of binding as thioredoxin (Trx) was also internalised, but to a much lesser extent. Uptake of galectin-8 was also seen in two other suspension cell lines, U937, a monocytic cell line, and HL-60, a promyeloblastic cell line as analysed by flow cytometry (not shown).

Internalisation of galectin-8 is not inhibited by amiloride, methyl-β-cyclodextrin, chlorpromazine or cytochalasin D

To determine which of the several possible cellular uptake mechanisms were responsible for galectin-8 internalisation, the effect of four different endocytosis inhibitors were tested. Experiments were done on both wt CHO (Figure 3A), Lec2 (not shown) and Jurkat cells (Figure 3B) and analysed with confocal microscopy and flow cytometry, respectively.

Macropinocytosis, internalisation of surrounding fluid phase proteins in a non-receptor mediated way, did not contribute significantly to galectin uptake, since 1 mM amiloride (Zhuang et al. 1984) had no effect. Uptake of fluorescently labelled dextran (a commonly used fluid phase uptake marker) did, however, decrease, as expected, after this treatment (Figure 3A).

The cholesterol and lipid raft-dependent caveolar endocytosis would be suggested by the high affinity of galectin-8 to glycolipids (Ideo et al. 2003; Carlsson et al. 2007). However, no effect on uptake was seen after pre-treatment of cells with up to 20 mM methyl-β-cyclodextrin (Figures 3A and B), a cholesterol depleting agent known to inhibit this pathway (Nabi et al. 2003), although the highest concentrations pushed Jurkat cells into apoptosis and decreased the height of the fluorescence histogram due to loss of cells in the viable cell gate (Figure 3B). Auto-fluorescent filipin-III was used to visualise cholesterol in the membrane and confirmed the effect of methyl-β-cyclodextrin (Figure 3A).

Classical clathrin mediated endocytosis is also unlikely, as pre-treatment of CHO and Jurkat cells with chlorpromazine (up to 50 μM) did not inhibit galectin-8 uptake (Figure 3), although such treatment significantly impaired endocytosis of human transferrin in Jurkat cells (Figure 3A). Uptake of human transferrin in CHO cells was too low to be reliably detected. However, chlorpromazine severely affected CHO cell morphology and thereby distinctly altered the pattern, with most endocytosed galectin-8 in one large perinuclear structure and much less along the cell membrane (Figure 3A).

Pre-treatment with up to 40 μM cytochalasin D, also did not inhibit galectin-8 uptake. However, like chlorpromazine, it grossly affected CHO cell morphology and altered cellular distribution of endocytosed galectin-8 into one coherent structure (Figure 3A). The effect of cytochalasin D in disrupting the actin cytoskeleton, was visualised by Alexa Fluor594-Phalloidin staining of F-actin (Figure 3A).

In Lec2 cells (not shown), the uptake of G8S and its typical distribution in these cells (see Figure 1A) also did not change upon treatment with amiloride or methyl-β-cyclodextrin. In cytochalasin D and chlorpromazine treated Lec2 cells endocytosed galectin was found in a more coherent structure as in wt cells, but less pronounced.

Little endocytosed galectin-8 was found inside intracellular structures with acidic pH, e.g. lysosomes, as there was hardly any co-localisation between galectin-8 and Lysotracker Red DND-99 (Figure 3A).

As found with galectin-8, galectin-3 uptake in CHO cells was not inhibited by amiloride or methyl-β-cyclodextrin (not shown). However, it was partially inhibited by 14 μM chlorpromazine (Figure 3C, middle panel) and completely by 40 μM (not shown). Cytochalasin D did not inhibit uptake/association with the cells, but caused an altered pattern, different from that seen with galectin-8 (Figure 3C, right panel). These data add to the evidence given in Figure 1B that the mechanism of uptake and intracellular targeting of galactin-3 is different from galectin-8.

Conclusion

Here we show that the pathway followed by externally added galectin-8 after endocytosis is determined by its fine specificity – a unique ability of its N-CRD to bind 3-sialylated galactosides. Previously we found, in contrast, that binding to the cell surface did not require this fine specificity (Patnaik et al. 2006), but appeared to be mediated by the combined interaction of the two CRDs with 'second best' ligands of moderate affinity (Carlsson et al. 2007). This suggests a scenario where galectin-8 first binds with broad specificity to *N*-linked glycans at the cell surface, and then during or soon after endocytosis shifts to other ligands, which determine the pathway taken. The mechanism of uptake is unknown and has to be classified as non-clathrin, non-caveolae mediated (Conner et al. 2003) as none of the inhibitors had any effect (Figure 3). *N*-linked glycans are required, however, perhaps for initial binding as indicated by the dramatically decreased surface binding (Patnaik et al. 2006) and endocytosis (Figure 1A) in Lec1 cells. The possible shift to other ligands is suggested by the preference of the N-CRD core binding site (C-D in Carlsson et al. 2007) for lactose and T-antigen (Galβ1-3GalNAc), as found in glycolipids and *O*-glycans, respectively, over LacNAc as found mainly in *N*-glycans. The high affinity for the best ligands may be necessary for this shift to occur, that is, to compete with the initial binding to lower affinity ligands.

Fairly subtle differences in ligand affinity appear to determine the intracellular pathway taken, illustrated by the striking effect seen in wt CHO cells with mutant G8S Q47A, which results from its just about 10 times weaker binding capacity to sialylated β-galactosides. The pathway also depends on the match between galectin specificity and carbohydrate structures of the cell, as illustrated by the altered targeting of the wt galectin in sialic acid deficient Lec2 cells and the reversed pattern with

mutant galectin (Figure 1A). This opens an interesting possibility of scenarios for the function of other galectins. Four bi-CRD galectins (-4, -8, -9, and -12) arose early in vertebrate development (Houzelstein et al. 2004), with different tissue distributions, and where known (-4, -8, and -9), with different fine specificities. This may reflect an adaptation of each galectin to cells with different glycan profiles and/or to targeting along different intracellular pathways. Also for other galectins, intracellular targeting may be determined by their fine specificity, as illustrated here for galactin-3, which differed from galactin-8 (Figure 1B). According to this hypothesis, there would be a complex system of intracellular sorting based on matches between galectin fine specificity and available glycan structures. This would predict different sorting of different galectins, and different sorting of the same galectin in different cell types.

Sorting by fine specificity may occur in vivo in both the exocytic and endocytic pathway, as the cytosolic galectins may reach either after non-classical translocation across membranes, into vesicles (Delacour et al. 2007) or out of the cell (Nickel 2005) followed by reuptake. Moreover, both the 'inbound' postendocytic vesicles and 'outbound' vesicles from the biosynthetic pathway (i.e. post-TGN), deliver the contents to the recycling endosome, which reorganise the cargo in new suitable carriers (Ang et al. 2004; Potter et al. 2006b) based on signals of both peptide and glycan types (Huet et al. 2003; Ellis et al. 2006; Potter et al. 2006a).

A galectin could be a 'driver' in such intracellular targeting, as indicated for galectin-3 and -4, since their removal in polarised cells distorts apical delivery of cargo (Delacour et al. 2005, 2006). A galectin could also be a 'passenger', and reflect the whereabouts of specific glycans in the cell. Some sialoglycoproteins recycle between the plasma membrane and Golgi via an endocytic pathway (Slimane et al. 2000; Potter et al. 2006b), and their interaction with the N-CRD could make galactin-8 a passenger and/or a driver in this process. Moreover, the C-CRD of galactin-8, then, could direct other glycoconjugates to the same pathway.

Materials and methods

Materials, cells and galectins

Alexa Fluor 488 and 594 labelling kits, LysoTracker red DND-99, Alexa Fluor594-Phalloidin, ProLong Gold antifade reagent with DAPI, fluorescein-dextran and Alexa Fluor594 human transferrin were from Molecular Probes, Inc (Invitrogen, Carlsbad, CA). All other chemicals were purchased from Sigma-Aldrich (St. Louis, MO) if not stated otherwise. The human cell lines Jurkat E6-1, a T-lymphoblast line, and U937, a monocyte cell line were cultured in RPMI-1640 medium supplemented with 10% fetal calf serum. Cell densities were kept between 0.1×10^6 and 1.5×10^6 cells/mL throughout cultivation. The promyeloblast cell line HL-60 was cultured as Jurkat cells but with RPMI-1640 containing GlutaMAX-I. Wild type Chinese hamster ovary (CHO-K1) cell line and the two mutants Lec1 and Lec2 were cultured in RPMI-1640 supplemented with 10% foetal calf serum and 50 μg/mL penicillin-streptomycin. Complete medium is growth medium for each cell line including supplements. Humidified 37°C incubators with 5% CO_2 were used for all cell incubations. Human galectin-8, its CRDs, and mutant were produced in *Escherichia coli* as fusion proteins with thioredoxin (Trx) and purified with lactosyl-sepharose as described in detail (Carlsson et al. 2007).

Endocytosis of galectin-8 in CHO cells for confocal analysis

CHO cells grown to 50–70% confluency in 12 or 24-well plates containing coverslips were first washed and then incubated for 30 min in serum-free medium. Following serum-free treatment cells were incubated with various proteins (Alexa Fluor488-labelled G8S, 0.5 μM; FITC-labelled G8S Q47A, 0.5 μM; Alexa Fluor594-labelled galectin-3, 0.5 μM with or without LysoTracker Red DND-99, 300 nM) for 30 min at 37°C before being washed in ice cold phosphate buffered saline (PBS, 118 mM NaCl, 63 mM Na/K-phopshate pH:7.2) and fixation in 2% formaldehyde (prepared fresh from a thawed stock solution of paraformaldehyde dissolved in PBS). Samples stained with Alexa Fluor594-Phalloidin were permeabilised in 0.1% Triton X-100 and staining performed according to the manufacturer's instructions. Coverslips were mounted using ProLong Antifade Reagent with DAPI and cured overnight before confocal microscopy using a Zeiss LSM510 (Carl Zeiss, Boston, MA).

Jurkat cells which had endocytosed galectin-8 (see endocytosis for FACS analysis) were fixed in 3.7% formaldehyde and let adhere to poly-L lysine coated glass slides before mounting and confocal microscopy.

Endocytosis of galectin-8 in jurkat, U937 or HL-60 for FACS analysis

Jurkat cells ($\sim 10^6$), undifferentiated U937 or undifferentiated HL-60 cells grown to mid log-phase were washed once in room temperature in complete medium before incubation with fluorescently labelled proteins: G8S (0.5 μM), G8L (0.5 μM), G8N (5 μM), G8C (5 μM), thioredoxin (Trx, 5 μM) or galectin-3 (0.5 μM). The total volume was 50–100 μL and incubation continued for 30 min at 37°C or on ice. Endocytosis was stopped by addition of ice cold PBS and cells were washed three times in ice cold PBS with or without 150 mM lactose. Analysis was performed on a FACS Calibur flow cytometer together with the Cell Quest Pro software (BD Biosciences, San Jose, CA). Specific activity (number of fluorescein molecules per number of protein molecules) of FITC-labelling was 0.5 for G8S, 0.5 for G8L, 0.7 for G8N, 0.4 for G8C and 0.9 for galectin-3.

The rate of galectin-8 internalisation was analysed as described above but endocytosis was stopped after 5, 10, 15, 20, 25 and 30 min, respectively.

Inhibitor treatment

CHO cells were grown, washed and incubated in serum-free medium as described for 'endocytosis for confocal microscopy' prior to inhibitor treatment. Cells were first pre-incubated with inhibitors for 30 min before endocytosis experiment was carried out in continued presence of inhibitor. Inhibitors used were amiloride (100 μM), methyl-β-cyclodextrin (5 mM), chlorpromazine (14 μM) and cytochalasin D (4 μM). As controls, fluorescein-labelled dextran at a concentration of 0.2 mg/mL, and Filipin-III at a concentration of 300 μM, were incubated with cells (inhibitor treated or not) for 30 min at room temperature and subsequently analysed with a Nikon TE2000-U epi-fluorescence microscope.

Jurkat cells were pre-treated with three inhibitor concentrations per inhibitor, amiloride (10, 100 and 1000 μM),

methyl-β-cyclodextrin (5, 10 and 20 mM), chlorpromazine (1, 14 and 50 μM) or cytochalasin D (0.4, 4 and 10 μM). As a control, AlexaFluor594-labelled human transferrin (200 μg/ml, 30 min at 37°C) was used for all concentrations of chlorpromazine and non-treated cells. After endocytosis (in presence of inhibitor) cells were chilled and washed in PBS with 150 mM lactose and fixed in 4% formaldehyde and analysed by flow cytometry.

Fluorescence polarisation

The glycan affinity of wt and mutant galectin-8 was calculated from the inhibition of fluorescence polarization by soluble saccharides (at three concentrations) as described (Carlsson et al. 2007), with 1 μM of galectin and 0.1 μM fluorescently labelled Neu5Acα2,3Galβ1,4Glc as probe (# 3 in Carlsson et al., 2007).

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Conflict of interest statement

None declared.

References

Ang AL, Taguchi T, Francis S, Folsch H, Murrells LJ, Pypaert M, Warren G, Mellman I. 2004. Recycling endosomes can serve as intermediates during transport from the Golgi to the plasma membrane of MDCK cells. J Cell Biol. 167:531–543.

Arbel-Goren R, Levy Y, Ronen D, Zick Y. 2005. Cyclin-dependent kinase inhibitors and JNK act as molecular switches, regulating the choice between growth arrest and apoptosis induced by galectin-8. J Biol Chem. 280:19105–19114.

Baptiste TA, James A, Saria M, Ochieng J. 2007. Mechano-transduction mediated secretion and uptake of galectin-3 in breast carcinoma cells: Implications in the extracellular functions of the lectin. Exp Cell Res. 313:652–664.

Bidon-Wagner N, Le Pennec JP. 2004. Human galectin-8 isoforms and cancer. Glycoconj J. 19:557–563.

Boura-Halfon S, Voliovitch H, Feinstein R, Paz K, Zick Y. 2003. Extracellular matrix proteins modulate endocytosis of the insulin receptor. J Biol Chem. 278:16397–16404.

Carcamo C, Pardo E, Oyanadel C, Bravo-Zehnder M, Bull P, Caceres M, Martinez J, Massardo L, Jacobelli S, Gonzalez A, et al. 2006. Galectin-8 binds specific β1 integrins and induces polarized spreading highlighted by asymmetric lamellipodia in Jurkat T cells. Exp Cell Res. 312: 374–386.

Carlsson S, Oberg C, Carlsson MAS, Nilsson U, Smith D, Cummings R, Almkvist J, Karlsson A, Leffler H. 2007. Affinity of galectin-8 and its carbohydrate recognition domains for ligands in solution and at the cell surface. Glycobiology. 17:663–676.

Conner SD, Schmid SL. 2003. Regulated portals of entry into the cell. Nature. 422:37–44.

Delacour D, Cramm-Behrens CI, Drobecq H, Le Bivic A, Naim HY, Jacob R. 2006. Requirement for galectin-3 in apical protein sorting. Current Biol. 16:408–414.

Delacour D, Gouyer V, Zanetta J-P, Drobecq H, Leteurtre E, Grard G, Moreau-Hannedouche O, Maes E, Pons A, Andre S, et al. 2005. Galectin-4 and sulfatides in apical membrane trafficking in enterocyte-like cells. J Cell Biol. 169:491–501.

Delacour D, Greb C, Koch A, Salomonsson E, Leffler H, Le Bivic A, Jacob R. 2007. Apical sorting by galectin-3-dependent glycoprotein clustering. Traffic. 8:379–388.

Ellis MA, Potter BA, Cresawn KO, Weisz OA. 2006. Polarized biosynthetic traffic in renal epithelial cells: Sorting, sorting, everywhere. Am J Physiol Renal Physiol. 291:F707–713.

Furtak V, Hatcher F, Ochieng J. 2001. Galectin-3 mediates the endocytosis of β-1 integrins by breast carcinoma cells. Biochem Biophy Res Commun. 289:845–850.

Hirabayashi J, Hashidate T, Arata Y, Nishi N, Nakamura T, Hirashima M, Urashima T, Oka T, Futai M, Muller WE, et al. 2002. Oligosaccharide specificity of galectins: A search by frontal affinity chromatography. Biochim Biophys Acta. 1572:232–254.

Houzelstein D, Goncalves IR, Fadden AJ, Sidhu SS, Cooper DN, Drickamer K, Leffler H, Poirier F. 2004. Phylogenetic analysis of the vertebrate galectin family. Mol Biol Evol. 21:1177–1187.

Huet G, Gouyer V, Delacour D, Richet C, Zanetta JP, Delannoy P, Degand P. 2003. Involvement of glycosylation in the intracellular trafficking of glycoproteins in polarized epithelial cells. Biochim. 85:323–330.

Ideo H, Seko A, Ishizuka I, Yamashita K. 2003. The N-terminal carbohydrate recognition domain of galectin-8 recognizes specific glycosphingolipids with high affinity. Glycobiology. 13:713–723.

Iglesias-Bartolome R, Crespo PM, Gomez GA, Daniotti JL. 2006. The antibody to GD3 ganglioside, R24, is rapidly endocytosed and recycled to the plasma membrane via the endocytic recycling compartment. Inhibitory effect of brefeldin A and monensin. Febs J. 273:1744–1758.

Lahm H, Andre S, Hoeflich A, Kaltner H, Siebert HC, Sordat B, von der Lieth CW, Wolf E, Gabius HJ. 2004. Tumor galectinology: Insights into the complex network of a family of endogenous lectins. Glycoconj J. 20:227–238.

Lau KS, Partridge EA, Grigorian A, Silvescu CI, Reinhold VN, Demetriou M, Dennis JW. 2007. Complex *N*-glycan number and degree of branching cooperate to regulate cell proliferation and differentiation. Cell. 129:123–134.

Leffler H, Carlsson S, Hedlund M, Qian Y, Poirier F. 2004. Introduction to galectins. Glycoconj J. 19:433–440.

Nabi IR, Le PU. 2003. Caveolae/raft-dependent endocytosis. J Cell Biol. 161:673–677.

Nagaoka MR, Strital E, Kouyoumdjian M, Borges DR. 2002. Participation of a galectin-dependent mechanism in the hepatic clearance of tissue-type plasminogen activator and plasma kallikrein. Thromb Res. 108:257–262.

Nickel W. 2005. Unconventional secretory routes: Direct protein export across the plasma membrane of mammalian cells. Traffic. 6:607–614–535.

Ochieng J, Furtak V, Lukyanov P. 2002. Extracellular functions of galectin-3. Glycoconj J. 19:527–535.

Partridge EA, Le Roy C, Di Guglielmo GM, Pawling J, Cheung P, Granovsky M, Nabi IR, Wrana JL, Dennis JW. 2004. Regulation of cytokine receptors by Golgi *N*-glycan processing and endocytosis. Sci. 306:120–124.

Patnaik SK, Potvin B, Carlsson S, Sturm D, Leffler H, Stanley P. 2006. Complex *N*-glycans are the major ligands for galectin-1, -3, and -8 on chinese hamster ovary cells. Glycobiology. 16:305–317.

Patnaik SK, Stanley P. 2006. Lectin-resistant CHO glycosylation mutants. Methods Enzymol. 416:159–182.

Potter BA, Hughey RP, Weisz OA. 2006a. Role of *N*- and *O*-glycans in polarized biosynthetic sorting. Am J Physiol Cell Physiol. 290:C1–C10.

Potter BA, Weixel KM, Bruns JR, Ihrke G, Weisz OA. 2006b. *N*-glycans mediate apical recycling of the sialomucin endolyn in polarized MDCK cells. Traffic. 7:146–154.

Slimane TA, Lenoir C, Sapin C, Maurice M, Trugnan G. 2000. Apical secretion and sialylation of soluble dipeptidyl peptidase IV are two related events. Exp Cell Res. 258:184–194.

Zhuang Y, Cragoe EJ Jr, Shaikewitz T, Glaser L, Cassel D. 1984. Characterization of potent Na^+/H^+ exchange inhibitors from the amiloride series in A431 cells. Biochem. 23:4481–4488.

Zick Y, Eisenstein M, Goren RA, Hadari YR, Levy Y, Ronen D. 2004. Role of galectin-8 as a modulator of cell adhesion and cell growth. Glycoconj J. 19:517–526.