Fungal Galectins, Sequence and Specificity of Two Isolectins from *Coprinus cinereus*

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Galectins are members of a genetically related family of β-galactoside-binding lectins. At least eight distinct mammalian galectins have been identified. More distantly related, but still conserving amino acid residues critical for carbohydrate-binding, are galectins in chicken, eel, frog, nematode, and sponge. Here we report that galectins are also expressed in a species of fungus, the inky cap mushroom, *Coprinus cinereus*. Two dimeric galectins are expressed during fruiting body formation which are 83% identical to each other in amino acid sequence and conserve all key residues shared by members of the galectin family. Unlike most galectins, these have no N-terminal post-translational modification and no cysteine residues. We expressed one of these as a recombinant protein and studied its carbohydrate-binding specificity using a novel nonradioactive assay. Binding specificity has been well studied for a number of other galectins, and like many of these, the recombinant *C. cinereus* galectin shows particular affinity for blood group A structures. These results demonstrate not only that the galectin gene family is evolutionarily much older than previously realized but also that fine specificity for complex saccharide structures has been conserved. Such conservation implies that galectins evolved to perform very basic cellular functions, presumably by interaction with glycoconjugates bearing complex lactoside carbohydrates resembling blood group A.

Galectins are animal lectins that are related in amino acid sequence and specifically bind to β-galactoside carbohydrates such as lactose (1). Members of this gene family all include a conserved carbohydrate-binding domain but vary in inclusion of other domains and in tissue expression patterns. More distantly related, but conserving critical amino acid residues involved in carbohydrate-binding, are galectins in chicken, eel, frog, nematode, and sponge (2).

Although galectins have been studied for 20 years now, physiological functions for these proteins have not yet been clearly established. Their affinity for oligosaccharides found on glycoconjugates on cell surfaces or in extracellular matrix has suggested that galectins function extracellularly by binding to such ligands. Indeed, certain galectins have particular affinity for specific glycoprotein ligands, such as polylactosamine chains on laminin (1, 3). When added to cells or overexpressed after transfection, galectins can have major effects on cell adhesion, proliferation, apoptosis, metastasis, and immune function (1–6). However, evidence has also been presented for intracellular functions of galectins, for instance in message splicing (7) or as nuclear proteins (8). Therefore, efforts are being directed at exploring the evolutionary origin of galectins in the hope that their functions will be easier to define in simple model organisms.

Here we report that a species of fungus, the inky cap mushroom, *Coprinus cinereus*, expresses two lectins related in sequence and carbohydrate-binding specificity to other galectins. This discovery means that the galectin gene family is even older than previously realized and must have evolved at least a billion years ago. Like many other galectins, recombinant *C. cinereus* galectin shows particular affinity for blood group A structures, suggesting that fine specificity for complex saccharide structures has also been conserved. Such conservation implies that galectins evolved to perform very basic cellular functions, presumably by interaction with glycoconjugates bearing complex lactoside carbohydrates resembling blood group A.

**Experimental Procedures**

**Lactose Affinity Chromatography**—Fruiting stage *C. cinereus* proteins were first extracted and partially purified using ammonium sulfate precipitation and ion exchange chromatography, as described previously (9). Lactose-binding proteins were then isolated by affinity chromatography from approximately 0.5 mg of protein in 1 ml of 50 mM Tris-HCl (pH 7.2), 150 mM NaCl (TBS) plus 10 mM mercaptoethanol. This partially purified fruiting body extract was passed through a 1-ml (bed volume) column of lactosyl-Sepharose, prepared as described previously (10). The column was washed with 10 ml of TBS, 10 mM mercaptoethanol, and eluted with another 5 ml by inclusion of 0.1 M lactose, while collecting 1-ml fractions. Protein concentrations were determined using a biycinchonic acid protein assay (BCA Protein Assay, Pierce). Fractions were then analyzed by SDS-PAGE (17% gel) and silver staining. Protein molecular weights were estimated by comparison with protein standards (SDS-PAGE Molecular Weight Standards—Low Range, Bio-Rad). It was subsequently found that the reducing agent, mercaptoethanol, was not required for purification of the lactose-binding proteins.

**Endonuclease Assay**—Endonuclease activity was measured basically...
as described previously (9) by incubating 23 μl of serially diluted (in 100 mM Tris-Cl (pH 7.4), 10 mM MgCl2) test fractions with 2 μl (0.5 μg) of supercoiled plasmid DNA for 30 min at 37 °C and then monitoring any DNA nicking by migration in a 1% agarose gel, stained with ethidium bromide.

**DNA Sequencing**—DNA was purified from overnight cultures of additional phage clones isolated during the original antibody screen of a *C. cinereus* agt11 DNA library (11), and the insert cDNA was removed by restriction digestion and subcloned into pGEM4Z plasmids (Promega Corp., Madison, WI). Both strands of the cDNA were then fully sequenced by standard techniques using Sequenase (U.S. Biochemical Corp.), T7 and T3 oligonucleotide primers flanking the cloning site, and subcloned into a pGEM4Z-2 vector (Promega Corp., Madison, WI). The library was screened in a colony hybridization system (CLONTEK). The resulting positive clones were identified by using lactose-Sepharose, all as described previously (13).

**Peptide Sequencing**—Partial amino acid sequences were determined by established protocols (12) from the N terminus of proteins separated by electrophoresis, ligation, bacterial transformation, and other basic molecular genetic manipulations all followed standard methods.

**Production and Purification of Recombinant Galectin**—To produce recombinant lectin in *Escherichia coli*, an Ndel site was engineered at the translation start site in the C. cinereus galectin-II clone. This plasmid was then purified by CsCl-Bu density equilibrium gradient ultracentrifugation, gelation, and the recombinant lectin was purified by affinity chromatography on lactosyl-Sepharose, all as described previously (13).

**Gel Filtration Chromatography**—To estimate approximate native molecular masses, proteins were analyzed by gel filtration chromatography using a Superdex 75 HR 10/30 molecular sieve column (bed volume approximately 24 ml) (Pharmacia Biotech Inc.) and a Perkin-Elmer Series 4 HPLC system with detection at 214 nm. Approximately 10 μg of purified recombinant protein or 0.5 μg of the partially purified fruiting body extract at 100 μg/ml in TBS, 4 mM mercaptoethanol, 0.1 M lactose was injected into the HPLC, equilibrated in the same buffer, and chromatographed at a flow rate of 0.5 ml/min. In some cases, the eluent was collected in 0.33-ml fractions for SDS-PAGE analysis. Approximate molecular masses of the test proteins were calculated by comparison with molecular mass standards (Sigma) and recombinant rat galectin-1 and human galectin-3 (13).

**Electrospray Ionization Mass Spectrometry (ESI-MS)**—Mass spectrometry was used to determine precise subunit molecular masses for the native galectins purified by lactose-affinity chromatography from fruiting body extracts. ESI-MS analysis was performed by the Biocytomass Mass Spectrometry Laboratory, Department of Chemistry, University of Western Ontario, London. Prior to analysis the purified lectins were extensively dialyzed against 2 mM ammonium acetate (pH 7.0). 400 pmoles of the protein was introduced into the ion source at a cone voltage of 22 V. The molecular mass spectrum was reconstructed from multiply charged ions in the m/z (mass to charge ratio) spectrum.

**Novel Carbohydrate-binding Assay**—Carbohydrate-binding specificity of recombinant lectin was determined using a novel assay and a panel of standard saccharides (as numbered in Table 1, numbers 1 and 18 kindly donated by Hakon Leffler, UCSF, numbers 8, 10, 11, and 20 from Oxford GlycoSystems, numbers 9 and 19 from Accurate Chemical and Scientific Corp., and the rest from Sigma). In brief, each saccharide was tested for inhibition of the binding of biotin-labeled asialofetuin (biotin-ASF) to lectin-Sepharose beads, detected by subsequent binding of streptavidin-peroxidase, and colorimetric quantification of bound peroxidase activity using soluble tetramethylbenzidine substrate.

Purified recombinant *C. cinereus* galectin-II or rat galectin-I were first conjugated to Sepharose by standard techniques (14). In brief, each protein was dialed into 75 mM NaCl, 75 mM NaHPO4/KHPO4 (pH 7.2) (PBS). The pH was raised by addition of 15% of 0.5 M NaHCO3 (pH 8.3), 1 M NaCl, 5 mM EDTA, and lactose was added to 0.1 M. Then 15 ml of this solution at approximately 1 mg/ml gelatinite was added to 15 ml (packed volume) of cyanogen bromide-activated Sepharose 4B beads (Pharmacia Biotech Inc.), which had been hydrated and washed with dilute HCl according to the manufacturer’s instructions. Coupling was allowed to proceed overnight at 4 °C with gentle rocking to keep the beads suspended. The beads were then pelleted by centrifugation; the supernatant was aspirated, and unreacted sites were blocked by resuspension and incubation for 2 h at 4 °C in 20 ml of 0.2 M ethanolamine in the above coupling buffer. The beads were then washed three times with 20 ml of TBS and stored at 4 °C.

Fetuin (Sigma) was desialylated by mild acid hydrolysis (15), diazotized against PBS, and biotinylated by addition of 0.5 ml at 20 mg/ml to an equal volume of 0.2 M NaCO3 (pH 0.8), and addition of 300 μl of 12 mg/ml horseradish peroxidase complex (Calbiochem) in dimethylformamide. After reaction in the dark for 1 h at room temperature, the reaction was quenched by addition of 100 ml of 1 M NH4Cl. The solution was then dialyzed twice against 1 liter of TBS and stored in frozen aliquots.

A competitive binding assay was developed in which 5 μl of 50 μg/ml biotin-ASF was added to 35 μl of 1:7 suspension of lectin-Sepharose beads in TBS, 1% bovine serum albumin (Sigma, RIA grade), 0.2% Triton X-100, and 10 μl of a given test saccharide. After incubating overnight at 4 °C with gentle rocking to maintain suspension, the beads with any bound biotin-ASF were pelleted by microcentrifuge, washed three times with 0.5 ml of the above buffer and then incubated for 1 h at 4 °C in 0.5 ml of 1,500 dilution of streptavidin-biotinylated horse-radish peroxidase complex (RPN 1051, Amersham Life Sciences Inc.) in the same buffer. Again, the beads carrying any streptavidin-peroxidase bound to biotin-ASF were pelleted and washed three times. The beads were then resuspended by vortexing in 100 ml of H2O, and 25 μl of suspension was placed in a microtiter plate well. 100 μl of tetramethylbenzidine reagent (TMB-Soluble, Intergen) was then added to each well, and the absorption at 620 nm of each well was recorded every minute for 30 min with mechanical agitation before each reading. From these readings the reaction velocity while in the linear range, reflecting the amount of bound peroxidase, was determined using Kinetical software (Bio-Tek Instruments, Inc., Winooski, VT). Values obtained in the presence of saccharide (averaged for triplicate experiments) were then calculated as a fraction (F) of that obtained in the absence of any inhibitor and plotted against the saccharide concentration (C) using SigmaPlot software (Jandel Scientific Software, San Rafael, CA). The approximate Kf for each saccharide, which should be approximately the concentration giving 50% inhibition of biotin-ASF binding to the lectin beads, was calculated by fitting the data to a simple competitive binding function for a trace ligand (F = 1/(1 + C/Kf)).
were almost completely retained and specifically eluted with lactose (Fig. 1, lanes 4–8). This indicates that the retained proteins possess lactose-binding activity. The endonuclease activity might be due to the lower band of the 15.5-kDa doublet or to some other minor component of the preparation. Regardless, all endonuclease activity is clearly separable from lactose-binding activity. Therefore, given its sequence similarity to galec-tins, the above cDNA sequence seemed quite likely to encode at least one of the lactose-binding proteins at 15.5 and 17 kDa and not an endonuclease.

Isolation of cDNAs for Two Closely Related C. cinereus Galectins—To confirm that the above C. cinereus cDNA encodes at least one of the lactose-binding bands identified here, we searched for a corresponding full coding length cDNA in order to produce and test the recombinant protein for lectin activity. Sequencing of additional clones isolated during the original library (11) yielded an apparently full coding length cDNA (Coprinus galectin-II, GenBank Accession No. U64676) 635 base pairs in length with a apparently full coding length cDNA (Coprinus lactose-Sepharose affinity chromatography from fruiting body extracts. ESI-MS revealed the predicted N-terminal sequence of the Cgl-II cDNA. The peptide sequence MLYRFVNNQIKQDDFKAE from the band migrating at approximately 15.5-kDa protein matches the predicted N-terminal sequence of the Cgl-I cDNA with the exception of histidine encoded by the cDNA as the fourth residue, instead of the arginine indicated by amino acid sequencing.

Biochemical Characterization of Recombinant Coprinus Galectin-II—Because N-terminal sequence matching the cDNA was obtained for both proteins by Edman degradation, it appeared that neither protein has a modified N terminus, retaining their initial methionines. This was confirmed by mass spectrometry of the Coprinus lectins purified by lactose-affinity chromatography from fruiting body extracts. ESI-MS revealed two major peaks with apparent masses of 16,408 (±1.2 Da) and 16,672 (±1.2 Da) (Fig. 4), corresponding exactly to the masses predicted from the cDNA sequences for Cgl-I and Cgl-II, respectively. Also apparent are minor peaks 96 Da larger than each of the major peaks and even smaller peaks 96 Da larger than those, but these appear to be ionization artifacts, because their presence and size varied depending on buffer conditions. These results confirm the deduced protein sequences and demonstrate that neither protein is post-translationally modified in any way.

To confirm the lectin activity indicated by similarity to galectins, the coding sequence for Cgl-II was subcloned into a prokaryotic expression vector and recombinant protein was produced in E. coli. This protein was purified from the lysed bacteria by affinity chromatography using a lactosyl-Sepharose column and confirmed to have lectin activity. SDS-PAGE revealed a single band in the purified recombinant protein matching in size the 17-kDa band in the partially purified extract (Fig. 1, lane 1). The purified recombinant lectin had no detectable endonuclease activity even at 2.0 mg/ml (Fig. 5, lane 6), whereas plasmid degrading activity was easily detectable in the partially purified fruiting body extract at 25 µg/ml (Fig. 5, lane 3).

Recombinant Cgl-II elutes as two distinct peaks on HPLC gel filtration chromatography under nondenaturing conditions.
Amino acid sequence comparison of galectins. Sequences for mammalian galectin-1 (16–18), galectin-2 (19), galectin-3 (20–22), galectin-7 (26, 27), and galectin-8 (29, based on 28) are human. The galectin-4 (23) and galectin-5 (24) sequences are rat. Galectin-6 has been completely sequenced in mouse but is not shown because it is 78% identical to galectin-4 (M. A. Gitt, C. Colnot, F. Poirier, K. J. Nani, S. H. Barondes, and H. Leffler, submitted for publication). Example sequences for amphipath, fish, nematode, and sponge are from *Xenopus laevis* (30), electric eel (31), chicken (32–34), *Caenorhabditis elegans* (35, 36), and *Geodiacydonium* (37), respectively. In some cases, only partial sequences covering the carbohydrate-binding domains are shown, as indicated by three periods preceding these sequence. For those galectins that include two carbohydrate-binding domains, the N-terminal domain is labeled A, the C-terminal domain is labeled B, and dashes indicate where these domains join.

Underlined gaps have been introduced to maximize alignment of conserved residues. Residue numbering above the sequences is based on the mammalian galectin-1 sequence. The most highly conserved residues are capitalized and shaded. Asterisks above the sequences designate residues found by x-ray crystallography to be directly involved in carbohydrate binding (38–40). Residues that are not identical in Cgl-I and Cgl-II are marked (#) under the Cgl-II sequence.

Characterization of Coprinus Galectin-II Carbohydrate-binding Specificity Using a Novel Assay—To compare the carbohydrate-binding specificity of *Coprinus* galectin with other galectins, a novel assay was designed based on saccharide inhibition of biotinylated asialofetuin binding to galectin protein immobilized on Sepharose beads. The advantage of this assay is that it is nonradioactive, whereas characterization of specificity for most other galectins has been based on saccharide inhibition of the binding of radiolabeled lectin to immobilized saccharide, such as lactosyl-Sepharose. To be sure that results from this assay are comparable with results reported for other assays, well characterized rat galectin-1 was tested simultaneously. For each saccharide tested, inhibition curves were plotted using a log scale for the saccharide concentration (as shown for lactose in Fig. 7). All curves for Cgl-II and rat galectin-1 were parallel, apparently reflecting simple noncooperative binding in each case. Based on these curves, the approximate \( K_i \) or the concentration giving 50% inhibition of biotin-ASF binding to the lectin beads was calculated for each saccharide. Results are shown in Table I for recombinant Cgl-II and rat galectin-1 in comparison with published results for inhibition of rat galectin-1 (43) binding to lactosyl-Sepharose. Clearly, the novel nonradioactive assay should be more practical, given the greater stability of its components.

The carbohydrate-binding specificity of recombinant Cgl-II is very similar to the specificities reported for other galectins (1). As with all other galectins, galactoside, and especially lactoside sugars competed for the lectin binding site. Also as with all other galectins, binding seems to require a free hydroxyl at the glucose 3-carbon of lactose (Gal \( \beta_1 \rightarrow 4 \) Glc), whereas binding seems only moderately affected by substitution at the galactose 2- or 3'-hydroxyl. Thus, substitution of lactose with fucose at position 3 (Table I, number 20) greatly reduces inhibitory potency, whereas substitution with fucose at position 2'
somewhat increases inhibitory potency compared with lactose (Table I, number 4). As for most galectins, N-acetyllactosamine (Table I, number 5) is a significantly more potent inhibitor than lactose. Like many other galectins, Cgl-II shows particular affinity for lactose substituted with GalNAc at the 3\textsuperscript{9} position on galactose (Table I, number 9). Blood group A tetrasaccharide (GalNAc\textsubscript{a}1–3[Fuc\textsubscript{a}1–2]Gal\textsubscript{b}1–4Glc), which has this structure, was the most potent competitive inhibitor tested (apparent $K_i$ 50.09 mM).

**DISCUSSION**

Identification of galectin expression in a fungus, *C. cinereus*, extends the known antiquity of this gene family. The *Coprinus* isolectins are the first galectins identified outside the animal kingdom, which diverged from the fungi approximately 1 billion years ago (44, 45). Such evolutionary conservation implies that galectins evolved to perform very basic biological functions. Physiological processes are considerably more limited in mushrooms than in most other species in which galectins have been studied, restricting the possible functions played by galectins as they originally evolved. Furthermore, because fungi (and sponges) diverged from the main phylogenetic tree before insects and animals diverged, it seems likely that galectins are also expressed in *Drosophila* and that, if these could be identified, the experimental advantages of that organism could be brought to bear on the question of galectin function.

Carbohydrate-binding specificity was only studied for one of the two *Coprinus* galectins, Cgl-II. Because they are so similar in amino acid sequence (83% identity), it seems unlikely that there are major differences in binding specificity between Cgl-I and Cgl-II. However, it is clear that these are not just alleles of each other, because preliminary studies have revealed tandem arrangement of the two corresponding genes.\(^2\) One possibility is that gene duplication has served to facilitate high level expression.

Some aspects of *Coprinus* galectin binding specificity can be interpreted from the crystal structures determined for mammalian galectin-1 (38, 39) and galectin-2 (40) complexed with saccharide. Those crystals reveal a carbohydrate-binding site composed of four adjacent $\beta$ strands (amino acids 31–83 of mammalian galectin-1), and all of the galectins, including both *Coprinus* galectins, conserve certain critical amino acid residues which directly interact with lactose, His-44 (except in the 32-kDa nematode galectin), Arg-48, Val-59 (except in the 32-kDa nematode C-terminal domain), Asn-61, Trp-68, and Glu-71 (amino acid residues numbered by position in mammalian galectin-1 as shown in Fig. 3). In the crystallized galectins, Asn-46 also contributes to lactose binding through water-mediated interaction with the 3-OH of lactose. However, this residue is not conserved in the *Coprinus* galectins, conserve certain critical amino acid residues which directly interact with lactose, His-44 (except in the 32-kDa nematode galectin), Arg-48, Val-59 (except in the 32-kDa nematode galectin C-terminal domain), Asn-61, Trp-68, and Glu-71 (amino acid residues numbered by position in mammalian galectin-1 as shown in Fig. 3). In the crystallized galectins, Asn-46 also contributes to lactose binding through water-mediated interaction with the 3-OH of lactose. However, this residue is not conserved in the *Coprinus* galectins, conserve certain critical amino acid residues which directly interact with lactose, His-44 (except in the 32-kDa nematode galectin), Arg-48, Val-59 (except in the 32-kDa nematode galectin C-terminal domain), Asn-61, Trp-68, and Glu-71 (amino acid residues numbered by position in mammalian galectin-1 as shown in Fig. 3).

\(^2\) R. P. Boulianne and B. C. Lu, unpublished observations.
partially purified endonuclease preparation (apparent dimer fractions (approximately 17.0 and 15.5 kDa (marked on the gel filtration) appear in the apparent monomer fractions (lanes 1–3) by SDS-PAGE and silver staining. Two bands of different molecular mass for peak 1 and peak 2 in the HPLC elution profile for the purified recombinant galectin-II were determined by comparison with protein standards (A, bovine serum albumin, 66 kDa; B, carbonic anhydrase, 29 kDa; C, recombinant rat galectin-1, 29 kDa; D, recombinant human galectin-3, 26 kDa; E, cytochrome c, 12.4 kDa; F, aprotinin, 6.5 kDa). C, HPLC fractions of the partially purified endonuclease preparation (lanes 2–11 covering elution volumes 8–13 ml) were compared with recombinant Coprinus galectin-II (lane 1) by SDS-PAGE and silver staining. Two bands of approximately 17.0 and 15.5 kDa (marked on the left) appear in the apparent dimer fractions (lanes 4–5, elution volumes 9–10 ml). These plus another band migrating slightly smaller than 15.5 kDa appear in the apparent monomer fractions (lanes 8–11, elution volumes 11–13 ml).

FIG. 6. Size estimation of native Coprinus galectins by HPLC gel filtration. A, HPLC molecular sieve (Superdex 10/30) elution profiles, detected by absorption at 214 nm, are shown for separation of purified recombinant Coprinus galectin-II (profile A) and the partially purified endonuclease preparation from fruiting bodies (profile B). Positions of the apparent dimer (peak 1) and monomer (peak 2) fractions are shown. B, approximate molecular masses for peak 1 and peak 2 in the HPLC elution profile for the purified recombinant galectin-II were determined by comparison with protein standards (A, bovine serum albumin, 66 kDa; B, carbonic anhydrase, 29 kDa; C, recombinant rat galectin-1, 29 kDa; D, recombinant human galectin-3, 26 kDa; E, cytochrome c, 12.4 kDa; F, aprotinin, 6.5 kDa). C, HPLC fractions of the partially purified endonuclease preparation (lanes 2–11 covering elution volumes 8–13 ml) were compared with recombinant Coprinus galectin-II (lane 1) by SDS-PAGE and silver staining. Two bands of approximately 17.0 and 15.5 kDa (marked on the left) appear in the apparent dimer fractions (lanes 4–5, elution volumes 9–10 ml). These plus another band migrating slightly smaller than 15.5 kDa appear in the apparent monomer fractions (lanes 8–11, elution volumes 11–13 ml).

thereby block binding of Galβ1–3GalNAc (46). Many galectins show enhanced binding to larger oligosaccharides, but the amino acid residues responsible have not yet been clearly established.

In general, the $K_i$ values measured for Cgl-II are higher than those reported for other galectins. $K_i$ is a measure of the competitive potency of a test saccharide for competitive inhibition of lectin binding to a standard ligand (asialofetuin in this case). However, these values are not necessarily directly comparable across studies, because they can be assay-dependent. $K_i$ values depend partly on the lectin’s affinity for the given standard ligand (unless the standard ligand concentration is much smaller than its dissociation constant), but values reported for various galectins have derived from assays using different standard ligands (in some cases with very different valencies). Even as measured here in the same assay, the fact that the $K_i$ values for Cgl-II are higher than those for rat galectin-1 could mean that Cgl-II has a higher affinity than rat galectin-1 for asialofetuin or that Cgl-II has lower affinity than rat galectin-1 for the tested saccharides. What should be comparable across different assays (assuming similar interaction valency) is the relative potency of test saccharides compared with a standard, such as lactose. Analyzed in this way, the 30-fold lower $K_i$ measured for blood group A tetrasaccharide compared with lactose for inhibition of Cgl-II is similar to the 25-fold difference found for rat galectin-3.

Preferential binding to blood group A structures has now been observed for a number of galectins, including mammalian galectins −3 (43, 47), −4 (24), and −5 (43) and a sponge galectin (48). The observation that blood group A tetrasaccharide is also the best of the tested competitors for the Cgl-II binding site marks such fine specificity as an evolutionarily old characteristic of galectins and suggests that it might be functionally important. At least some fungi incorporate GalNAc into glycoproteins and polysaccharides (49), but there has not yet been enough structural characterization of fungal glycoconjugates to use this information to identify any candidate ligands. It is also possible that relevant ligands are not fungal, but perhaps animal, insect, or nematode glycoconjugates mediating, for instance, spore adhesion to those organisms (50).

While evidence has been presented for both intra- and extracytoplasmic functions of galectins, the complex carbohydrate structures preferred by galectins are extracytoplasmic, and in many cases galectins have been shown to be secreted and accumulated extracellularly (1, 2). However, none of the galectins yet described, including the Coprinus galectins, include a secretion signal sequence. Nevertheless, mammalian galectin-1 and galectin-3 have been shown to be exported from mammalian cells by nonclassical mechanisms (51–56), and galectin-1 can even be exported when expressed as a recombinant protein in Saccharomyces cerevisiae (57). Thus, it is likely that the Coprinus galectins, too, are secreted proteins.

The Coprinus galectins share a number of additional characteristics with other galectin family members. Like most other galectins, the Coprinus galectins form non-disulfide bonded dimers and are thus functionally divergent with the potential to cross-link glycoconjugates on cell surfaces or in extracellular matrix. Like many other galectins, the Coprinus galectins are expressed at very high levels (11), a characteristic of proteins with more structural roles, as opposed to catalytic or signaling
roles. Also like many other galectins, the \textit{Coprinus} galectins show marked developmental regulation, in this case during fruiting body formation (11). These characteristics reinforce previous suggestions that galectin functions may be particularly important in regulating developmental changes in cell-cell or cell-matrix interactions.

Unlike other galectins, the \textit{Coprinus} galectins have unmodified N termini. It is not clear why the mobilities of Cgl-I and Cgl-II diverge on SDS-PAGE, because the molecular masses, as predicted from deduced sequence and confirmed by mass spectrometry, are very close, 16,408 and 16,671, respectively. Therefore, these proteins have no post-translational modifications. In contrast, all other galectins for which this has been studied have acetylated N termini (after cleavage of the initiator methionine in most cases) (2). Mammalian galectin-3 also undergoes regulated phosphorylation (58).

At one time galectins were referred to as S-type lectins (59), because they were thought to require reduced thiols to retain carbohydrate-binding activity. While this is true for some galectins, including mammalian galectin-1 (41, 42), it is not true for many other galectins. The \textit{Coprinus} galectins fall into the latter category, and like a galectin in nematodes (36) and one in electric eel (30), Cgl-I and Cgl-II have no cysteine residues and retain carbohydrate-binding activity in the absence of reducing conditions. If, as has been proposed (41), oxidative inactivation serves to limit the extracellular lifespan of some galectins, this is not the case for the \textit{Coprinus} galectins.

Small, saline-soluble lectins have been found in many other fungal species (50, 60), and almost all are developmentally regulated with high expression in fruiting bodies and little or no expression in vegetative mycelia. Most show binding specificity for GalNAc, Galβ1–3GalNAc, or asialomucin. Some of these could be galectins, such as the β-galactosyl-specific lectin isolated from fruiting bodies of another basidiomycete mushroom, \textit{Ischnoderma resinosum} (61). However, the few fungal lectins that have been sequenced are clearly not members of the galectin family. For instance, the galactosamine-binding lectins from the nematode-trapping fungus \textit{Arthrobotrys oligospora} and the common edible mushroom \textit{Agaricus bisporus} are related to each other in sequence and form a novel lectin gene family (62, 63), whereas the fucose-binding lectin from the mushroom \textit{Aleuria aurantia} has an unrelated sequence (64).

It is notable that galactoside-binding lectins are also specifically expressed during fruiting body formation in other organisms, such as the cellular slime mold, \textit{Dictyostelium discoideum} (65), and the bacterium, \textit{Myxococcus xanthus} (66). These lectins are clearly unrelated in sequence to the galectins but like the galectins have been shown to accumulate extracellularly despite the lack of classical secretion signal sequences (67, 68). The fact that in each of these organisms small, soluble galactoside-binding lectins are synthesized at high levels at a time when the organisms are starving and that such lectins seem to have evolved independently several times might indicate that galactoside-binding lectins play some important role in fruiting. Indeed, suppression of lectin expression in \textit{D. discoideum} (69) or in \textit{M. xanthus} (70) resulted in impaired fruiting body formation, apparently due to a defect in the cells’ ability to migrate into an aggregate, the initial step in fruiting body formation for these species. Fruiting in basidiomycetes, including \textit{C. cinereus}, involves a similar aggregation of cells (from the vegetative mycelium) to form a more complex tissue, the mushroom. However, this is believed to involve only changes in cell adhesion and elongation, not cell migration (71). We hope to better define the function of the \textit{C. cinereus} galectins by using homologous recombination to eliminate expression of both \textit{C. cinereus} galectin genes.

**REFERENCES**

1. Barondes, S. H., Cooper, D. N. W., Gitt, M. A., and Leffler, H. (1994) \textit{J. Biol. Chem.} \textbf{269}, 20807–20810
2. Hirabayashi, J., and Kasai, K. (1993) \textit{Glycobiology} \textbf{3}, 297–304
3. Hughes, R. C. (1984) \textit{Glycobiology} \textbf{4}, 5–12
4. Lotan, R., Belleni, P. N., Tressler, R. J., Lotan, D., Xu, X.-C., and Nicolson, G. L. (1994) \textit{Glycoconjug. J.} \textbf{11}, 462–468
5. Yang, R.-Y., Hsu, D. K., and Liu, F.-T. (1996) \textit{Proc. Natl. Acad. Sci. U. S. A.} \textbf{93}, 6737–6742
6. Perillo, N. L., Pace, K. E., Seilhamer, J. J., and Baum, L. G. (1995) \textit{Nature} \textbf{378}, 736–739
7. Dagher, S. F., Wang, J. L., and Patterson, R. J. (1995) \textit{Proc. Natl. Acad. Sci. U. S. A.} \textbf{92}, 1213–1217
8. Wang, L., Inohara, H., Pienta, K. J., and Raz, A. (1995) \textit{Biochem. Biophys. Res. Commun.} \textbf{217}, 292–303
9. Lu, B. C., Wong, W., Fanning, L., and Sakaguchi, K. (1988) \textit{Eur. J. Biochem.} \textbf{174}, 725–732
10. Leffler, H., Masiarz, F. R., and Barondes, S. H. (1989) \textit{Biochemistry} \textbf{28},

**TABLE I**

| Saccharide Inhibition of Coprinus Galectin-II compared to Rat Galectin-1 |
|-----------------|-----------------|-----------------|
|                  | Cgl-II          | Rat Gal-1       | Lactosyl-Sepharose* |
| Saccharide       | Apparent IC50 (mM) | Apparent IC50 (mM) | Apparent IC50 (mM) |
| Galactose        | 150             | 70              | 65               |
| GalNAc           | 200             | 200             | NT               |
| GalNAcβ1–3Gal    | 10              | NT              | 17               |
| Galβ1–4Glc       | 2.8             | 0.5             | 0.5              |
| Galβ1–4Fuc       | 3.9             | 3.5             | 3.5              |
| Galβ1–4Glc       | 2.2             | 0.3             | 0.3              |
| Fuca1–2Galβ1–4Glc| 1.8             | 0.3             | 0.3              |
| Galα1–3Glc       | 2.2             | 1.0             | 1.2              |
| Galβ1–4Glcα1–3Galβ1–4Glc| 0.09 | NT | 1.5 |
| Galβ1–3Glcα1–3Galβ1–3GlcNAc| 1.2 | NT | 0.3 |
| Galβ1–4Glcα1–3Galβ1–4GlcNAc| 1.6 | NT | NT |

*Published results for inhibition of soluble rat galectin-1 binding to lactosyl-Sepharose (43) are included for comparison.
