Structure–Function Relationship of a Recombinant Human Galactoside-Binding Protein†

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ABSTRACT: A galactoside-binding lectin (hL-31) containing a collagen-like sequence was identified in human tumor cells. It was found to be the homologue of the IgE-binding protein, the macrophage cell-surface Mac-2 antigen, and the murine CBP35, RL-29, and mL-34 lectins. Here we report on the expression in *Escherichia coli* and functional analysis of recombinant hL-31 (rhL-31). The rhL-31 was purified in one step through an asialofetuin affinity column. The rhL-31 was reactive to anti-lectin antibodies and retained its lactose-dependent hemagglutination of trypsin-treated glutaraldehyde-fixed rabbit erythrocytes. The rhL-31 elutes from an affinity column as a 31-kDa monomer and undergoes homodimerization at relatively high protein concentrations, comparable to those used to mediate hemagglutination. Electron microscopy showed that the rhL-31 appears as a Y-shaped structure. Lactoperoxidase-catalyzed iodination of murine tumor cell-surface proteins followed by collagenase treatment revealed that the lectin is probably a peripheral membrane protein whereby both the amino and the carboxy termini are exposed on the outer cell membrane. These results point to the membrane disposition and orientation of the lectin and suggest a mechanism for a structure–function relationship of lectin activity.

Diverse carbohydrate-binding proteins of cellular and extracellular matrices are important in the specification of cell–cell recognition and cell–ligand(s) interaction [for a review, see Sharon and Lis (1989)]. These molecules have distinct functions and recognition sites involving different cell lineages at different developmental and pathological stages. Families of carbohydrate-binding proteins that share common sugar-binding specificity like galactose do exist, despite their functional and structural diversity. These include a group of 14-kDa galactoside-binding lectins (Leffler et al., 1989), a 67-kDa component of the elastin receptor (Mecham et al., 1989), the 55-kDa ectosialyltransferase of Hodgkin disease (Paietta et al., 1986), the 43-kDa human actin-binding brain lectin (Joubert et al., 1992), the 50-kDa rat testis galactosyl receptor (Abdullah & Keirzenbaum, 1989), the murine and human tumor-associated 34-kDa (mL-34)[1] and 31-kDa (hL-31) galactoside-binding lectins, respectively (Raz et al., 1989; 1991), the 35-kDa fibroblast carbohydrate-binding protein (CBP35) (Jia & Wang, 1988), the IgE-binding protein (ϵBP) (Albrandt et al., 1987), the 32-kDa macrophage non-integrin laminin-binding lectin (Mac-2) (Woo et al., 1990), and the rat, mouse, and human 29-kDa (L-29) galactoside-binding lectin (Oda et al., 1991). Molecular cloning studies have revealed that the latter five (mL-34, hL-31, CBP35, ϵBP, and Mac-2 and L-29) polypeptides share significant homology and are designated herein as ~30-kDa galactoside-binding lectins (GBL-30). The GBL-30 polypeptide contains two distinct structural domains; the amino-terminal half is composed of G-X-Y tandem repeats characteristic of the collagen supergene family, and the carboxy-terminal half contains a globular structure which encompasses the galactoside-binding site (Raz et al., 1989, 1991; Jia & Wang, 1988; Albrandt et al., 1987; Woo et al., 1990; Oda et al., 1991).

Independent immunohistochemical studies, cell-surface labeling, and ligand binding (Raz et al., 1984; Moutsatsos et al., 1986; Gritzmacher et al., 1988; Weis et al., 1991; Frigeri & Liu, 1992) have repeatedly shown the GBL-30 to be localized in the cytosol, in the nucleus, and on the cell surface, where they are presumed to function by interacting with complementary glycoconjugates. However, since they lack a classical signal peptide and an obvious transmembrane-spanning domain, their membrane disposition and orientation remain unknown. The involvement of GBL-30 in cell–cell recognition and cell laminin interaction and its ability to agglutinate red blood cells in a divalent cation independent fashion raise the following major question: Is GBL-30 a monomer, dimer, or multimer?

In this paper, we demonstrate that the GBL-30 is a cell-surface component and that part of the collagen-like sequence is exposed to the outer cell surface; we also report on the expression of a recombinant human GBL-30 (rhL-31) and its biochemical and structural properties, as well as an electron microscopic analysis.

EXPERIMENTAL PROCEDURES

Construction of the Expression Vector pET-5ahL-31. The vector pET-5a was obtained from Novagen, Madison, WI. To construct the recombinant expression plasmid pET-5ahL-31, a 0.85-kb *Eco*RI fragment containing the hL-31 cDNA was

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[1] Abbreviations: GBL-30, 30-kDa galactoside-binding lectins; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; mL-34, mouse galactoside-binding protein; hL-31, human galactoside-binding protein; PBS, phosphate-buffered saline; PMSF, phenylmethanesulfonyl fluoride.

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isolated from plasmid pUC-13. The fragment was directionally cloned into the unique *Eco*RI site of pET-5a, and the plasmid was used to transform competent *Escherichia coli* strain HMS174 using the method of Hanahan (1983). The transformants were identified by their ability to synthesize hL-31 as screened by slot and Western blotting techniques utilizing a monoclonal antibody to the Mac-2 antigen (obtained from R. Lotan) which has a similar antigenic epitope to hL-31 (Jia & Wang, 1988). Two clones, H5 and H8, were found to express hL-31 after induction with isopropyl β-D-thiogalactopyranoside (IPTG) (Promega, Madison, WI).

Isolation and Purification of rhL-31. One liter of LB medium containing 10 mM $MgCl_2$ and 100 μg/mL ampicillin was inoculated with 5 mL of an overnight culture of HMS-174 containing the recombinant plasmid. The bacterial cells were grown in an incubator shaker at 37 °C until an optical density of 0.5, at which point IPTG (1 mM) was added and cells were then incubated for an additional 4 h and harvested by centrifugation at 1250*g* at 4 °C. The pellet was washed with PBS and suspended in 100 mL of ice-cold lysis buffer (150 mM NaCl, 1% NP-40, 0.5% DOC, 0.1% SDS, 50 mM Tris, pH 8, 0.241 unit/mL aprotinin, 1 μg/mL leupeptin, 1 μg/mL pepstatin, and 0.2 mM PMSF). The buffer also contained 2 mM dithiothreitol (DTT) for isolation in reducing conditions.

The bacterial cells were disrupted by a probe-type sonicator with multiple short bursts of maximum intensity (30 s × 4), keeping the cells at 4 °C between bursts. The lysate was centrifuged at 40000*g* for 20 min and the precipitate resuspended in lysis buffer (20 mL) and disrupted once more. The supernatants obtained from the 40000*g* spins were combined and passed through an asialofetuin affinity column (100 × 22 mm inner diameter). The column was made by linking the asialofetuin to Affigel-15 (Bio-Rad, Richmond, CA) according to the manufacturer's protocol, and was well equilibrated in phosphate buffer (10 mM phosphate, with or without 2 mM DTT, 1 mM $MgSO_4$, 0.2 mM PMSF, and 0.2% NaN_3, pH 7.2). The pass-through from the column was reloaded and the column washed with 3–5 column volumes of phosphate buffer, and the bound protein was eluted with 0.2 M lactose, collecting 2-mL fractions. Eluted fractions were quantitated by the Bio-Rad protein assay kit (Bio-Rad) and analyzed by SDS–PAGE.

Electrophoresis. SDS–PAGE was performed under reducing and nonreducing conditions, using a 12.5% polyacrylamide separating gel and a 3.5% stacking gel. Samples were dissolved in sample buffer and boiled for 5 min. The gels were stained with Coomassie blue, and the protein standards were prestained low molecular weight markers (Bio-Rad). For Western transfer, the ^{14}C-labeled molecular weight markers (Amersham) were mixed with prestained protein standards to help in tracking the efficiency of transfer.

Immunoblotting and Immunoprecipitation. The samples from the slab gels were electroblotted to either nitrocellulose or Immobilon-P. The membranes were quenched in 5% nonfat dried milk in PBS for 4 h and then incubated with the primary antibody for 1 h (23 °C) in the same quench solution. The membrane was washed 5 times (10 min each) with the quench solution containing 0.1% Tween, and subsequently incubated with the secondary antibody (iodinated goat anti-rat IgG) for 1 h (23 °C) and washed as above. Cells labeled by surface iodination or by incorporation of [^{35}S]methionine were extracted at 10^6 cells/mL in a lysis buffer consisting of 10 mM NaH_2PO_4, 100 mM NaCl, 1% Triton X-100, 0.5% sodium deoxycholate, 0.1% SDS, and 1 mM phenylmethanesulfonyl fluoride. Fractions of 0.5 mL of cell lysates were immunoprecipitated with preimmune serum or anti-lectin antibodies. Antigen–antibody complexes were collected by centrifugation after binding to *Staphylococcus aureus*. The immunoprecipitates were subjected to reducing SDS–PAGE and analyzed by fluorography as described (Zvibel & Raz, 1985).

Hemagglutination Assay. Trypsin-treated, glutaraldehyde-fixed rabbit erythrocytes were prepared as described (Raz & Lotan, 1981). The erythrocytes were stored at 4 °C as a 10% suspension in Ca^{2+}- and Mg^{2+}-free PBS (CMF-PBS) containing 0.02% sodium azide. Prior to the assay, they were diluted with 0.1 M glycine in PBS, resulting in a 4% suspension. Hemagglutination was determined by serially diluting protein samples in microtiter plates with a V-shaped bottom such that the highest concentration was 10 μg/mL per well. The lowest concentration capable of hemagglutination was noted in each case. Each well contained 50 μL of lectin sample diluted in CMF-PBS, 50 μL of 1% bovine serum albumin in CMF-PBS, and 25 μL of erythrocyte suspension. The samples were incubated for 1 h at 23 °C. The effects of various treatments on the hemagglutination activity of the lectin samples were in the absence or presence of an 80 mM final concentration of lactose. To show whether the lectin required reducing conditions for hemagglutination activity, the lectin was tested in the presence or absence of 5 mM DTT.

Electron Microscopy. Approximately 0.1 mg/mL aliquots of the recombinant hL-31 were dialyzed extensively against 0.15 M ammonium acetate, pH 6.8, sprayed onto freshly cleaved mica, dried in vacuum, and shadowed with tungsten by means of electron-bombardment heating, essentially as described by Slayter (1976). The metal replicas were removed to atmospheric pressure, coated with 0.25% parlodion in *n*-butyl acetate, and air-dried for 10 min. The coated mica was scored in 2-mm squares; the edges were trimmed and inserted into a distilled water surface. The floating replicas were picked in 200-mesh copper grids and blotted dry. The grids were then examined in a Zeiss 10C transmission electron microscope at an accelerating voltage of 60 kV.

Gel Filtration Chromatography. hL-31 was eluted by gel filtration chromatography through a precalibrated Sephacryl-100 column in 10 mM phosphate buffer (pH 7.2) with a flow rate of 0.12 mL/min. The standards used were β-galactosidase (116 kDa), phosphorylase B (97.4 kDa), bovine serum albumin (66 kDa), ovalbumin (45 kDa), and carbonic anhydrase (29 kDa). The protein concentration of the fractions was determined by the Bio-Rad protein assay kit (Bio-rad Laboratories).

A Study of Surface Expression: Cell-Surface Labeling. Exposed cell-surface proteins were radioiodinated using the lactoperoxidase technique as described (Moutsatsos et al., 1986). UV-2237-IP3 fibrosarcoma cells were harvested using 2 mM EDTA, in CMF-PBS, then washed, and counted. One million cells were suspended in 100 μL of PBS. The reaction was started by adding 10 μL of a 1 mg/mL lactoperoxidase stock solution (Sigma, St. Louis, MO), 10 μL of a 0.03% H_2O_2 solution, and 500 μCi of carrier-free $Na^{125}I$ (New England Nuclear, Boston, MA). The reaction was allowed to proceed for 4 min at room temperature, after which 10 μL of lactoperoxidase and 10 μL of H_2O_2 were again added for 4 min. Finally, 10 μL of H_2O_2 was added for a further 4 min.

Collagenase Digestion. (A) Intact Viable Cells. Aliquots of ^{125}I-labeled cells (2 × 10^5 UV 2237-IP3) were incubated for 1 h at 37 °C in phosphate-buffered saline (pH 7.2) (containing 2 mM PMSF, with or without 0.05% Triton

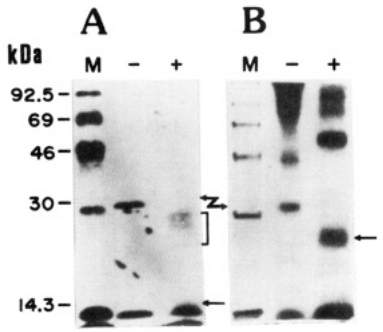

FIGURE 1: Immunoprecipitation of UV-2237-IP3 fibrosarcoma cells untreated (–) or treated (+) with collagenase by anti-lectin antibodies. (A) [^{35}S]Methionine labeling; (B) ^{125}I-labeled cell-surface labeling. The double arrow between panels A and B points to the location of the nondegraded 34-kDa lectin molecules. The lower single arrow to the right of panel A points to the location of the 14.5-kDa gel lectin. The location of collagenase-degraded [^{35}S]lectin polypeptides is bracketed in panel A. The single arrow to the right of panel B points to the location of the collagenase-degraded [^{125}I]lectin molecule. M, migration of ^{14}C-labeled protein markers.

X-100) with or without 10 μg/mL collagenase (EC 3.4.24.3; Boehringer). The cells were then solubilized for immunoprecipitation as described above.

(*B*) *Cell Extracts*. Aliquots of ^{35}S-labeled cells were first solubilized with lysis buffer and then subjected to collagenase digestion as described above.

RESULTS

Cell-Surface Expression of mL-34. We and others have shown by ligand- and antibody-binding studies that GBL-30 is expressed on the cell surface of diverse cells and that the carboxy terminus of the molecules which encompass the galactoside-binding domain is exposed to the outer cell membrane. However, since GBL-30 lacks a classical signal peptide and an obvious transmembrane domain (Drickamer, 1988; Foddy et al., 1990; Frigeri et al., 1990; Frigeri & Liu, 1992; Gritzmacher et al., 1988), its membrane disposition remains largely unknown. In an effort to address this question, we decided to take advantage of the susceptibility of soluble GBL-30 to collagenase cleavage (Raz et al., 1989) to study the possible membrane orientation of GBL-30. The murine UV-2237-IP3 fibrosarcoma intracellular proteins were metabolically labeled with [^{35}S]methionine and exposed cell-surface proteins labeled by lactoperoxidase-catalyzed iodination. Labeled cell extracts (^{35}S) or viable cells (^{125}I) were exposed to bacterial collagenase, followed by solubilization, immunoprecipitation with polyclonal anti-lectin antibodies (Raz et al., 1987), and protein separation by SDS–PAGE. The anti-lectin antibody used here recognizes both mL-14.5 and mL-34 lectin species, and was chosen for recognizing any alteration in the migration pattern of mL-14.5 following enzymatic treatment as a control for the specificity of the enzyme only to the collagenase-like sequences of the GBL-30.

As seen in Figure 1A, most of the intact ^{35}S-mL-34 polypeptides were cleaved by the collagenase with the appearance of lower molecular mass polypeptides ranging from 23 to 29.5 kDa. The bacterial collagenase preferentially cleaves polypeptides after proline in the sequence Gly-X-Pro-Gly (Seifer & Harper, 1971), and there are four such sites in the mL-34 sequence (Raz et al., 1989). It is therefore likely that the four predicted sites were accessible for digestion at random. The intact mL-14.5 (Figure 1A) demonstrates that only the collagen-like sequence in the amino terminus of mL-34 is the target for collagenase with no apparent nonspecific cleavages. In contrast, collagenase treatment of viable ^{125}I-labeled cells resulted in degradation of ^{125}I-mL-34 to a single polypeptide with an apparent molecular mass of 26.5 kDa (Figure 1B), suggesting that either one or both of the first two cleavage sites, amino acid residues 21–24 and 36–37 (from the first methionine), are either exposed or accessible for collagenase degradation on the cell surface. Furthermore, mL-34 is not released from the membrane to the medium following enzyme treatment.

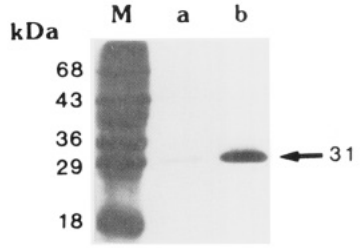

FIGURE 2: SDS–PAGE and immunoblot analysis of rhL-31. Bacterial lysate supernatant (100 mL) was applied on an asialofetuin–Affigel-15 column (0.9 × 20 cm). The column was washed at 4 °C in phosphate buffer until the OD_{280} was <0.002. The bound protein was eluted with 0.2 M lactose in the same buffer and subjected to 12.5% reducing SDS–PAGE. Lane a, Coomassie staining; lane b, immunoblotting with anti-lectin antibodies; M, molecular mass markers.

Expression and Functional Analysis of Recombinant (r) hL-31. We have expressed and purified the rhL-31 by inserting the cDNA coding for hL-31 into an inducible plasmid vector (pET-5a) in both sense and antisense orientations relative to the lacV5 gene. Time course analysis revealed maximum rhL-31 expression 4 h after 1 mM IPTG induction with a yield of about 2 mg of rhL-31/L of bacterial culture. The rhL-31 was purified by single-step affinity chromatography using asialofetuin covalently bound to Affi-Gel-15 and elution with 200 mM lactose in PBS containing 2 mM DTT. The eluted protein fraction was separated by SDS–PAGE and consisted of a single band of 31 kDa as visualized by Coomassie staining (Figure 2a). The corresponding immunoblot analysis (Figure 2b) confirmed the identity of the 31-kDa band as the rhL-31 gal-lectin. No rhL-31 was detected either in non-transformed *E. coli* lysates or in the lysate of *E. coli* transformed with the anti-sense hL-31 cDNA (not shown).

Next, we tested whether the rhL-31 exhibits the biological activity of lectins, namely, the ability to agglutinate trypsin-treated, glutaraldehyde-fixed rabbit erythrocytes. As depicted in Figure 3a, the rhL-31 induced in a dose-dependent fashion hemagglutination at a concentration as low as 2.5 μg/mL. The hemagglutination was completely abolished by lactose (80 mM) (Figure 3a′). Previously, the homologous murine lectin was designated as S-type lectin as it was shown to be Ca^{2+} independent and to require reducing agents for hemagglutinating activity (Drickamer, 1988). To test the dependence of the rhL-31 on reducing agents for hemagglutinating activity, the polypeptide was reextracted and purified from *E. coli* extracts in the absence of DTT. Figure 3b depicts a serial dilution hemagglutination assay, revealing the rhL-31 potency was retained and inhibited by lactose in the absence of reducing agent (Figure 3b′). Similar observations were obtained by testing the hemagglutinating activity of the IgE-binding protein, ϵBP (Frigeri et al., 1990). In addition, other gal-lectins such as kidney asialofetuin-binding lectin (Foddy et al., 1990), the lung lectin (Cerra et al., 1985), and rCBP35 (Woo et al., 1991) also bind carbohydrates under nonreducing conditions. Furthermore, the rhL-31 also binds to immobilized laminin (Ochieng et al., 1992) in a similar fashion to other

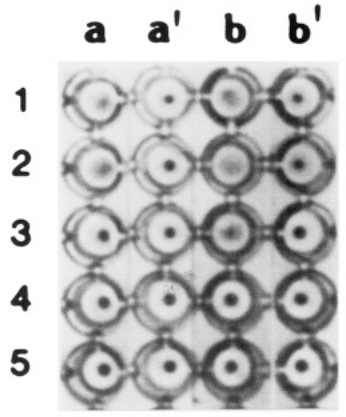

FIGURE 3: Hemagglutinating activity of recombinant hL-31. The rhL-31 was diluted from 10 μg/mL by serial 2-fold dilutions in CMF-PBS. The assay was done in V-shaped-bottom microtiter plates. Lane a, double-dilution hemagglutinating activity of rhL-31 in the presence of 2 mM DTT; lane a′, same as in lane a but with 80 mM lactose in each well; lanes b and b′, same as lanes a and a′, respectively, but without DTT.

native gal-lectins (Zhou & Cummings, 1990; Cooper et al., 1991; Woo et al., 1990).

Self-Association of rhL-31. By definition, hemagglutination activity mediated by a lectin requires it to be bi- or polyvalent in order to form a bridge between adjacent erythrocytes and produce agglutination. However, the affinity chromatography eluted polypeptide appeared as a single band of 31 kDa (Figure 2), similar to the results obtained for rϵBP (Frigeri et al., 1990). It was recently reported, however, that rCBP35 purified by laminin affinity chromatography is a disulfide-linked homodimer of 67 kDa and that the failure of other investigators to detect it stemmed from the presence of reducing agents in one or more of the purification steps (Woo et al., 1991). We had also previously reported that affinity purification of murine tumor gal-lectins may result in the appearance of two polypeptides species of 34 and 68 kDa (Raz et al., 1984). However, subsequent experiments revealed inconsistency and reproducibility problems in the detection of the higher molecular mass lectin species. Thus, the recent reports on the dimerization of CBP35 (Woo et al., 1991) and the lack of observed dimerization of rϵBP (Frigeri et al., 1990), and the relatively high concentration dependence of lectin-mediated hemagglutination, prompted the question of whether the dimerization of gel-lectins is concentration-dependent, covalent-binding, or both. In an attempt to answer this question, we isolated, purified, and eluted 1 mg of rhL-31 through a gel filtration column in the presence and absence of DTT, collecting 2-mL fractions. In both cases, only one protein peak was observed, eluting in the region of the 30-kDa band (Figure 4). The peak fractions in both cases (reducing and nonreducing) were collected, their protein concentrations determined, and were then subjected to reducing and non-reducing SDS–PAGE and analyzed by Western blotting. This analysis showed the 31-kDa band in the peak fractions (Figure 4 inset), but unexpectedly, a second band of 62 kDa appeared in the peak fractions (analyzed by nonreducing SDS–PAGE). Apparently, the rhL-31 undergoes dimerization in the tubes with high concentration of the monomer (>20 μg/mL; protein concentration of each tube not shown). When the peak fractions were analyzed by SDS–PAGE/Western under reducing conditions, the dimer band (62 kDa) was hardly visible (data not shown).

The electron microscopic analysis of rhL-31 showed that the majority of molecules consist of Y-shaped structures (Figure 5). The overall dimension and structure of rhL-31

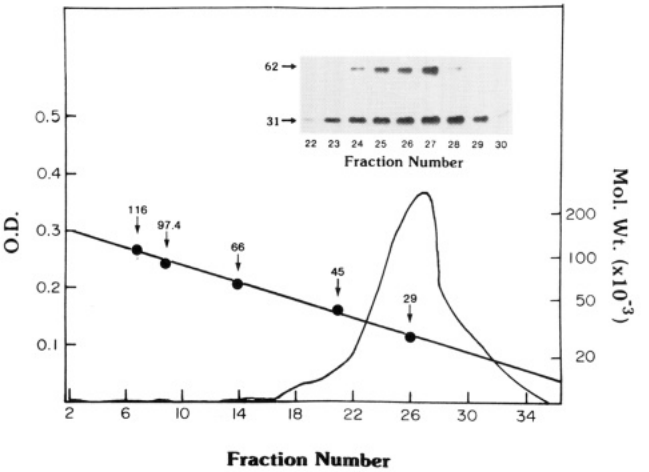

FIGURE 4: Gel filtration and concentration-dependent dimerization of rhL-31. One milligram of rhL-31 was passed through a Sephacryl-100 gel filtration column which had been calibrated with molecular mass markers (β-galactosidase, 116 kDa; phosphorylase B, 97.4 kDa; bovine serum albumin, 66 kDa; ovalbumin, 45 kDa; carbonic anhydrase, 29 kDa). The peak fractions were collected; their protein concentrations were determined and then subjected to SDS–PAGE under nonreducing conditions. After electroblotting, the fractions were immunodetected using anti-lectin antibodies (inset). The positions of the 31- and 62-kDa species as determined by molecular mass standards are shown by arrows at the left (inset). Numbers of fraction in the Western analysis correspond to those of the gel filtration.

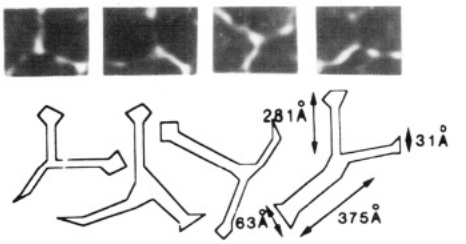

FIGURE 5: Electron micrographs of rhL-31. The bottom view represents a schematic representation of the rhL-31 molecules, and the numbers relate to the dimensions in angstroms (595000×).

are similar to the previously reported collagen-like molecules, i.e., Clq, SP-A, and MBP (Thiel & Reid, 1984). To prove that the structures we observed were actually rhL-31 and not artifacts, metal replicas made under the same conditions but without rhL-31 showed only a clear background. It is likely that the carbohydrate binding and hemagglutination are mediated by the "arms" of the Y, and the rhL-31, like the Clq, the SPA-A, and the MPB (Thiel & Reid, 1985), shows the characteristic bend halfway along the molecule, pointing to the departure from the collagen-like "stem" to the carbohydrate-binding half of the molecule.

DISCUSSION

In this report, we have attempted to determine (a) the membrane expression and disposition of GBL-30 and (b) the structure–function relationship of a recombinant GBL-30.

Although the GBL-30 was previously shown to be a cell-surface constituent, it became apparent from the predicted amino acid sequence of the lectin that the molecule lacks both an obvious leader and transmembrane domain sequences (Drickamer, 1988; Foddy et al., 1990; Frigeri et al., 1990; Frigeri & Liu, 1992; Gritzmacher et al., 1988), and thus the question concerning their transport to and disposing on the cell surface remains largely unknown. On the basis of cell-surface labeling, immunohistochemical staining, and ligand binding, a cell-surface orientation was proposed whereby the

carboxy terminus, which encompasses the galactoside-binding site, is facing the outside of the cell while the membrane orientation of the amino-terminal half of the molecule has not been described to date. Here we show that part of the amino terminal of the lectin, containing the collagen-like sequence, is also exposed on the cell surface, as determined by its susceptibility to collagenase cleavage. The results of the immunoprecipitation show that the cleavage of the lectin by collagenase is restricted to the collagen-like domain. Collagenase degradation of soluble mL-34 resulted in the appearance of at least four polypeptides ranging from 23 to 29.5 kDa (Figure 1), while the membrane-bound mL-34 was cleaved only to one or two polypeptides with an apparent molecular mass of 26.5 kDa (Figure 1). These results suggest that only the first, the second, or both of the collagenase cleavage sites of mL-34, amino acid residues 21–24 and 36–37 (from the first methionine) (Raz et al., 1989), are accessible to enzyme degradation on the cell surface. Taken together, it appears that, at least in part, both the amino and the carboxy termini are facing the outer cell surface. From the sequence of mL-34, it is evident that the first 11 amino acid residue (from the initiation codon) domain is of noncollagenous origin (Raz et al., 1989) and may act as a leader sequence. There are 2 distinct hydrophobic domains in the molecule (134–152 and 214–235 amino acid residues) which may interact with the membrane lipid bilayer. If the first domain [134–152 amino acid (aa) residues] is used to anchor the mL-34 to the plasma membrane, its size does not permit a full turn in the membrane, and therefore it cannot span the membrane twice, allowing both ends to be exposed outside. However, this domain may interact with the external half of the plasma membrane bilayer, allowing the lectin to behave as a peripheral rather than a integral membrane protein. The second hydrophobic domain (214–235 aa residues), although not a classical transmembrane-spanning domain, may be utilized for such a function, thus explaining the results showing that both the amino terminus and the galactoside-binding sites are exposed on the outer cell surface, suggesting that the carboxy terminus (236–264 aa residues) may be facing the cytoplasm. At this stage, however, we cannot rule out the possibility that the lectin is associated by a noncovalent interaction with other membrane protein(s) or glycoconjugates. It was recently suggested that εBP found on the surface of mast cells may be attached to glycolipids or glycoproteins, probably via the galactoside-binding site, as the binding is reversed by lactose. This observation raised the question as to how the galactoside-binding site if occupied for cell-surface anchoring may participate in IgE-binding and cell-rosette formation (Frigeri & Liu, 1992), unless these functions are mediated by different domain(s) of the molecule. An important serendipitous observation was the failure of collagenase treatment to release the lectin from the viable fibrosarcoma cell surface (not shown), as previously reported for the collagenase treatment of *Klebsiella pneumonia* bacteria, which resulted in the release of pullulanase from the cell surface (Charalamboas et al., 1988). This enzyme, like GBL-30, is a cell-surface polypeptide having six G-X-Y collagen-like repeats at its amino terminus, with a functional carboxy terminus. It is still unclear how GBL-30 is associated with the cell surface. In order to resolve this puzzle, we intend to generate antibodies against synthetic peptides representing the amino and carboxy termini of the molecule and use them to directly establish the membrane structural orientation of GBL-30.

To further study the structure–function relationship of GBL-30 activities, the human homologue (hL-31) recombinant protein was isolated. The recombinant lectin was purified by one-step affinity chromatography on immobilized asialofetuin, and eluted with lactose, resulting in one molecular mass protein species of 31 kDa. The protein was identified as rhL-31 by immunoblotting analysis either with monoclonal antibodies to the Mac-2 antigen, which is the murine lectin homologue (Woo et al., 1990), or with polyclonal antibodies directed against a specific sequence of the lectin (Raz et al., 1989). In addition to its lactose-dependent asialofetuin binding, the rhL-31 also induces hemagglutination and binds to immobilized laminin (Ochieng et al., 1992), and both processess are inhibited by lactose. These results imply that the rhL-31 maintained a similar structurally active conformation as the native polypeptide. It has been previously assumed that GBL-30 requires reducing conditions for its biological activity. However, the thiol dependence of this family of protein activity was recently questioned, as it was shown that both rat εBP and CBP35 in either native or recombinant forms do not require reducing conditions for carbohydrate-binding function (Frigeri et al., 1990; Woo et al., 1991). Here we show that rhL-31 binds carbohydrates and induces hemagglutination under both reducing and nonreducing conditions, and thus we concur with the notion that the GBL-30 group of proteins do not necessarily need reducing conditions for biological activity (Frigeri et al., 1990; Woo et al., 1991).

Studies on the role of reducing conditions in the function of GBL-30 point to the question concerning the S–S dimerization of the molecules (Woo et al., 1991). Previously it was shown that GBL-30 molecules eluted from size-exclusion columns as monomers (Raz et al., 1989, 1991; Jia & Wang, 1988; Albrandt et al., 1987; Woo et al., 1990; Oda et al., 1991). These observations were, however, recently challenged as it was shown that rCBP35 forms functional dimers using cysteine-186 (Woo et al., 1991). We previously showed that lectins purified from murine tumor cells may appear as two polypeptide species of 34 and 68 kDa (Raz et al., 1984). However, we could not consistently detect the 68-kDa species in subsequent experiments. Taken together, we speculated that the apparent conflict in the molecular sizing of the lectins may not be due to experimental deficiency but rather from purification yields and the concentration of the lectin in solutions. Indeed, we show (Figure 4) that rhL-31 may appear as a dimer at a concentration exceeding 20 μg/mL. Since the analysis presented in Figure 4 was done under nonreducing conditions, the question as to why the dimer was not eluted between tubes 12 and 16 where it was expected arises. As the lectin is eluted from the column, due to the dilution and spreading of the "dimer peak", the equilibrium is shifted in favor of the monomer, and hence the dimer peak is not observed. Thus, it appears that formation of the rhL-31 dimer is favored mainly by a high monomer concentration. The dimerized molecule, however, appears to be unstable with equilibrium quickly shifting to the monomer in dilute solutions. Therefore, other yet undefined interactions, such as disulfide bond formation as suggested by Woo et al. (1991), may be necessary to stabilize the dimerized molecules. Recently, Hsu et al. (1992) showed that εBP, a member of the GBP-30, can be chemically cross-linked at relatively high monomer concentration to form dimers and higher molecular weight aggregates. We have also shown that both rhL-31 and cellular hL-31 can be easily cross-linked by bis(sulfosuccinimidyl suberate) to form dimers and higher molecular weight aggregates (data not shown), implying that these molecules

are closely associated with each other. It is conceivable that these molecules can use their amino-terminal end for this association as suggested by Hsu et al. (1992), so that their carbohydrate-binding domain is free for biological activity such as hemagglutination. These results are not surprising in light of the observations that other molecules which contain collagen-like sequences and globular domains such as pullulanase, Clq, SPA, and MBP undergo a noncovalent self-assembly (Thiel and Reid, 1984; Charalambous et al., 1986). The Y-shaped molecules observed in the electron micrographs most probably represent the dimerized forms of hL-31 since visualization was done after preparation of a concentrated protein sample (100 μg/mL).

From the results presented above and the fact that the 14-kDa galactoside-binding lectins are devoid of collagen-like sequences, a question concerning the functional role of this sequence in lectin physiological function arises. It is possible that all or at least part of the collagen-like sequence (the collagenase-resistant part) might serve to stabilize the dimer either as soluble or as insoluble forms or can act as a "spacer" separating the functional, globular domain of GBL-30 from the cell surface, similar to its proposed role in pullulanase (Tong et al., 1988). Alternatively, this domain may serve as an additional functional domain of the molecule not associated with its galactoside-binding property. Several other proteins, Clq, MBP, SP-A, and conglutinin, were reported to have similar structural similarities to GBL-30. They contain a short NH_2-terminal of non-collagen-like sequence, followed by a collagenous region of repeating Gly-X-Y sequence, and a COOH-terminal containing the recognition domain (Voss et al., 1988). All of these molecules were found to be recognized by the Clq receptor through their collagenous domain. Thus, it was proposed that these molecules and probably GBL-30 can serve dual functions as receptor (for ligands) and as ligand for receptor(s) recognizing collagen- and collagen-like sequences. Binding of molecules containing collagen-like sequences to a common receptor, or to a family of receptors, could be of physiological significance as a general clearance mechanism of the ligands of these molecules (Voss et al., 1988).

The concept that carbohydrate-binding proteins may have more than one function is not new. Macrophage asialoglycoprotein-binding protein I has a carbohydrate-binding site and a distinct cell recognition signal, R-G-D (Li et al., 1990). Insulin-like growth factor II (Tong et al., 1988), elastin/laminin receptor (Mecham et al., 1989), and human lymphocyte low-affinity Fc receptor (Bettler et al., 1989) have distinct sites for protein–protein and protein–carbohydrate interactions. In addition, the gene family of selectins contains an amino terminal of a lectin-like domain followed by an epidermal growth factor-like motif and consensus repeats related to the complement-regulatory proteins (McEver, 1991). The results reported here should facilitate the understanding of the physiogiological role(s) of GBL-30 and contribute to the understanding of the structure–function relationship of the various domains of the lectin.

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