Cell surface counter receptors are essential components of the unconventional export machinery of galectin-1

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Galectin-1 is a component of the extracellular matrix as well as a ligand of cell surface counter receptors such as β-galactoside-containing glycolipids, however, the molecular mechanism of galectin-1 secretion has remained elusive. Based on a nonbiased screen for galectin-1 export mutants we have identified 26 single amino acid changes that cause a defect of both export and binding to counter receptors. When wild-type galectin-1 was analyzed in CHO clone 13 cells, a mutant cell line incapable of expressing functional galectin-1 counter receptors, secretion was blocked. Intriguingly, we also find that a distant relative of galectin-1, the fungal lectin CGL-2, is a substrate for nonclassical export from Chinese hamster ovary (CHO) cells. Alike mammalian galectin-1, a CGL-2 mutant defective in β-galactoside binding, does not get exported from CHO cells. We conclude that the β-galactoside binding site represents the primary targeting motif of galectins defining a galectin export machinery that makes use of β-galactoside-containing surface molecules as export receptors for intracellular galectin-1.

Introduction

Galectins are β-galactoside–specific lectins being associated with components of the extracellular matrix and counter receptors on the cell surface of mammalian cells (Liu and Rabinovich, 2005). Provided they contain β-galactosides, both glycoproteins and glycolipids can be clustered through interactions with multivalent galectins, a key event in galectin signal transmission to downstream targets (Brewer et al., 2002). Galectins have been implicated in a wide range of physiological processes such as apoptosis, tumor progression, inflammation, cell adhesion, and others (Perillo et al., 1995; He and Baum, 2004). However, galectins do not contain NH2-terminal signal peptides for efficient and quantitative secretion but rather appear to be tightly balanced between intra- and extracellular populations (Hughes, 1999; Nickel, 2003). Even though it has been known for more than 15 yr that Gal-1 export is mediated by an unconventional secretary mechanism (Cooper and Barondes, 1990; Muesch et al., 1990), the molecular machinery involved has remained elusive. However, based on various experimental systems, evidence is accumulating that Gal-1 export is mediated by direct translocation from the cytoplasm across the plasma membrane into the extracellular space (Schäfer et al., 2004; Nickel, 2005).

So far, it has been assumed that galectins do not interact with their counter receptors until they have been released into the extracellular space. In the current study, we provide direct evidence that these interactions are an integral part of the export mechanism itself. We report that both Gal-1 mutants deficient in β-galactoside binding and mutant cell lines deficient in the biogenesis of galectin counter receptors are defective with regard to Gal-1 secretion. These data are further emphasized by the finding that CGL-2, a distant relative from the multicellular fungus Coprinopsis cinerea with only limited similarity to mammalian galectin-1 at the level of both primary and quaternary structure (Lobsanov et al., 1993; Walser et al., 2004) and, therefore, not being expected to be recognized as an export substrate in mammalian cells, is found to be secreted from CHO cells. Alike mammalian Gal-1, CGL-2 export from CHO cells is blocked...
Identification of Gal-1 mutants deficient in binding to β-galactosides

To elucidate the export targeting motif we generated more than 100 single amino acid mutants of human Gal-1. The individual mutants were selected in three different ways: (1) a random mutagenesis using a low fidelity PCR; (2) targeted mutagenesis of surface residues based on the crystal structure of galectin-1 (Lobsanov et al., 1993); and (3) targeted mutagenesis of residues conserved between human Gal-1 and CGL-2 from C. cinerea. All mutants were stably expressed as GFP fusion proteins in CHO cells using a doxycycline-dependent transactivator system (Engling et al., 2002). All mutant galectin proteins were analyzed by SDS-PAGE and Western blotting using affinity-purified anti-GFP antibodies. For further details see Materials and methods.

Results

Table I. Single amino acid changes in human Gal-1 that cause β-galactoside binding deficiency

| Mutation | Selection procedure/reference |
|----------|-------------------------------|
| N34A     | Random mutagenesis/conserved between Ga-1 and CGL-2 |
| D38K     | Surface exposure               |
| N41A     | Random mutagenesis/conserved between Ga-1 and CGL-2 |
| L44A     | Conserved between Ga-1 and CGL-2 |
| L44D     | Conserved between Ga-1 and CGL-2 |
| L44S     | Conserved between Ga-1 and CGL-2 |
| H45A     | Random mutagenesis/conserved between Ga-1 and CGL-2; Scott and Zhang, 2002 |
| F46A     | Conserved between Ga-1 and CGL-2 |
| R49A     | Conserved between Ga-1 and CGL-2; Scott and Zhang, 2002; Ford et al., 2003 |
| H53A     | Random mutagenesis/surface exposure; Lopez-Lucendo et al., 2004 |
| H53E     | Random mutagenesis/surface exposure; Lopez-Lucendo et al., 2004 |
| H53G     | Random mutagenesis/surface exposure; Lopez-Lucendo et al., 2004 |
| G54A     | Conserved between Ga-1 and CGL-2 |
| N57A     | Random mutagenesis              |
| V60A     | Random mutagenesis              |
| W69G     | Hirabayashi and Kasai, 1991     |
| E72A     | Conserved between Ga-1 and CGL-2; Hirabayashi and Kasai, 1991 |
| R74A     | Conserved between Ga-1 and CGL-2; Hirabayashi and Kasai, 1991 |
| F80K     | Random mutagenesis/conserved between Ga-1 and CGL-2 |
| F80S     | Random mutagenesis/conserved between Ga-1 and CGL-2 |
| R112A    | Conserved between Ga-1 and CGL-2 |
| Y120D    | Random mutagenesis/conserved between Ga-1 and CGL-2 |
| F127D    | Random mutagenesis/conserved between Ga-1 and CGL-2 |
| H1129    | Surface exposure                |
| V132E    | Random mutagenesis/surface exposure |
| V132R    | Random mutagenesis/surface exposure |

Based on the procedures and/or references shown in the right column, a total of 26 amino acid changes are listed that cause a complete block of binding to β-galactoside-containing glycolipids and glycoproteins. For details see text.

Gal-1 mutants deficient for binding to β-galactosides are also deficient for export from CHO cells

In previous studies, we established both a cell surface biotinylation assay and a FACS-based assay system designed to quantitatively assess secretion of Gal-1 and other unconventional secretory proteins from CHO cells (Seelenmeyer et al., 2003; Stegmayr et al., 2005). To quantitatively study export of Gal-1 mutants deficient in binding to β-galactosides, we combined
these assays with immunoprecipitation of Gal-1–GFP fusion proteins from the medium of the expressing cells using affinity-purified anti-GFP antibodies. As shown in Fig. 2 A, most of the extracellular Gal-1–GFP population was found to be associated with the cell surface of CHO cells (lane 2) with only a minor portion being found in the medium (lane 3). As controls, both GFP without Gal-1 tag (Fig. 2 F) and endogenous Gal-1 (Fig. 2 G) were compared with Gal-1–GFP. As expected, GFP could not be detected on the cell surface (Fig. 2 F, lane 2) and only small amounts were detectable in the medium (Fig. 2 F, lane 3). By contrast, endogenous Gal-1 was found both on the cell surface and in the medium of CHO cells (Fig. 2 G). The overall efficiency of Gal-1 versus Gal-1–GFP secretion was similar with ~10% of each reporter molecule being localized to the extracellular space under steady-state conditions. These data indicate that tagging of Gal-1 with GFP does not interfere with its secretion. Consistent with the biochemical data, the wild-type form of Gal-1–GFP was detected on the cell surface using the FACS assay as shown in Fig. 3. As expected, mutant forms of Gal-1–GFP such as W69G and E72A, which are defective with regard to binding to β-galactosides, could not be detected on the cell surface using both the biotinylation (Fig. 2, B and C, lane 2) and the FACS assay (Fig. 3). Strikingly, however, Gal-1–GFPW69G and Gal-1–GFP E72A were also absent from the medium suggesting that they are not secreted from CHO cells (Fig. 2, B and C, lane 3). We then tested whether these Gal-1 mutants are stable in conditioned medium derived from CHO cells in order to analyze whether protein degradation causes their absence from the supernatants of Gal-1–GFPW69G and Gal-1–GFP E72A-expressing cells. As demonstrated in Fig. 4, both the wild-type form of Gal-1–GFP (A) and the β-galactoside-binding mutants (B and C, respectively) are not degraded when incubated in conditioned CHO medium at 37°C for 48 h (compare lanes 2 and 4). Thus, the absence of the reporters from both the cell surface and the medium of Gal-1–GFPW69G- and Gal-1–GFP E72A-expressing CHO cells demonstrates that they are no substrates for the Gal-1 export machinery. Intriguingly, the combined phenotypes for Gal-1–GFPW69G and Gal-1–GFP E72A shown in Figs. 2–4 were also

Figure 2. Biochemical analysis of export of various galectin–GFP fusion proteins from CHO cells using cell surface biotinylation and immunoprecipitation from cell culture supernatants. The fusion proteins indicated were expressed in CHO cells for 48 h at 37°C (six-well plates; 70% confluency). The medium was removed and subjected to immunoprecipitation using affinity-purified anti-GFP antibodies. Cell surfaces were treated with a membrane-impermeable biotinylation reagent. After detergent-mediated cell lysis biotinylated and nonbiotinylated proteins were separated using streptavidin beads. Aliquots from the input material (lane 1; 1%), the biotinylated fraction (lane 2; 10%) and the immunoprecipitate from the cell culture medium fraction (lane 3; 50%) were analyzed by SDS-PAGE and Western blotting using affinity-purified anti-GFP antibodies. In G, affinity-purified anti-Gal-1 antibodies were used to detect endogenous Gal-1. For further details see Materials and methods.

Figure 3. Quantitative analysis of export of various galectin–GFP fusion proteins from CHO cells using flow cytometry. CHO cells were grown on six-well plates and induced with doxycycline for 48 h at 37°C to express the fusion proteins indicated. After removal of the medium, cells were labeled with affinity-purified anti-GFP antibodies while they were still attached to the culture dishes. Primary antibodies were labeled with APC-conjugated secondary antibodies followed by detachment of the cells using PBS/EDTA. GFP (expression level; green) and APC-derived fluorescence (cell surface; blue) were quantified by flow cytometry using a FACSCalibur system (Becton Dickinson; n = 4). For further details see Materials and methods.
found for all other β-galactoside–binding mutants of Gal-1 listed in Table I (unpublished data). These findings imply that binding to β-galactosides of Gal-1 is a prerequisite to enter the export pathway.

**CGL-2**, a distant relative of Gal-1 from the multicellular fungus *C. cinerea*, is exported from CHO cells depending on its ability to bind to β-galactosides

To challenge the hypothesis that the binding capacity of Gal-1 to β-galactosides is a requirement for its export we analyzed whether the fungal galectin ortholog CGL-2 is a substrate for the Gal-1 export pathway in mammalian cells. Because homologies between Gal-1 and CGL-2 are extremely weak at the level of both the primary and the quaternary structure (Lobsanov et al., 1993; Walser et al., 2004) and, in addition to the typical galectin fold, efficient binding to β-galactosides is a common denominator of Gal-1 and CGL-2, we analyzed whether CGL-2 is an export substrate in CHO cells. In both cell surface biotinylation (Fig. 2 D) and flow cytometry (Fig. 3) experiments, a CGL-2–GFP fusion protein was indeed found to be an export substrate with similar efficiency as compared with Gal-1. Intriguingly, a single-site mutation in a tryptophan residue (W72G; analogous to W69G in Gal-1) essential for β-galactoside binding (Walser et al., 2004; and Fig. 1, A and B) results in a complete export block from CHO cells of CGL-2–GFP (Fig. 2 E and Fig. 3). These data are consistent with the idea

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**Figure 4.** Stability of galectin–GFP fusion proteins in conditioned media derived from CHO cells. The fusion proteins indicated were expressed in CHO cells for 48 h at 37°C (six-well plates; 70% confluency). From each cell line, a cell-free supernatant was prepared. Normalized amounts (GFP fluorescence) were incubated in conditioned medium derived from CHO cells for the times indicated followed by immunoprecipitation (lanes 2–4) using affinity-purified anti-GFP antibodies. The samples were analyzed by SDS-PAGE and Western blotting using antibodies directed against GFP. Lane 1, input (no IP, 10%); lane 2, no incubation (IP, 10%); lane 3, incubation for 48 h at 4°C (IP, 10%); lane 4, incubation for 48 h at 37°C (IP, 10%). For further details see Materials and methods.

**Figure 5.** Comparative analysis of export of galectin–GFP fusion proteins from CHO wild-type and CHO clone 13 cells using cell surface biotinylation and immunoprecipitation from cell culture supernatants. The fusion proteins indicated were expressed in both CHO wild-type (A–F) and CHO clone 13 cells (G–L) for 48 h at 37°C (six-well plates; 70% confluency). The medium was removed and subjected to immunoprecipitation using affinity-purified anti-GFP antibodies. Cell surfaces were treated with a membrane-impermeable biotinylation reagent. After detergent-mediated cell lysis, biotinylated and non-biotinylated proteins were separated using streptavidin beads. Aliquots from the input material (lane 1; 0.25%), the biotinylated fraction (lane 2; 25%) and the immunoprecipitate from the cell culture medium fraction (lane 3; 25%) were analyzed by SDS-PAGE and Western blotting using affinity-purified anti-GFP antibodies. For further details see Materials and methods.
that the β-galactoside binding site is the primary export targeting motif of galectins and that interactions of galectins with their counter receptors are essential for export.

Wild-type forms of both Gal-1 and CGL-2 fail to be exported from CHO cells lacking functional counter receptors for galectins

To conclusively address the question whether counter receptors play a direct role in the export mechanism of galectins, we generated cell lines expressing various forms of Gal-1- and CGL-2–GFP fusion proteins based on a CHO mutant termed clone 13 (Briles et al., 1977; Deutscher and Hirschberg, 1986). In this mutant cell line, the Golgi apparatus–resident transporter for UDP-galactose is defective and hence, these cells do not produce galectin counter receptors as galactosyltransferases in the Golgi lumen do not receive activated galactose residues as substrates (Deutscher and Hirschberg, 1986). As a result, both glycoproteins and glycolipids derived from clone 13 cells do not contain β-galactosides in their sugar moieties and, therefore, do not bind galectins on their cell surface (unpublished data).

As demonstrated in the cell surface biotinylation experiments shown in Fig. 5, both Gal-1–GFP (A, lanes 2 and 3) and CGL-2–GFP (D, lanes 11 and 12) are efficiently exported from CHO wild-type cells (combined signals for cell surface and medium fractions). By contrast, when expressed in CHO clone 13 cells, the wild-type forms of Gal-1- and CGL-2–GFP fusion proteins fail to get access to the extracellular space as the combined signals for cell surface and medium fractions (Fig. 5 G [lanes 2 and 3] and J [lanes 11 and 12], respectively) do not differ significantly from the negative control (GFP; Fig. 5, G, F, and L [lanes 17 and 18]) and are largely reduced as compared with those observed in CHO wild-type cells (A and D, respectively). As expected, export of β-galactoside binding-deficient mutants (as exemplified by W69G and E72A for Gal-1 as well as W72G for CGL-2) is not only blocked in CHO wild-type cells (Fig. 2, B, C, and E; Fig. 5, B, C, and E) but also in CHO clone 13 cells (Fig. 5, H, I and K, respectively).

In Fig. 6, export from both CHO wild-type and CHO clone 13 cells of the various galectin–GFP fusion proteins (as measured by cell surface biotinylation and immunoprecipitation from the cell culture supernatants) was quantified using fluorescent secondary antibodies combined with a LI-COR Odyssey imaging system. For each fusion protein, the combined signals derived from cell surface biotinylation and material from the cell culture supernatant were expressed as a percentage of the overall expression level of a given fusion protein. To compare export of the various fusion proteins, secretion of Gal-1–GFP from CHO wild-type cells was set to 100%. As demonstrated in Fig. 6, the wild-type forms of both Gal-1–GFP and CGL-2–GFP are secreted from CHO wild-type cells, however, export is largely reduced when Gal-1–GFP and CGL-2–GFP are expressed in CHO clone 13 cells. The various mutant forms of Gal-1 and CGL-2 are neither exported from CHO wild-type nor from CHO clone 13 cells to a significant extent.

Figure 6. Quantitation of export of galectin–GFP fusion proteins from CHO wild-type and CHO clone 13 cells using cell surface biotinylation and immunoprecipitation from cell culture supernatants. The fusion proteins indicated were expressed in both CHO wild-type and CHO clone 13 cells for 48 h at 37°C (six-well plates; 70% confluency). The medium was removed and subjected to immunoprecipitation using affinity-purified anti-GFP antibodies. Cell surfaces were treated with a membrane-impermeable biotinylation reagent. After detergent-mediated cell lysis, biotinylated and nonbiotinylated proteins were separated employing streptavidin beads. Aliquots from the input material (0.25%), the biotinylated fraction (25%) and the immunoprecipitate from the cell culture medium fraction (25%) were analyzed by SDS-PAGE and Western blotting using affinity-purified anti-GFP antibodies. Primary antibodies were detected with Alexa 680-coupled anti-rabbit secondary antibodies. Signals for galectin–GFP fusion proteins and GFP were quantified using a Odyssey imaging system (LI-COR Biotechnology). The combined signals for the cell medium and the material associated with the cell surface were calculated as a percentage of the total amount of galectin–GFP fusion protein expressed in each case. These data were corrected for unspecific release as monitored by GFP present in the medium of the cells. The extracellular population of Gal-1–GFP secreted from CHO wild-type cells was set to 100%. For further details see Materials and methods.

Subcellular distribution of Gal-1–GFP and CGL2–GFP reporter molecules in CHO wild-type and CHO clone 13 cells

To compare the subcellular distribution of Gal-1 and CGL-2 mutant forms with the corresponding wild-type proteins, we analyzed the cell lines described above by confocal microscopy (Fig. 7). When living CHO wild-type cells were imaged, all reporters were found in the cytoplasm as well as to some extent in the nucleus (Fig. 7, first column). When CHO wild-type and CHO clone 13 (unpublished data) were compared, no differences in live cell imaging could be observed for any of the reporter molecules being analyzed. In general, a similar picture was ob-
served after fixation both for CHO wild-type and CHO clone 13 cells (Fig. 7, second and fourth column, respectively), however, in some cases aggregates or particulate structures were observed that apparently represent fixation artifacts. Consistent with the FACS experiments shown in Fig. 3, cell surface staining of all cell lines using affinity-purified anti-GFP antibodies revealed an extracellular population in CHO wild-type cells only for the wild-type forms of Gal-1–GFP and CGL2–GFP (Fig. 7, third column). In CHO clone 13 cells, cell surface staining could not be detected for any of the reporter molecules including the wild-type forms of Gal-1 and CGL-2 (Fig. 7, fifth column).

Discussion

Galectins are extracellular modulators of a broad variety of biomedically relevant cellular processes such as tumor progression and apoptosis (Liu and Rabinovich, 2005). For example, galectin-1 is overproduced and secreted in large amounts from tumor cells, a strategy of immune suppression by inducing apoptosis of activated T cells (Perillo et al., 1995). However, the export mechanism does not rely on the classical secretory pathway as galectins neither contain signal peptides nor is the efficiency of galectin export affected by brefeldin A (Hughes, 1999), a drug that blocks ER/Golgi apparatus–dependent secretory transport (Misumi et al., 1986; Lippincott-Schwartz et al., 1989; Orci et al., 1991). Galectins have therefore been termed unconventional secretory proteins, however, the molecular machinery mediating galectin export from mammalian cells has remained elusive (Hughes, 1999; Nickel, 2003).

In this study we identify galectin counter receptors (i.e., β-galactoside–containing cell surface glycolipids and/or glycoproteins) as essential components of the overall process of Gal-1 secretion. Based on an unbiased screen, we have identified 26 single-site mutations in Gal-1 that cause both binding deficiency to counter receptors and export deficiency. In fact, we could not identify a single Gal-1/β-galactoside binding mutant that retained export competence. To put this finding one step further we analyzed Gal-1 secretion in a somatic CHO mutant cell line defective in a Golgi apparatus–resident transporter that is required for translocation of activated galactose from the cytoplasm into the lumen of the Golgi apparatus, a process essential for the generation of β-galactoside–containing glycolipids and glycoproteins (Deutscher and Hirschberg, 1986). Intriguingly, we found that wild-type Gal-1 fails to get exported from this mutant cell line demonstrating that indeed secretion of Gal-1 from mammalian cells strictly depends on functional interactions between Gal-1 and its counter receptors. These data are emphasized by our findings on CGL-2, a distant relative of
Gal-1 from the multicellular fungus *C. cinerea*. Even though similarities between CGL-2 and human Gal-1 are very weak at both the level of the primary and the quaternary structure (Lobsanov et al., 1993; Walser et al., 2004), CGL-2 is recognized by mammalian cells as an export substrate. Strikingly, a single-site mutation (W72G) that is known to cause CGL-2 binding deficiency to β-galactosides (Walser et al., 2004), results in a block of export from CHO cells of CGL-2, again consistent with our finding that functional interactions with counter receptors are essential for the overall export process.

Regarding the molecular mechanism of Gal-1 export from mammalian cells, there are two possible scenarios that would be consistent with the data presented in this study. On the one hand, galectin counter receptors on the cell surface might be part of a molecular trap through which secreted Gal-1 molecules would be removed from an equilibrium between an intracellular and an extracellular pool of Gal-1. In principle, the extracellular galectin trap could be necessary for sustained Gal-1 export of the cytoplasmic pool. However, in the absence of functional interactions between Gal-1 and its counter receptors, Gal-1 export is apparently fully blocked (Fig. 2). Therefore, the trapping mechanism does not satisfactorily explain our observations as Gal-1 transport should be observable at least to some extent until a certain equilibrium between intra- and extracellular pools is reached. Therefore, in a variation of this model, β-galactoside–containing cell surface molecules might be tightly coupled to the translocation machinery. In this way, counter receptors might function by exerting a pulling force at the extracellular side of the putative translocation pore required for directional transport of galectin-1 across the plasma membrane.

An alternative explanation of our results would be that galectin counter receptors act as export receptors for Gal-1. It is indeed tempting to speculate that Gal-1 interactions with counter receptors are not restricted to the extracellular space but rather already occur at the cytoplasmic side of the plasma membrane. This assumption does, of course, not comply with the established view, namely that for example glycolipids are exclusively localized to the extracellular leaflet of the plasma membrane with the glycan moieties being exposed to the extracellular space. However, it appears possible that a limited number of, for example, β-galactoside–containing glycolipids gets translocated to the inner leaflet of the plasma membrane catalyzed by a plasma membrane-resident flippase. This subpopulation probably would not be detectable under steady-state conditions. In this model, retrotranslocation of counter receptors occupied by Gal-1 would mediate export to the extracellular space.

Although both models are certainly speculative at this point, they are consistent with several observations that have been made previously. First, Gal-1 membrane translocation has been reported to occur at the level of the plasma membrane (Cooper and Barondes, 1990; Mehul and Hughes, 1997; Hughes, 1999; Nickel, 2003; Schäfer et al., 2004). Second, Huet and colleagues recently reported pharmacological evidence that galectin-4 secretion is impaired in epithelial cells after treatment with 1-benzyl-2-acetamido-2-deoxy-α-D-galactopyranoside, an inhibitor of glycosylation (Delacour et al., 2005). Third, it has been reported that membrane translocation of both FGF-2 and Gal-1 occurs in a folded state (Backhaus et al., 2004). In the context of the current study, this finding is of particular interest as both models described above can only be true if the β-galactoside binding site of Gal-1 remains functional during membrane translocation. In this regard, the putative translocation machinery might be functionally related to the bacterial twin-arginine translocation system through which proteins are secreted in a fully folded state (Robinson and Bolhuis, 2004; Nickel, 2005).

The findings presented in this study also provide a potential explanation for the apparent nonexistence of a linear targeting motif in Gal-1. Our data conclusively point to a direct role of counter receptors as export adaptors and the β-galactoside binding motif of Gal-1 as the primary targeting element. In this context, it is interesting to note that secretion from *Saccharomyces cerevisiae* of both rat galectin-1 and *C. cinerea* CGL-2 has been reported (Cleves et al., 1996; Boulianne et al., 2000). This is because this organism does not contain endogenous galectins and the existence of glycolipids and glycoproteins containing β-galactosides has not been clarified. It, therefore, remains to be investigated whether secretion of galectins from *S. cerevisiae* occurs by a molecular mechanism similar to that of mammalian cells. Finally, it is of note that the secretion mechanism being postulated in this study provides a functional basis for quality control in the overall process of Gal-1 secretion. As the β-galactoside binding motif of Gal-1 is shown to be the primary targeting element for secretion, quality control is in place since only properly folded Gal-1 will be recognized by the export machinery (Nickel, 2005).

### Materials and methods

#### Antibodies

For flow cytometry analyses, immunoprecipitation experiments, and Western blot detection of galectin–GFP fusion proteins affinity-purified anti-GFP antibodies were used (Engling et al., 2002). Secondary antibodies used for flow cytometry (allophycocyanin [APC]–conjugated anti-rabbit antibodies) were from Invitrogen, HRP-coupled secondary antibodies used for Western blot detection (ECL) of immunoprecipitates were from Sigma-Aldrich (clone KG-16). Alexa 680–coupled anti–rabbit antibodies were used for quantitative analyses of antigens processed by Western blotting (Fig. 6; Odyssey system; LI-COR Biotechnology) were purchased from Invitrogen.

#### Generation of stable cell lines expressing both wild-type and mutant forms of galectin–GFP fusion proteins in a doxycycline-dependent manner

Mutant forms of Gal-1 and CGL-2 were generated by PCR-based site-directed mutagenesis and cloned as GFP fusion constructs into the retroviral vector pREV-TRE2. CHO cell lines expressing the various fusion proteins in a doxycycline-dependent manner were generated as described previously (Engling et al., 2002; Seelenmeyer et al., 2003).

#### Quantitative analysis of galectin binding to cell surfaces using flow cytometry

Both wild-type and mutant forms of Gal-1–GFP and CGL-2–GFP fusion proteins were expressed in CHO cells by incubating the cells in the presence of doxycycline (1 μg/ml) for 48 h at 37°C. Cell-free supernatants were prepared by homogenization combining freeze–thaw cycles with sonication. Membranes were removed in two steps by centrifugation at 1,000 g$_{av}$ (10 min at 4°C) and 100,000 g$_{av}$ (1 h at 4°C). The resulting supernatants were analyzed for the amounts of fusion protein based on GFP fluorescence as measured with a fluorescence plate reader (SpectraMax Gemini XS; Molecular Devices Corp.). Normalized amounts of cell-free supernatants (150 GFP units corresponding to ~1.5 μg GFP) were then...
incubated with CHO cells not expressing galectin–GFP fusion proteins (CHO/GF146; Engling et al., 2002) for 48 h at 37°C. After detachment of cells from the culture dishes using PBS/EDTA, cells were sedimented and solubilized in PBS/TX-100. Normalized amounts of the detergent lysates (50 GF units corresponding to ~0.5 μg GFP; SpectraMax Gemini XS; Molecular Devices Corp.) were then incubated with lactose beads (Sigma-Aldrich) for 1 h at 4°C. After extensive washing in TX-100–containing buffer, bound material was eluted using SDS sample buffer. Both the flow-through fraction and the SDS eluates were analyzed by SDS-PAGE and Western blotting using anti-GFP antibodies as described in the legend of Fig. 1.

Biochemical analysis of galectin export from CHO cells using cell surface biotinylation and immunoprecipitation from cell culture supernatants

Galectin–GFP fusion proteins were expressed in the corresponding CHO cell lines by incubation in the presence of doxycycline (1 μg/ml) for 48 h at 37°C (six-well plates; 70% confluency). The medium was removed and the cells washed once with PBS. Medium and PBS wash buffer were combined and subjected to immunoprecipitation using affinity-purified anti-GFP antibodies. To analyze secretion of endogenous Gal-1, affinity-purified anti-Gal-1 antibodies (Seelenmeyer et al., 2003) were used for immunoprecipitation experiments. Cell surfaces were treated with a membrane-impermeable biotinylation reagent (EZ-Link Sulfo-NHS-SS-Biotin; Pierce Chemical Co.). After detergent-mediated cell lysis, the cells were scrapped off the culture plates, insoluble material was then removed by low speed centrifugation. Biotinylated and nonbiotinylated proteins were separated using streptavidin beads (Ultralink immobilized streptavidin; Pierce Chemical Co.). Material bound to streptavidin beads was eluted with SDS sample buffer. Samples from the input material and the nonbound fraction and the streptavidin-bound fraction were analyzed by SDS-PAGE and Western blotting as indicated in the legends to Figs. 2 and 5. For the biotinylation and immunoprecipitation experiments depicted in Fig. 6, secondary antibodies coupled to a fluorophor (Alexa 680) were used to allow quantitation of the signal using a Odyssey imaging system (LI-COR Biotechnology).

Quantitative analysis of galectin export from CHO cells using flow cytometry

Galectin–GFP fusion proteins as well as GFP were expressed in the corresponding CHO cell lines by incubation in the presence of doxycycline (1 μg/ml) for 48 h at 37°C (six-well plates; 70% confluency). The medium was removed and the cells washed once with PBS. Both primary (anti-GFP; 1 h) and secondary [APC-conjugated anti–rabbit; 30 min] antibody labeling was conducted at 4°C with the cells being attached to the culture plates. The cells were then detached from the culture plates using cell dissociation buffer (Life Technologies). Before the flow cytometry analysis, propidium iodide (1 μg/ml) was added in order to detect damaged cells. GFP (expression level) and APC-derived fluorescence (cell surface population) were analyzed using a FACScalibur flow cytometer (Beckton Dickinson). Background fluorescence was determined by measuring CHO/MACTAM5 cells (Engling et al., 2002), which were treated with both primary and secondary APC-conjugated secondary antibodies. GFP- and APC-derived fluorescence were measured simultaneously without compensation.

Stability analysis of both wild-type and mutant forms of galectin–GFP fusion proteins in conditioned media derived from CHO cells

Cell-free supernatants of both wild-type and mutant forms of Gal-1–GFP and CGL2–GFP fusion proteins as well as GFP as a control were obtained as described above. Normalized amounts (50 GF units corresponding to ~0.5 μg GFP; SpectraMax Gemini XS; Molecular Devices Corp.) were then treated with conditioned medium derived from CHO cells. Samples were then either directly subjected to immunoprecipitation using anti-GFP antibodies (Fig. 4, lane 2), incubated for 48 h at 4°C followed by immunoprecipitation (Fig. 4, lane 3) or incubated for 48 h at 37°C followed by immunoprecipitation (Fig. 4, lane 4). In each case, bound material was eluted with SDS sample buffer. The samples were then analyzed by SDS-PAGE and Western blotting using anti-GFP antibodies and anti–rabbit secondary antibodies (clone RG-16; see above) coupled to HRP (ECL detection).

Confocal microscopy

CHO cells transduced with the reporter constructs indicated were grown on glass coverslips for 36 h at 37°C in the presence of 1 μg/ml doxycycline (70% confluency). The cells were then either processed for live imaging or subjected to paraformaldehyde fixation (3% wt/vol, 20 min at 4°C) without permeabilization followed by antibody processing as indicated. Alexa546-coupled secondary antibodies were used for cell surface staining experiments. The specimens were mounted in Fluoromount G (Southern Biotechnology Associates, Inc.) and viewed with a confocal microscope (LSM 510; Carl Zeiss Microlmaging, Inc.).

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