The Mushroom *Marasmius oreades* Lectin Is a Blood Group Type B Agglutinin That Recognizes the Galα1,3Gal and Galα1,3Galβ1,4GlcNAc Porcine Xenotransplantation Epitopes with High Affinity*

Received for publication, January 7, 2002, and in revised form, February 6, 2002
Published, JBC Papers in Press, February 8, 2002, DOI 10.1074/jbc.M200161200

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A blood group B-specific lectin from the mushroom *Marasmius oreades* (MOA) was investigated with respect to its molecular structure and carbohydrate binding properties. SDS-PAGE mass spectrometric analysis showed it to consist of an intact (H; 33 kDa) and truncated (L; 23 kDa) subunit in addition to a small polypeptide (P; 10 kDa). Isolation in the presence of EDTA produced only the H subunits, indicating that the latter two are formed by metalloclopease cleavage of the intact H subunit. Tryptic digestion of the H, L, and P polypeptide chains followed by mass spectral analysis supports this view. The lectin strongly precipitated blood group type B substance, was nonreactive with type A substance, and reacted weakly with type H substance. Carbohydrate binding studies reveal a high affinity for Galα1,3Gal (but not for the isomeric α1,2, α1,4, and α1,6-disaccharides); Galα1,3Galβ1,4GlcNAc, and the type B branched trisaccharide. MOA also reacts strongly with murine laminin from the Engelbreth-Holm-Swarm sarcoma and bovine thyroglobulin, both of which contain multiple Galα1,3Galβ1,4GlcNAc end groups. This linear B trisaccharide is a component of porcine tissues and organs, preventing their transplantation into humans. MOA also shares carbohydrate recognition of this trisaccharide with toxin A elaborated by *Clostridium difficile*.

Blood group-specific lectins have served as serological reagents for typing blood for over 65 years (1–3). Among the useful reagents are *Dolichos biflorus* lectin for human type A1 erythrocytes (4, 5), the *B. Griffithia simplicifolia* isolectin for type B erythrocytes (6), and the *Ulex europaeus I* (7, 8) and *eel* (*Anguilla anguilla*) serum agglutinins for type O(H) erythrocytes (1, 2). Of all the blood group-specific lectins studied, group B-specific lectins are probably fewest in number.

Presently, the fruiting bodies of mushrooms are being studied as sources for lectins with novel carbohydrate binding activity. Several surveys have been published that classify these and react with non-enumerated groups. This paper is available online at http://www.jbc.org

**MATERIALS AND METHODS**

Most sugars were available from previous studies. Galα1,3Gal, Galα1,3(Fuc1,2)Gal, and l-Fuc1,2Galα1,4Glc were purchased from Dextra Laboratories (Reading, United Kingdom). Galβ1,6Gal was from Sigma, and Galα1,3Galβ1,4GlcNAc (linear B-2 trisaccharide) was from Calbiochem. Galα1,2Gal was from Toronto Research Chemicals, Inc. (New York, Ontario, Canada). Soluble GalNAcα1,3Galβ-polyacrylamide conjugate was purchased from Glycotech Corp. (Rockville, MD).

Galα1,6Gal was a gift of Dr. Paul Kovac (Laboratory of Medicinal Chemistry, Section on Carbohydrates, National Institutes of Health). Blood group types A (368 PI/WS), B (hnmad BGS 531 PI/WS, pepsin treated), and H (BGS PI/WS) substances were the gift of Dr. Ronald Poreitz (Rutgers University). Pigeon ovalbumin was a gift from Dr. Y. C. Lee (Department of Biology, Johns Hopkins University). Synsorbs A and B were purchased from Chembiomed Ltd. (Edmonton, Alberta, Canada).

*Electrophoresis*—SDS-PAGE was carried out in 12.5% gels (0.8% cross-linked) in Tris/glycine buffer, pH 8.8, by the method of Laemmli (16). Unless otherwise indicated, samples were denatured by boiling in buffer containing 1% SDS and 1% 2-mercaptoethanol. *HPLC*—Size exclusion chromatography was performed using a high performance liquid chromatography; GS I-B 4 and -A 4 isolectin,
Fluorescein-labeled MOA Staining—MOA was fluorescently labeled using fluorescein isothiocyanate (Sigma) at pH 9.5 in the presence of 0.2 M lactose, followed by exhaustive dialysis. A solution of fluorescein-labeled MOA (8 μg/ml) in PBS containing 2% goat serum and 0.2% Triton X-100, in the absence or presence of 20 μM linear B6 trisaccharide, was applied to a cryostat section (10 μm, fixed in paraformaldehyde) of porcine skeletal muscle. After standing at room temperature for 2 h, the staining solution was removed, and the section was washed with PBS and examined under a fluorescence microscope.

Preparation of Lectin—Fruiting bodies of M. oreades mushrooms were harvested in June and from August to October 2000 from grassy plots in Ann Arbor, Michigan, or were purchased from American Mushrooms. Fruiting bodies were cleaned of soil and debris and chopped into small pieces. Initially, fresh tissue (45 g) was homogenized at 4 °C in 250 ml of PBS containing 10 mM thiourea, 1 g/liter ascorbate, 50 mg/liter phenylmethylsulfonyl fluoride, and 1–2 g of insoluble polyvinylpolypyrrolidone (Sigma) in a Waring Blender at high speed. The homogenate was stirred 3–4 h in the cold, strained through four layers of cheesecloth, and centrifuged at 13,000 × g for 20 min. The supernatant solution was made 20% saturated with (NH₄)₂SO₄, and the resulting solution was centrifuged to remove a small amount of precipitate. This supernatant solution was adjusted to 80% saturation with solid (NH₄)₂SO₄ and stirred overnight, and the precipitate was collected by centrifugation, redissolved in 20 ml of PBS, and dialyzed. An affinity column (2.5 × 15 cm) of melibiose-Sepharose gel, prepared using divinyl sulfone coupling, was loaded with the dialyzed fraction and washed with PBS until the absorbance of the effluent at 280 nm became <0.1. Lactose (0.1 M) in PBS was then used to displace bound protein. The protein solution so eluted was dialyzed against PBS and passed through a second affinity column, Synsorb, consisting of type B trisaccharide (Galα1,3(β-Fucα1,2) Galβ(1→3)GlcNAc) linked to diatomaceous earth. The bound lectin was eluted with 20 mM d-mannopropionic acid containing 0.15 M NaCl, pH 6.0. The “pass-through” and displaced protein fractions were combined separately and assayed against a panel of human erythrocytes. Finally, the protein fraction displaced from the Synsorb B column was passed through a column of Synsorb A, which contains covalently bound type A trisaccharide. A very small amount of material was bound (<5%); it was eluted with 20 mM d-mannopropionic acid, 0.15 M NaCl. Both fractions were assayed against types A, B, and O erythrocytes.

It was later found (see below) that proteolysis and possibly oxidation was taking place during the isolation and purification steps. Subsequently, the extraction procedure was modified by including a protease inhibitor mixture (protein P8215; Sigma) at the level of 1 μl/mg of extract buffer in place of phenylmethylsulfonyl fluoride, eliminating Ca²⁺ and including 1.25 mM EDTA in all buffers, and carrying out the extraction and ammonium sulfate precipitations under an atmosphere of argon.

RESULTS

By employing a series of chromatographic affinity columns, we have isolated a lectin that strongly agglutinates human B erythrocytes with a concomitant low activity against O cells. Successive passage through Synsorb B and Synsorb A columns removed a slight activity against type A erythrocytes that was present in some preparations. The hemagglutinating activity against types A, B, and O cells and several other cell types is presented in Table I. Hemagglutination activity was unchanged by external dialysis of the lectin and assay in metal-free buffer containing EDTA, indicating the absence of a divalent metal ion requirement (data not shown). Subsequently, metal-free buffer containing EDTA was used routinely to prevent metalloclopeptide degradation of the lectin.

SDS-PAGE analysis of the lectin revealed the presence of two major bands at 33 and 23 kDa (designated H and L, respectively), as was previously reported (14). A significant band, not noted previously, was also observed at ~10 kDa, designated P (Fig. 1). Upon heavy loading of the gel, several minor bands migrating between the L and P bands were also observed. The absence of 2-mercaptoethanol in the sample preparation buffer did not alter the pattern of bands, indicating
the absence of interchain disulfide links. Samples prepared without heating also showed a band at \(-55 \text{ kDa}\), streaking between the bands, and lesser amounts of the other bands, suggesting that bands H, L, and P are associated into a heteromeric structure that is dissociated slowly by SDS at room temperature.

The native protein was subjected to size exclusion chromatography by HPLC, wherein it migrated as a single, symmetric peak having the same mobility as *Pisum sativum* and *Xanthosoma sagittifolium* lectins, both known to be of \(-50 \text{ kDa}\) (20, 21). In the absence of a haptenic sugar, all three lectins appeared to be of much lower molecular mass based on calibration of the column with cytochrome c, ovalbumin, and bovine serum albumin. In the presence of 0.1 M lactose, but not methyl \(\alpha\)-mannoside, MOA migrated slightly faster than ovalbumin (Fig. 2), at an apparent mass of 49.5 kDa, suggesting that the lectin interacted weakly with the column matrix, despite its being a silica-based noncarbohydrate matrix. Lectins frequently are observed to migrate through size exclusion matrices at slower rates than expected by their molecular masses, both because of weak interaction with the matrix (generally polysaccharide-based matrices) or because of their compact structure, usually with a high amount of \(\beta\)-structure.\(^2\) Interestingly, neither sugar used altered the migration of the other two lectins, both of which bind mannose. These results do not rule out the possibility that native MOA is somewhat larger than 49.5 kDa, but it is clear from the HPLC results it exists in solution as a single molecular weight species composed of at least three different sized subunits.

The native lectin was subjected to MALDI-TOF mass spectrometric analysis, wherein mass ions corresponding to the three bands were detected (data not shown). Pairs of masses corresponding to the 23- and 32-kDa subunits, separated by \(-180\) and 260 Da, respectively, were observed (probably due to binding of the matrix), whereas the 10-kDa subunit exhibited a single molecular mass of \(-9800 \text{ Da}\). Within the limits of the mass calibration, the sum of the two smaller molecular masses (corresponding to L and P subunits) approximates the larger molecular mass of band H. Mass spectrometric analysis of tryptic peptides from the various bands and total amino acid analysis (data not shown) also support the conclusion that the two lighter bands, L and P, are fragments of the intact band H.

No significant amounts of any amino acids were obtained during several cycles of automated amino acid sequencing of the native protein or of the H and L bands, indicating that they possess blocked N-termini. The P band released small amounts of several amino acid derivatives at some of the cycles (similar to traces seen in the native protein also), but no single sequence was detected, suggesting that it is also largely blocked and may be heterogeneous.

\(^2\) W. J. Peumans, personal communication.

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**Fig. 1.** SDS-PAGE gels of MOA. Lanes B, C, and E, affinity-purified MOA at approximately 5, 10, and 20 \(\mu\)g, respectively; lanes A and D, low molecular mass protein standard (2–80 kDa).

**Fig. 2.** Size exclusion chromatography of MOA by HPLC. Elution buffer (PBS) contained 0.1 M lactose. Elution positions of standards are indicated by the arrows. BD, blue dextran (\(>1000 \text{ kDa}\)); BSA, bovine serum albumin (66 kDa); OV, hen ovalbumin (45 kDa); Cyt, cytochrome c (12.5 kDa); NA, nicotinic acid (<1 kDa).

Proceeding from the observation that recombinant MOA is a homodimer consisting of two identical subunits of 32 kDa (15) whose solutions are completely colorless (no absorbance above 320 nm), we set out to attempt to isolate a similar protein from the mushroom, taking care to inhibit any protease activity that could give rise to the “clipped” lectin (the 23- and 10-kDa polypeptide chains) as well as prevent possible oxidation of aromatic side chains. The modified procedure described above indeed led to the isolation of a homodimer similar to the recombinant lectin in lacking any 23- and 10-kDa subunits and having no absorbance above 320 nm (data not shown).

To further study the blood group specificity of the *M. oreades* lectin, we conducted quantitative precipitation assays of the lectin with soluble cyst blood group substances. Fig. 3 shows that MOA reacts strongly with human blood type B substance, not at all with type A substance, and rather weakly with type H substance. The explanation for these reactions will be discussed in terms of the specificity studies described below.

Sugar ligand binding to MOA was conducted using three approaches: inhibition of type B hemagglutination, hapten inhibition of MOA-type B substance precipitation, and isothermal titration calorimetry. All three techniques gave approximately the same results, with the calorimetric data being the most precise. Being a blood type B agglutinin, MOA was assayed primarily against \(\beta\)-galactosyl-terminated sugars and oligosaccharides. As shown in Fig. 4 and Table II, lactose, \(N\)-acetyllactosamine, and melibiose (Gala1,6Glc) were very poor ligands. Methyl \(\alpha\)-galactopyranoside was similarly very poor. The blood group B disaccharide, Gala1,3Gal, was an excellent ligand, with \(K_\text{d} = 6.0 \times 10^{-3} \text{ M}^{-1}\), whereas the isomeric disaccharides Gala1,2Gal, Gala1,4Gal, and Gala1,6Gal bound poorly or not at all. The addition of a GlcNAc group to the reducing end of Gala1,3Gal to give Gala1,3Gal\(\beta\)1,4GlcNAc increased the binding by \(-50\%\) to \(K_\text{d} = 9.7 \times 10^{-3} \text{ M}^{-1}\). Similarly, adding an \(\alpha\)-fucosyl group to the disaccharide to afford the blood group B branched trisaccharide (Gala1,3(\(\alpha\)-Fuc\(\alpha\)1,2)Gal) enhanced its affinity to MOA 4-fold (\(K_\text{d} = 3.6 \times 10^{-4} \text{ M}^{-1}\)). Finally, we assayed the trisaccharide \(\alpha\)-Fuc\(\alpha\)1,2Gal\(\beta\)1,4Glc (fucosyllactose), related to the blood group H trisaccharide; it had a \(K_\text{d} = 548 \text{ M}^{-1}\) by isothermal titration calorimetry. It is apparent that the \(\alpha\)-fucosyl residue makes a significant contribution to the binding affinity of the essentially inactive lactose (\(K_\text{d} = 185 \text{ M}^{-1}\)). This also is probably the reason that MOA recognizes and agglutinates human O erythrocytes to a limited extent and gives a weak precipitin curve with blood group H substance.

On the basis of MOA’s recognition of blood type B disaccha-
rides and linear trisaccharides, we believed that the lectin should precipitate both with laminin from the Engelbreth-Holm-Swarm murine sarcoma and with bovine thyroglobulin. Indeed, as shown in Fig. 5, MOA reacted strongly with laminin, which we have shown contains Galα1,3Galβ1,4GlcNAc end groups (22, 23), as well as with bovine thyroglobulin, which has the same determinants (24). Significantly, the lectin did not give a precipitin reaction with pigeon ovalbumin, which contains multiple Galα1,4Gal end groups (25), thus demonstrating its specificity for Galα1,3Gal groups. Also of significance, MOA did not recognize the blood group type A disaccharide (GalNAcα1,3Gal), as shown by its failure to precipitate with this disaccharide-polyacrylamide glycoconjugate. MOA readily agglutinated Ehrlich ascites tumor cells, which contain the same epitopic end groups in their cell membranes (26).

Fluorescein-labeled MOA stained porcine striated skeletal muscle (Fig. 6), endothelial cells lining the capillaries being the significant structures stained by the lectin. Incubation of the staining solution in the presence of 20 mM linear B6 trisaccharide essentially abolished staining (data not shown), indicating specific binding of the fluorescein-labeled lectin to the porcine tissue.

**DISCUSSION**

Results presented in this paper indicate that the *M. oreades* lectin, as isolated previously and in the initial stages of this work, was a dimer composed of an intact and a truncated subunit in addition to a small polypeptide presumably generated by proteolytic cleavage of the intact H subunit. Both the intact H and truncated L subunits are blocked at their N termini. Subsequently, we isolated an intact form of the lectin and an intact recombinant lectin (15), which have very similar binding properties, indicating that cleavage is due to the presence of contaminating metalloprotease activity and is not relevant to protein function.

The most interesting aspect of this study concerns the high specificity of the lectin for Galα1,3Gal end groups; the α1,2-, α1,4-, and α1,6-linked disaccharides do not bind to the lectin. These data indicate that MOA has an extended binding site that accommodates the Galα1,3Gal disaccharide. To the best of our knowledge, this is the first lectin shown to exhibit this exclusive specificity. Additional contributions to the binding energy are made by the addition of β1,4GlcNAc and α1,2-fucosyl groups to the reducing Gal unit of Galα1,3Gal. These additions account for the blood group B activity of the lectin.

The structures of these oligosaccharides, related to human blood group B substance and assayed for their activity in this study, are presented in Fig. 7.

It is instructive to compare MOA with the *Griffonia simplicifolia* I-B4 isolectin (GS I-B4), a highly specific α-D-galactosyl-binding lectin, with respect to the size of their combining sites. A great deal of binding data indicate that the GS I-B4 isolectin has a very restricted binding site that does not appear to extend significantly beyond the monosaccharide unit (i.e. the α-D-galactosyl group) (27, 28). This concept of a restricted site was substantially verified by the recently completed x-ray crystallographic structure of the B4 isolectin complexed with Galα1,3Gal in which it is shown that only the nonreducing α-galactosyl group makes contact with the lectin (29). Moreover, Kirkeby and Moe (30), using an enzyme-linked lectin assay, showed that the α-galactosyl group and the α1,2, α1,3, and α1,4-galactobiosyl groups as well as the linear B-trisaccharide (Galα1,3Galβ1,4GlcNAc) linked to human serum albumin were very similar in their binding affinity to GS I-B4. On the contrary, MOA, which binds only very weakly or not at all to methyl α-D-galactopyranoside or to the α1,2-, α1,4-, and α1,6-linked galactobioses, has an extended binding site, which accommodates the blood type B disaccharide and linear trisaccharide (Galα1,3Gal and Galα1,3Galβ1,4GlcNAc, respectively) and the branched chain blood group B trisaccharide determinant with high affinity.

Taking advantage of these carbohydrate binding properties of MOA, we demonstrated that it gave a very strong precipitation reaction with laminin from the Engelbreth-Holm-Swarm murine sarcoma. This highly glycosylated glycoprotein contains the linear type B blood group trisaccharide at many of its chain ends (22, 23). It also recognizes and precipitates bovine thyroglobulin, which also contains the linear B trisaccharide (24). Interestingly, this lectin will not precipitate the galactomannan from *Cassia alata* that contains multiple α-galactosyl end groups, illustrating its lack of reactivity with single sugar residues (Fig. 5). Nor did MOA bind to cross-linked guaran, also a galactomannan. Conversely, both the galactomannan (not shown) and pigeon ovalbumin precipitated strongly with GS I isolectins (Fig. 5). MOA does not recognize the blood group type A disaccharide (GalNAcα1,3Gal), as shown by its failure to precipitate with this disaccharide-polyacrylamide glycoconjugate, whereas the GS I-A4 isolectin, which is specific for αGalNAc end groups (27), reacted strongly with it (Fig. 5).

MOA should be a valuable reagent for the glycobiologist for the detection and preliminary characterization of glycoconjugates...
Galα1,3Gal Recognition by M. oreades Mushroom Lectin

### Table II

| Saccharide                  | Subunit concentration | Binding sites, n<sup>ab</sup> | \( K_d \) | \( K_a \) | \(-\Delta H \) | \(-\Delta G \) | TDS
|-----------------------------|----------------------|-----------------------------|---------|---------|----------------|----------------|-------|
| Galα1,3Gal                  | 0.10                 | [1.0]<sup>c</sup>           | 10.13   | 0.123   | 5.88           | 2.82           | 3.06  |
| Lactose                     | 0.08                 | 1.02                        | 5.08    | 0.197   | 8.86           | 3.1            | 5.76  |
| LacNac                      | 0.092                | 0.99                        | 3.45    | 0.290   | 14.22          | 3.33           | 10.9  |
| LactoNhosojMe<sup>d</sup>   | 0.08                 | 1.01                        | 4.67    | 0.214   | 6.64           | 3.15           | 3.49  |
| Galβ1,4Gal                  | 0.08                 | 1.01                        | 3.74    | 0.288   | 6.12           | 3.28           | 2.84  |
| Galα1,2Gal                  | 0.10                 | >10                         | <0.1    |         |                 |                 |       |
| Galα1,3Gal                  | 0.10                 | [1.0]                       | 0.182   | 5.48    | 20.1           | 5.05           | 15.0  |
| Galα1,4Gal                  | 0.10                 | >10                         | <0.1    |         |                 |                 |       |
| Galα1,6Gal                  | 0.01                 | >10                         | <0.1    |         |                 |                 |       |
| Linear B2 trisaccharide     | 0.095                | 0.95                        | 0.103   | 9.753   | 20.6           | 5.39           | 15.2  |
| B trisaccharide             | 0.071                | 0.68                        | 0.036   | 27.8    | 20.6           | 6.01           | 14.6  |
| 2'-Fucosylactose            | 0.115                | 0.99                        | 1.83    | 0.548   | 10.1           | 3.70           | 6.4   |

*Based on a subunit mass of 33 kDa.

Based on 1 binding site for mono- or oligosaccharide per subunit.

Parameter fixed at bracketed value.

<sup>d</sup>Galα1,3GalNacβ1,4Me.<sup>c</sup>

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Fig. 5. Quantitative precipitation assay of MOA with glycoconjugates. Varying amounts of glycoconjugates, ranging from 0 to 100 µg were incubated with 20 µg of lectin in a total volume of 150 µl of PBS (pH 7.2). After 48 h at 4°C, the amounts of protein precipitated were quantified. ●, laminin; ●, bovine thyroglobulin; ▲, C. alata galactomannan; ▲, pigeon ovalbumin; ○, GalNacα1,3Galβ1-polyacylamide; □, GS I interaction with pigeon ovalbumin; ▼, GS I interaction with GalNacα1,3Galβ1-polyacylamide.

Fig. 6. Section of porcine striated skeletal muscle stained with fluorescein-labeled M. oreades lectin. Image size is 370 x 260 µm.

World monkeys but not in humans, apes, or Old World monkeys. It is also possible that this carbohydrate antigen is present on the surface of the malaria parasite, Plasmodium falciparum (36). Another interesting application of this lectin could be its use in the possible competition with toxin A elaborated by Clostridium difficile, which is responsible for antibiotic-induced diarrhea (37). Both MOA and toxin A recognize the Galα1,3Galβ1,4GlcNAc trisaccharide epitope.

Acknowledgments—We thank the following for gifts of reagents: Dr. Ronald Poretz for blood group A, B, and H substances; Dr. Donald MacCallum for Ehrlich ascites tumor cells; Dr. Paul Kovac (National Institutes of Health) for a sample of Galα1,4GlcNAc; Dr. Ronald Poretz for blood group A, B, and H substances; Dr. Donald MacCallum for Ehrlich ascites tumor cells; Dr. Paul Kovac (National Institutes of Health) for a sample of Galα1,4GlcNAc; Dr. Paul Kovac (National Institutes of Health) for a sample of Galα1,4GlcNAc.

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