L-29, an Endogenous Lectin, Binds to Glycoconjugate Ligands with Positive Cooperativity†

Stephen M. Massa,‡ Douglas N. W. Cooper,§ Hakon Leffler,§ and Samuel H. Barondes*,§

Department of Psychiatry and Langley Porter Psychiatric Institute and Department of Neurology, University of California, San Francisco, California 94143

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ABSTRACT: The soluble mammalian lactose-binding lectins L-14-I and L-29 are both secreted and bind to oligosaccharides on laminin, a large extracellular matrix glycoprotein containing polylactosamine chains. Because of the potential functional significance of these lectin–laminin interactions, we compared quantitative aspects of L-14-I and L-29 binding to immobilized laminin using recombinant lectins labeled with ^{125}I. We report that the concentration-dependent binding of L-29 exhibits positive cooperativity whereas binding of L-14-I does not. Cooperative binding of L-29 can also occur on glycoconjugate substrates other than laminin and is not dependent on cystine bond formation or aggregation in solution. L-29 contains repetitive sequences within the N-terminal domain not present in L-14-I. This domain is not required for binding activity, but is required for positive cooperativity. Though the precise mechanism of interaction of L-29 with laminin remains to be determined, it apparently results in assembly of a lectin aggregate on the substrate surface, which could have important functional consequences.

Soluble lactose-binding lectins (S-Lac)[1] are a family of vertebrate proteins which are soluble without detergents, share homologous lactose-binding domains, and do not require divalent cations for carbohydrate binding (Drickamer, 1988; Leffler et al., 1989). Beyond these basic similarities, they are distinct with regard to fine carbohydrate specificity (Leffler & Barondes, 1986), cellular and tissue localization (Barondes, 1984; Crittenden et al., 1984; Harrison, 1991; Wasano et al., 1990), and putative functions (Harrison, 1991; Hamann et al., 1991; Frigeri & Liu, 1992; Mercurio & Shaw, 1991; Cooper et al., 1991). The most extensively studied of these are L-14-I and L-29. Both are found in the cytoplasm (Moutsatsos et al., 1986; Cooper & Barondes, 1990; Harrison, 1991) and are secreted despite the lack of any known secretion-mediating sequences (Cooper & Barondes, 1990; Lindstedt et al., 1991). For L-14-I, evidence for an alternative mechanism of secretion has been presented (Cooper & Barondes, 1990), involving accumulation in the cytosol beneath the plasma membrane followed by pinching off of lectin-rich vesicles. For L-29, the mechanism of secretion is unknown.

L-29 exhibits a number of structural and functional characteristics which differentiate it from L-14-I: (1) it possesses N-terminal domains not present in L-14-I, the first with some similarity to a serum response factor and the second containing a repeating sequence rich in proline and glycine (Albrandt et al., 1987; Jia & Wang, 1988; Oda et al., 1991; Cherayil et al., 1989, 1990; Robertson et al., 1990; Raz et al., 1989); (2) it is monomeric in solution in reduced form (Woo et al., 1991), while L-14-I forms a homodimer (Harrison, 1991); (3) it is phosphorylated in a regulated fashion (Cowles et al., 1990; Hamann et al., 1991), a modification not observed in L-14-I (Tracey et al., 1992); and (4) it has a higher relative affinity for branched polylactosamine chains (Leffler & Barondes, 1986; Sparrow et al., 1987).

Laminin, a large multifunctional basement membrane glycoprotein containing numerous polylactosamine chains (Arumugham et al., 1986; Fujiwara et al., 1988; Knibbs et al., 1989), has been implicated as a ligand for both L-14-I (Cooper et al., 1991; Zhou & Cummings, 1990) and L-29 (Woo et al., 1990; Mercurio & Shaw, 1991). The functional significance of interactions of laminin with lectins has been the subject of several recent studies (Dean et al., 1990; Cooper et al., 1991; Bouzon et al., 1990; Sato & Hughes, 1992). These have suggested that lectins may modulate cell–matrix interactions by interfering with cell-surface binding to laminin, perhaps by sterically hindering association of a cell-surface receptor (e.g., an integrin) with a laminin peptide epitope adjacent to an oligosaccharide.

To evaluate possible differences in the interaction of the lectins with this critical glycoconjugate component of the extracellular matrix, we examined binding of ^{125}I-labeled lectins to laminin substrates. We report here that, in contrast to L-14-I, L-29 binding to laminin exhibits striking positive cooperativity. We also present evidence that this property depends on an intact N-terminal domain but does not involve disulfide-mediated dimerization.[2]

EXPERIMENTAL PROCEDURES

Materials. Unless otherwise noted, chemicals and proteins were purchased from Sigma Chemical Co. (St. Louis, MO),

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* To whom correspondence should be addressed at the Langley Porter Psychiatric Institute, Box F-0984, University of California, San Francisco, CA 94143.

‡ Department of Neurology.

§ Department of Psychiatry and Langley Porter Psychiatric Institute.

[1] Abbreviations: S-Lac lectins, soluble lactose-binding lectins; L-14-I, lectin of subunit molecular weight about 14 000, also known under the names L-14, galaptin, bovine heart lectin, human placenta lectin, human spleen galaptin, and others; L-29, lectin also known under the names CBP-35, L-34, Mac-2, and IgE-binding protein (EBP); BSA, bovine serum albumin; LacNAc–BSA, *N*-acetyllactosamine–BSA; PCR, polymerase chain reaction; PBS, phosphate-buffered saline; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; CD, circular dichroism.

[2] This work was presented at the Second Keystone Symposium on Glycobiology, Park City, UT, March 21–27, 1992 (Massa et al., 1992).

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restriction enzymes were from Bethesda Research Laboratories (Gaithersburg, MD), and Taq DNA polymerase was from Perkin-Elmer Cetus (Norwalk, CT). (LactNAC-β-*O*-CET-E)$_n$–bovine serum albumin (LacNAc–BSA) was obtained from Research Diagnostics Inc. (Flanders, NJ). ^{125}I-labeled Bolton–Hunter reagent was obtained from New England Nuclear/Dupont (Boston, MA).

Recombinant Lectins. Recombinant rat L-14-I was produced in *Escherichia coli*, treated with iodoacetamide, and purified as previously described (Cooper et al., 1991). To prepare recombinant L-29, a plasmid containing the complete coding sequence of human L-29 (Oda et al., 1991) was used as template for PCR, with primers designed to create a 5′ *Nde*I site at the initiating ATG (5′-GCCAACGAGCG-GCATATGGCAGAC-3′) and a *Bam*HI site 3′ to the terminating sequences (5′-ACACAGGATCCAGCTAT-GACC-3′). The PCR product was ligated to pCR1000 (TA cloning system; Invitrogen, San Diego, CA) and excised with *Nde*I and *Bam*HI, and the fragment was ligated into *Nde*I- and *Bam*HI-digested pET-3C (Novagen Inc., Madison, WI). *E. coli* strain BL21(DE3) transformed with this construct was stimulated with 0.1–0.5 mM isopropyl β-D-thiogalactopyranoside and lysed by sonication in phosphate-buffered saline (PBS: 20 mM sodium phosphate/150 mM NaCl, pH 7.4) with 0.5 mg/mL phenylmethanesulfonyl fluoride, and lectin was isolated by affinity chromatography on lactosyl-Sepharose and eluted with lactose as previously described (Leffler et al., 1989).

Collagenase Digestion. L-29 was incubated overnight at 37 °C with type VII collagenase (Sigma) with a lectin: collagenase ratio of 20:1 by weight in 20 mM Tris-HCl, pH 7.4, 150 mM NaCl, and 2 mM $CaCl_2$. The degree of proteolysis was assessed by SDS–PAGE electrophoresis utilizing the PHAST system (Pharmacia LKB Biotechnology, Uppsala, Sweden) with 8–25% gradient gels as described by the manufacturer.

^{125}I Labeling. L-14-I and L-29 were labeled with ^{125}I-labeled Bolton–Hunter reagent by a modification of the method described by Harlow and Lane (1988). The protein solution was incubated with the Bolton–Hunter reagent overnight at 4 °C and the lectin repurified by affinity chromatography on lactosyl-Sepharose with 1 mg/mL BSA added as carrier. It was then dialyzed extensively against PBS–BSA to remove lactose. Specific activities obtained were 1.8–7.1 μCi/μg for L-29 and 1.9 μCi/μg for L-14.

Binding Assay. Immulon 2 Removawells (Dynatech Laboratories Inc., Chantilly, VA) were coated with laminin and other proteins by incubation overnight at 4 °C with 50 μL of protein in PBS without Ca^{2+} or Mg^{2+}. The amount of laminin bound to wells at varying concentrations was estimated by using ^{125}I-labeled laminin. Unless otherwise noted, the amount of laminin added per well was 110 ng. Wells were then saturated with protein by incubation with 1 mg/mL BSA in PBS for 3 h at room temperature.

For the assessment of concentration-dependent binding of the lectin, a constant amount of radiolabeled lectin (usually between 15 000 and 30 000 cpm) was added to a series of wells, with increasing amounts of unlabeled lectin. The radiolabeled lectins and other test materials in PBS with 1 mg/mL BSA and 0.05% Tween-20 were combined in the protein-coated wells to a final volume of 20 μL and incubated with rocking for 75 min at room temperature. For sugar inhibition experiments, a constant amount of radiolabeled lectin of appropriate specific activity was added to a series of wells, with increasing concentrations of lactose. At the end of the incubation period, the sample wells were washed with four 150-μL aliquots of PBS containing 0.05% Tween-20. The wells were separated and individually counted in a Beckman Gamma 4000 counter. Particular care was taken to wash rapidly (0.5–1 s/wash) and reproducibly, to accurately reflect equilibrium binding. Percentage bound was calculated as 100[(cpm bound to test substance – cpm bound to BSA)/(cpm added to well)]. Background binding of ^{125}I-labeled lectin to BSA alone was usually less than 150 cpm. All experiments were performed in triplicate and are generally reported as mean ± standard error. In some graphs, data from multiple experiments are pooled.

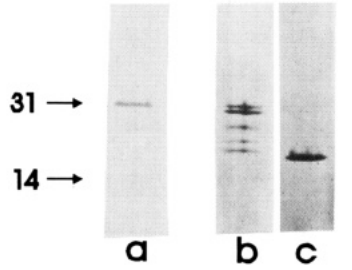

FIGURE 1: SDS–PAGE of recombinant L-29 preparations. (Lane a) Recombinant L-29 purified by affinity chromatography; (lane b) L-29 stored at 4 °C for 1 month; (lane c) L-29 digested with bacterial collagenase. Molecular weight standards: M_r 14 400, hen egg white lysozyme; M_r 31 000, bovine carbonic anhydrase.

Lectin Blots. Laminin was electrophoresed under reducing conditions in a 5% polyacrylamide gel, and blotted to nitrocellulose as described by Sambrook et al. (1989). The nitrocellulose blots were blocked with PBS containing 2% bovine hemoglobin and 0.05% Tween-20, incubated with radiolabeled lectins (approximately 0.2 μg/mL) with or without lactose in the same solution for 75 min, and washed with PBS/0.05% Tween-20 before fluorography on Kodak X-omat film.

Spectroscopy and Gel Filtration Chromatography. Circular dichroism spectra were recorded between 200 and 300 nm at room temperature with a Jasco J500A spectropolarimeter. A SPEX Fluorolog 1680 double spectrometer was used to record fluorescence emission spectra between 275 and 400 nm, using an excitation wavelength of 272 nm. Spectra from lectin concentration series were normalized and compared, and plots of concentration versus E_{302} and E_{348} were evaluated for linearity. Molecular sieve chromatography was performed with a Superdex 75 HR 10/30 column (Pharmacia) and a Perkin-Elmer Series 4 liquid chromatography system and LC-95 spectrophotometer/detector (Perkin-Elmer Corp.).

RESULTS

Recombinant L-29 isolated by affinity chromatography on lactosyl-Sepharose was more than 95% pure (Figure 1a). Some preparations contained substantial amounts of protein migrating in the M_r 17 000–26 000 range (as in Figure 1b). These proteins were demonstrated, by Edman sequencing, to be proteolytic products of L-29 that had been cleaved in the N-terminal region.

The relative lability of the N-terminal region was demonstrated by studies of the effects of exhaustive collagenase (Sigma type VII) digestion of several samples, which gave rise to a single protein band with a molecular weight of about 17 000 (Figure 1c). The collagenase-insensitive M_r 17 000 product was identified by Edman sequencing as the L-29 C-terminal region beginning at Gly^{107}. It was also noted that some purified lectin preparations underwent slow breakdown

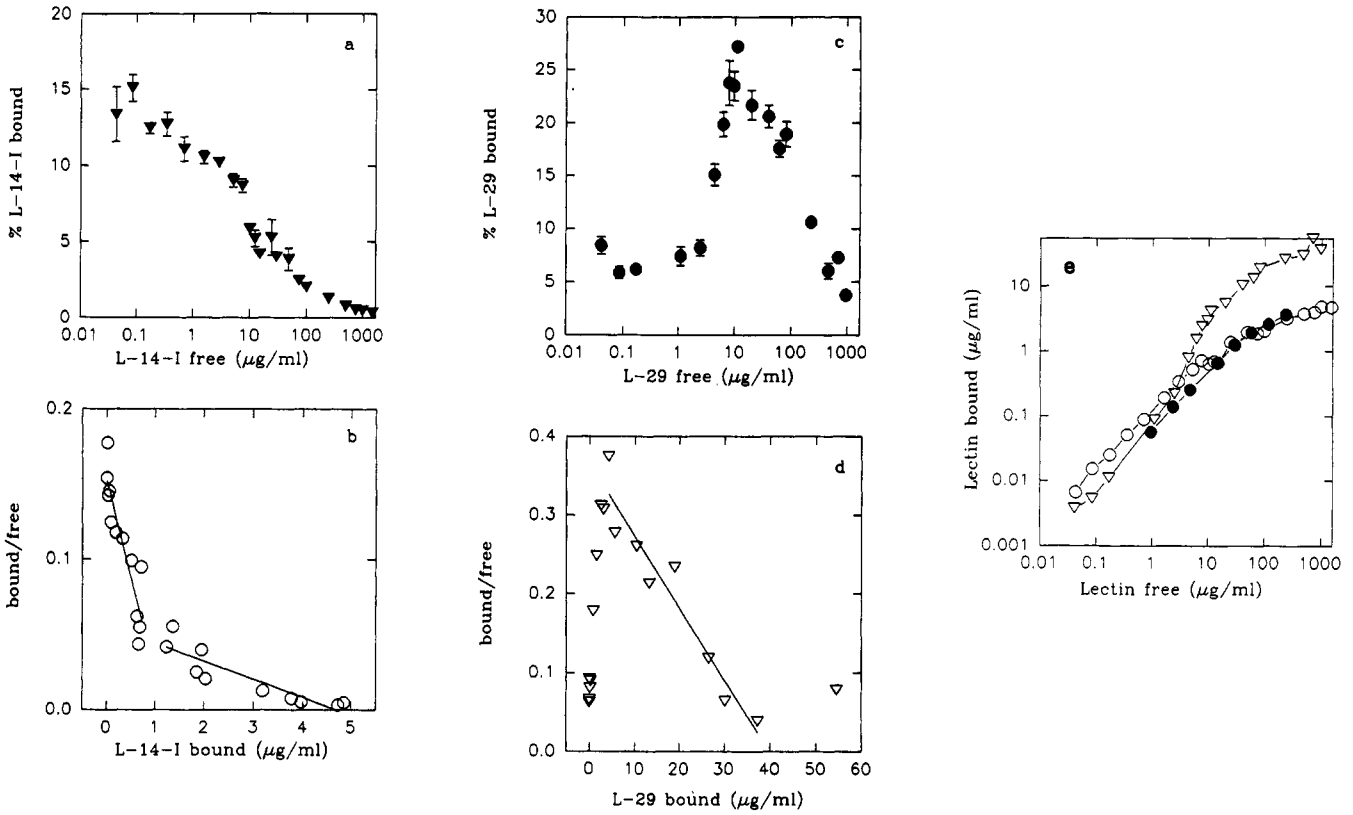

FIGURE 2: Binding of a series of concentrations of recombinant L-14-I and L-29 to a laminin substrate. A fixed tracer concentration of labeled lectin (represented by the leftmost data point in panels a, c, and e) was mixed with a range of concentrations of the same unlabeled lectin. The same data were plotted in three different ways: panels a and c show percent lectin bound vs concentration of free lectin; panels b and d show the data plotted according to Scatchard, and straight lines in the Scatchard plots represent linear regression performed with the program Sigmaplot (Jandel Scientific, Corte Madera, CA), with a two-component model (b) or simple regression (d); panel e shows the absolute amount of lectin bound vs free; here the data for L-14-I (open circles), L-29 (open triangles), and collagenase-treated L-29 (closed circles) are compared in the same plot. The other plot types for the collagenase-treated L-29 are shown in Figure 6 (open triangles).

to an M_r 17 000 product, suggesting the presence of a copurifying collagenase activity. Since proteolytic degradation of the lectin was found to influence the binding assays (see below), only L-29 preparations containing less than 1% degraded lectin, as assessed by SDS–PAGE, were used for the reported studies.

The concentration dependence of L-14-I and L-29 binding to laminin was examined using ^{125}I-labeled lectin incubated with laminin immobilized in plastic microtiter wells in the presence of various concentrations of cold lectin. The curves describing L-14-I binding revealed a monotonically diminishing percentage bound (Figure 2a), as saturation of the binding sites was approached (Figure 2e, open circles). Scatchard analysis of these data revealed a concave-upward curve with at least two components, consistent with multiple independent binding site classes, or negative cooperativity (Figure 2b). This result differed from the more unimodal curve for L-14-I binding to immobilized laminin reported by Zhou and Cummings (1990). The reasons for this difference are not entirely clear, but might result from their use of a narrower concentration range or a different laminin preparation.

L-29 binding to laminin was more complex. The percentage bound (Figure 2c) rose rapidly between 2 and 15 μg/mL added lectin, with approximately 30% of the lectin binding at the maximum. At concentrations higher than 15–25 μg/mL, the binding fraction declined though saturation was not achieved even at 1000 μg/mL (Figure 2e, open triangles). Direct comparison of L-14-I- and L-29-binding curves reveals a marked divergence between the two beginning at approximately 5 μg/mL (Figure 2e). Scatchard representation of L-29 binding (Figure 2d) shows a concave-downward curve of at least two components, consistent with positive cooperativity. The curve at higher bound concentrations was approximately linear, suggesting a single binding site class, though the presence of additional low-affinity sites could not be ruled out (Klotz, 1982). Extrapolation of the Scatchard curve to obtain an estimate of maximum binding suggests that, on average, greater than 200 L-29 molecules can bind to each laminin molecule.

The demonstration of a possible intermolecular interaction between L-29 molecules prompted consideration of the possibility that dimerization due to disulfide bonding might be involved. This had been suggested by the work of Woo et al. (1991), who reported the formation of disulfide-linked L-29 dimers on oxidation of recombinant lectin. To examine this possibility, binding assays were done in the presence of the reducing agents β-mercaptoethanol and dithiothreitol, and with lectin treated with iodoacetamide (Figure 3). In each case, the positive cooperativity was unaffected by the treatment, suggesting that disulfide cross-links are not involved.

To investigate potential differences in the binding of the lectins to different components of laminin, we examined binding of radiolabeled L-14-I and L-29 to laminin chains that had been separated by SDS–PAGE and blotted onto nitrocellulose (Figure 4). This revealed lactose-sensitive binding of each lectin to both A and B chains. The B1 and B2 chains were not resolved.

To examine the possibility that there were unique sites for L-14-I and L-29 in laminin, we studied the binding of mixtures

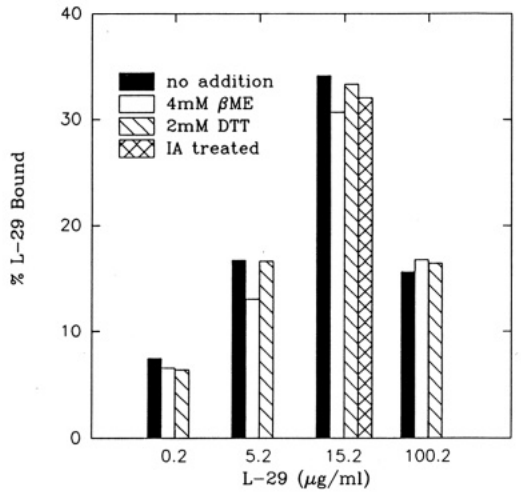

FIGURE 3: Effect of reducing agents and iodoacetamide treatment on binding of a series of concentrations of L-29 to laminin. Reducing agents: 4 mM β-mercaptoethanol or 2 mM dithiothreitol was included during the binding incubation. IA treated: L-29 was treated with a 50-fold molar excess of iodoacetamide at 4 °C overnight and purified by gel filtration. Iodoacetamide-treated lectin was tested only at 15 μg/mL.

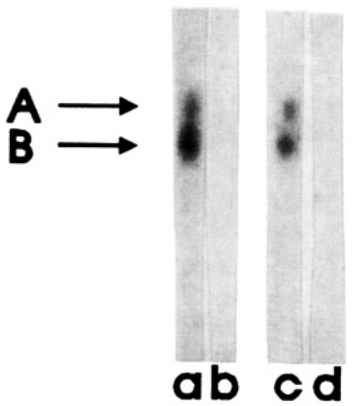

FIGURE 4: Autoradiograms of blots of separated laminin A and B chains reacted with [^{125}I]L-29 or [^{125}I]L-14-I in the presence or absence of 50 mM lactose. A and B, positions of laminin A and B chains. (a) [^{125}I]L-29; (b) [^{125}I]L-29 plus lactose; (c) [^{125}I]L-14-I; (d) [^{125}I]L-14-I plus lactose.

of the two lectins (Figure 5). L-29 readily inhibited [^{125}I]L-14-I binding and, as expected from its positive cooperativity, was more effective above 10 μg/mL than a molar equivalent concentration of L-14-I dimer (Figure 5a). L-14-I competitively inhibited [^{125}I] L-29 binding with no evidence for cooperative interaction of the two lectins (Figure 5b). These data suggest that the binding sites for L-14-I and L-29 overlap extensively.

To determine which region of L-29 was important for cooperative binding, we took advantage of the fact that bacterial collagenase digests only the proline–glycine-rich N-terminal domain, leaving intact the lactose-binding C-terminal domain. We digested ^{125}I-labeled L-29 with collagenase and assessed its binding to laminin (Figure 6). At low concentrations, binding of this fragment was similar to the intact lectin. However, in the presence of increasing concentrations of unlabeled C-terminal fragment, there was no evidence for cooperativity (Figure 6, inset), and the binding curve resembled that of L-14-I (Figure 2e, closed circles). The same lack of cooperativity was found for binding of the labeled fragment in the presence of increasing concentrations of unlabeled intact L-29. These results contrast strikingly with those obtained above for binding of intact L-29, and

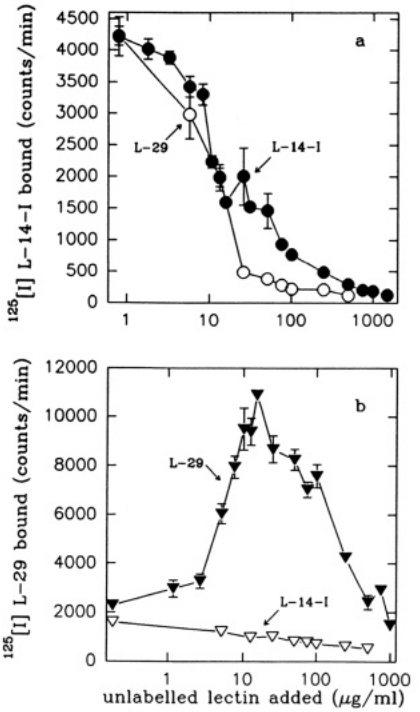

FIGURE 5: Effect of a series of concentrations of unlabeled L-14-I or L-29 on binding of fixed tracer concentrations of [^{125}I]L-14-I or [^{125}I]L-29 to immobilized laminin. (a) [^{125}I]L-14-I binding: filled circles, in the presence of increasing unlabeled L-14-I; open circles, in the presence of increasing unlabeled L-29. (b) [^{125}I]L-29 binding: filled triangles, in the presence of increasing unlabeled L-29; open triangles, in the presence of increasing unlabeled L-14-I.

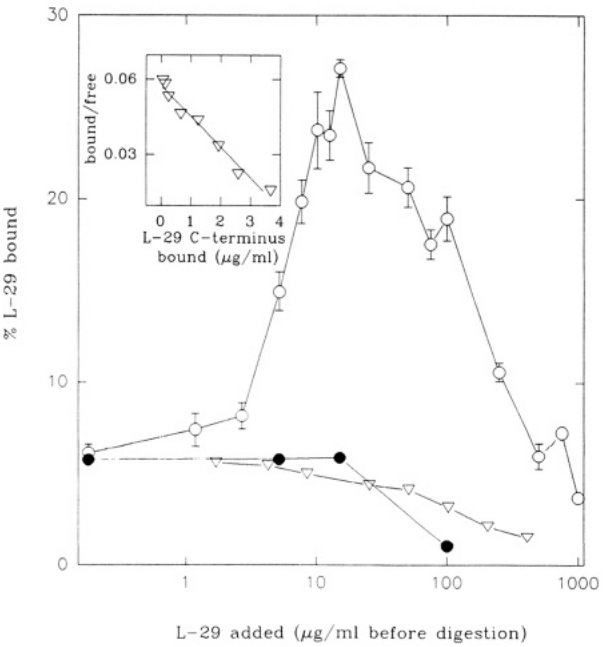

FIGURE 6: Collagenase treatment abolishes cooperative L-29 binding. Open circles, intact [^{125}I]L-29 binding in the presence of increasing intact unlabeled L-29. Closed circles, collagenase-treated [^{125}I]L-29 binding in the presence of increasing intact unlabeled L-29. Open triangles, collagenase-treated [^{125}I]L-29 binding in the presence of collagenase-treated unlabeled L-29 that had been repurified by gel filtration. Inset: Scatchard representation of the latter data.

suggest that the N-terminus plays an important role in the concentration-dependent cooperative binding of this lectin.

We were also interested in determining whether specific glycoconjugate or protein domains within laminin were required for cooperative L-29 binding. To assess this, we examined L-29 binding to a neoglycoprotein, LacNAc–BSA,

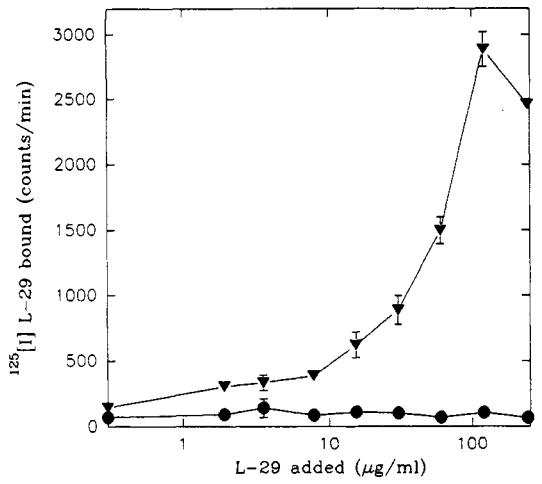

FIGURE 7: L-29 binding to LacNAc–BSA. LacNAc–BSA or BSA was incubated in sample wells, each at a concentration of 1 mg/mL, before washing and blocking with BSA as described under Experimental Procedures. Conditions were otherwise the same as with laminin-binding experiments. Filled triangles, binding to LacNAc–BSA. Filled circles, binding to BSA.

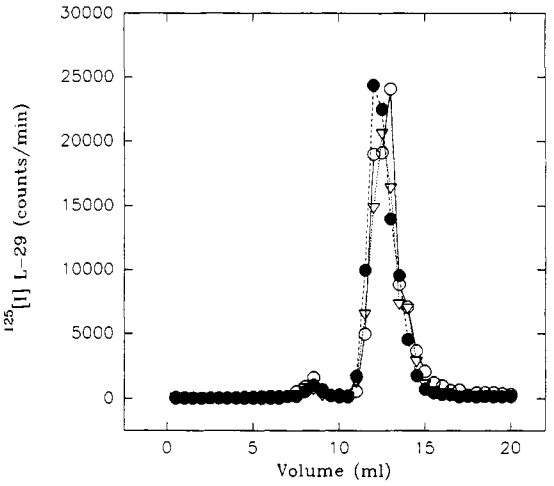

FIGURE 8: Molecular sieve chromatography of L-29. In all cases, [^{125}I]L-29 (0.2 μg/mL) was injected with BSA and eluted in PBS, with the following additions: open circles, none; open triangles, addition of 500 μg/mL unlabeled L-29 to the injected solution; filled circles, addition of 500 μg/mL unlabeled L-29 plus 2.2 mM *N*-acetyllactosamine to the injected solution and of 2.2 mM *N*-acetyllactosamine to the elution medium.

which has neither complex polylactosamine chains nor the peptide sequences of laminin (Figure 7). We found that L-29 binding to LacNAc–BSA also showed positive cooperativity. Therefore, neither laminin-specific protein structures nor polylactosamine or other complex polysaccharides are required for cooperative binding of L-29.

Since cooperativity could involve aggregation of the lectin before binding to the substrate, several physical methods were applied to investigate the possibility that such protein–protein interactions may occur in solution. Fluorescence spectra were obtained at concentrations of L-29 ranging from 0.39 to 500 μg/mL, and CD spectra were obtained at concentrations ranging from 0.5 to 500 μg/mL (data not shown). These studies yielded no evidence of concentration-dependent changes in the chemical environment of tyrosine and tryptophan residues or changes in polypeptide backbone secondary structure, respectively, arguing against molecular interactions of L-29 in solution even at high concentration. Since the interaction of L-29 with carbohydrate ligand might be necessary to promote a conformational change necessary for oligomerization, we tested for spectral shifts in the presence of thiodigalactoside, the highest affinity simple saccharide ligand for L-29. The addition of thiodigalactoside to L-29 in PBS elicits a downward shift in peak emission intensity and wavelength (from 348 to 338 nm), consistent with a change in the chemical environment of lectin tryptophan residues due to saccharide binding. However, even in the presence of saturating concentrations of thiodigalactoside (1.6 mM for the fluorescence studies and 4 mM for the CD studies), no lectin concentration-dependent spectral changes suggestive of protein–protein interactions were found (data not shown).

We also examined possible multimerization of L-29 by gel filtration (Figure 8). [^{125}I]L-29 behaved primarily as a monomer. A small amount (less than 3%) of radioactive material eluted with a retention time appropriate for a dimer. This is likely to be an artifact arising during labeling or storage, since no such peak was observed by UV absorption on gel filtration of unlabeled lectin. The elution profile of [^{125}I]L-29 was not affected by incubation with a high concentration of unlabeled lectin (up to 500 μg/mL). Thus, there is no detectable formation of L-29 oligomers in solution. Since interaction of L-29 with carbohydrate ligand might be necessary to promote oligomerization and since cooperativity

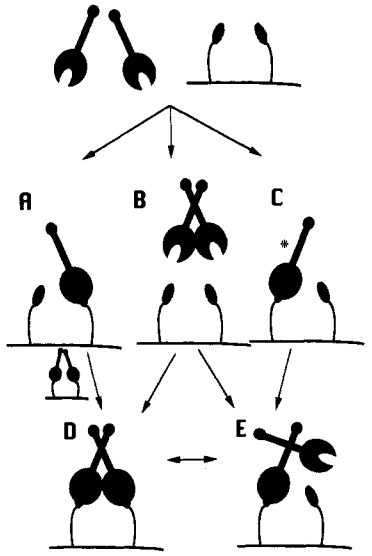

FIGURE 9: Schematic of possible mechanisms of interaction between L-29 and a polyvalent ligand. Filled figure, L-29; stippled figure, a polyvalent ligand, e.g., laminin. Though diagrammed as connected, binding sites might exist on different laminin molecules. Though depicted as dimers, L-29 aggregates are likely larger structures. The asterisk indicates a putative conformational change in L-29 structure, induced by binding to a glycoconjugate, that favors its binding to another L-29 monomer.

could be demonstrated on an *N*-acetyllactosamine-bearing substrate, we also tested effects of 2.2 mM *N*-acetyllactosamine on lectin elution (Figure 8). Again, there was no change in the elution profile, arguing against self-association in solution.

Since there experiments suggested that oligomerization was dependent on interaction of L-29 with immobilized as opposed to soluble glycoconjugate ligands, we considered possible explanations for this behavior. Perhaps when a monomer of L-29 binds to an oligosaccharide in laminin, it undergoes a conformational change allowing it to then bind to other L-29 monomers, as diagrammed in Figure 9C,E. An alternative is that independent binding of two or more L-29 molecules to adjacent glycoconjugates promotes protein–protein interactions, as diagrammed in Figure 9D. Were the latter possibility the case, L-29 binding to laminin should be more resistant to

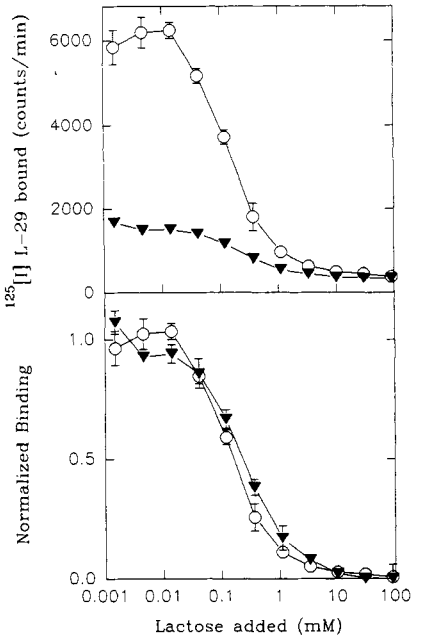

FIGURE 10: Effect of lactose on binding of high and low concentrations of L-29 to laminin. (Top) Samples at 0.18 μg/mL (filled triangles) or 15.18 μg/mL L-29 (open circles) were incubated with increasing concentrations of lactose, and binding was assessed. The K_i for each curve was approximately 0.3 mM. (Bottom) Curves from the top panel normalized to their average maximum and minimum values, as $(cpm - cpm_{min})/(cpm_{max} - cpm_{min})$.

inhibition by lactose under conditions in which there is positively cooperative binding, because the hypothesized multivalent interaction between binding sites on an L-29 oligomer and oligosaccharides on the laminin matrix should be more stable than monovalent binding. However, L-29 binding to a laminin substrate at high and low concentrations of L-29 showed no difference in sensitivity to lactose inhibition over a range of lactose concentrations (Figure 10).

DISCUSSION

We have compared the binding of the lactose-binding lectins L-29 and L-14-I to laminin. As expected, the binding characteristics of L-14-I were consistent with the binding of a divalent molecule to a polyvalent ligand (Dower et al., 1984). In such a model, high-affinity binding occurs at low concentrations due to divalent binding, while at higher concentrations lower affinity binding predominates due to disruption of potentially divalent binding sites by lectin binding in a univalent manner (i.e., with one subunit unliganded). This is consistent with the concordance of the binding curves of L-14-I and the univalent L-29 C-terminus at higher lectin concentrations (Figure 2e). However, this type of binding may also occur as the result of other mechanisms of negative cooperativity, or when there are multiple binding site classes (Norby et al., 1980). In contrast, the binding characteristics of L-29 were surprising, with a 4-fold increase in binding percentage over a relatively narrow concentration range (2–15 μg/mL), indicating positive cooperativity (Figure 2). In addition, the amount of lectin bound at saturation was remarkable, as each laminin molecule bound on average nearly 6 times its weight of L-29.

Cooperativity, positive or negative, is particularly prominent at control points in biological systems, where it may contribute an "on–off" effect, completely opening or closing a reaction pathway over a very narrow concentration range (VanHolde, 1971). For L-29 binding to cell surfaces or basement membrane components, the exquisite sensitivity of potentially cooperative binding to local lectin concentration could provide a finely balanced switch controlling local matrix organization, cell adhesion, or other functions. For example, generation of a multimer could make possible cross-linking between laminin and a cell-surface glycoconjugate. Cooperative interaction of L-29 with putative intracellular ligands in the cytosol or in the nucleus (Laing & Wang, 1988; Wang et al., 1991) might also be functionally important.

Given the potential functional role of this phenomenon, we sought to further characterize it by examining (1) structural features of the lectin required for cooperativity, (2) structural characteristics of the substrate necessary for cooperativity, and (3) potential mechanisms for the phenomenon.

Structural Features of the Lectin Required for Cooperativity. We were interested in the possibility that cystine bond formation, reported to result in dimerization of the lectin (Woo et al., 1991), was important for the cooperative effect. However, since cooperative binding was unaffected by reducing agents, it seems unlikely that disulfide bridge formation has a major role in cooperativity.

The N-terminal half of L-29 includes a region, beginning at amino acid 40 in human and amino acid 39 in mouse, with repeats of the consensus sequence PGAYPG (7 repeats in human, 8 in mouse or rat), with spacing varying from 1 to 7 residues between repeats (Albrandt et al., 1987; Jia & Wang, 1988; Oda et al., 1991; Cherayil et al., 1989, 1990; Robertson et al., 1990; Raz et al., 1989). The function of this region is unknown, but the results reported here suggest that it may be involved in cooperative interactions between L-29 molecules. This proline- and glycine-rich region is susceptible to collagenase digestion, and such cleaved L-29 still binds to laminin, but without cooperativity (Figure 6, inset).

The repeating PGAYPG residues are reminiscent of repeating regions found in elastin, for which structural studies using synthetic polypeptides [e.g., $(VPVPG)_n$] have suggested the formation of a β-spiral (Chang et al., 1989; Urry et al., 1989; Bhandary et al., 1990; Arad & Goodman, 1990). This is an extended structure containing type II β-turns and multiple stabilizing intrachain hydrogen bonds. Like tropoelastin, these synthetic polypeptides may polymerize to form a network of filaments (Urry et al., 1989). Therefore, as with other elastin-like polypeptides, the repeating regions of L-29 might associate to form multimeric structures. L-29 molecules could potentially associate in parallel and/or antiparallel fashion, providing multivalent sugar-binding bridges to other regions of the substrate or more distant sites. However, it is also possible that the cooperative binding depends, in part or completely, on other parts of L-29.

The sensitivity of L-29-binding characteristics to removal of the N-terminus raises the possibility that L-29 function could be regulated by proteolysis. It is notable, in this regard, that metalloproteinases which digest many types of collagen and elastin as well as L-29 (J. Hermman, S. Fisher, and H. Leffler, unpublished data) are secreted by a number of cell types which also contain L-29, including alveolar and peritoneal macrophages, and fibroblasts (Moutsatsos et al., 1986; Sparrow et al., 1987; Ho & Springer, 1982; Emonard & Grimaud, 1990).

Structural Characteristics of the Substrate Necessary for Cooperativity. To define substrate components required for cooperative binding of L-29 to laminin, we examined L-29 binding to LacNAc–BSA and underivatized BSA. The results indicate that binding to a simple uniform polyvalent glycoconjugate ligand is sufficient to induce marked cooperativity

and that the structural properties of laminin are not essential for the effect. Thus, a mechanism based on specific lectin-induced changes in laminin configuration (e.g., uncovering of more binding sites, or altering the affinity of adjacent sites) is unlikely.

Possible Mechanisms of Cooperativity. As discussed above, cooperativity of L-29 binding to laminin requires a portion of the lectin not directly involved in carbohydrate binding and does not depend on structures specific to laminin. Therefore, we have further considered two possible mechanisms for the positive cooperativity: (1) L-29 might undergo concentration-dependent oligomerization in solution, with consequent changes in valency and binding affinity; (2) ligand binding might induce interaction between lectin molecules.

The first possibility (diagrammed in Figure 9B,D) is an unlikely explanation for the cooperative effect, since there was no evidence for significant intermolecular interactions in solution, over broad concentration ranges as measured by gel filtration (Figure 8), CD, and fluorescence spectra.

The second possibility might occur in two ways: (a) The binding of an L-29 monomer to an oligosaccharide ligand might induce a conformational change in the monomer which then favors its binding of other free L-29 molecules (diagrammed in Figure 9C,E). The result is a multimer in which one subunit is bound to a laminin glycoconjugate and others are held simply by protein–protein bonds. This possibility is consistent with the lack of change in sensitivity of L-29–laminin binding to lactose over a wide range of L-29 concentrations. However, the lack of aggregation of L-29 in the presence of free ligand (*N*-acetyllactosamine) argues against this hypothesis. (b) The binding of L-29 monomers to adjacent laminin oligosaccharides might allow high local concentrations or long interaction times necessary for the formation of stable lectin complexes (diagrammed in Figure 9A,D). However, it is difficult to reconcile this possibility with the finding that cooperative binding is not especially resistant to lactose inhibition.

The positively cooperative binding of L-29 to laminin is only seen at relatively high concentrations of the lectin (above 5 μg/mL), which raises the question of whether such concentrations are achieved physiologically. There is evidence that L-29, like other soluble lactose-binding lectins, may exist at concentrations far greater than that required for positive cooperativity. For example, Madin-Darby canine kidney cells contain approximately 135 μg/mL packed cell volume (Lindstedt et al., 1991). However, this lectin is concentrated in the cytosol and separated from extracellular or cell-surface glycoconjugates with which it could bind cooperatively. L-29 is secreted by some cell types (Moutsatsos et al., 1986; Lindstedt et al., 1991) and is a major surface-associated protein of activated macrophages (Ho & Springer, 1982). This suggests the possibility that cooperative binding of L-29 to extracellular glycoconjugates may be physiologically significant.

After this work was completed, Hsu et al., (1992) described binding to immobilized IgE of a protein they call ϵBP (Robertson et al., 1990) which is identical with L-29 (Oda et al., 1991). As in the present work, increasing concentrations of lectin resulted in increased fractional binding, and this positive cooperativity required the N-terminal domain. In contrast with the present work, Hsu et al. found some evidence that lectin oligomers formed in solution; but in both cases, oligomerization was greatly augmented by binding to a substrate. Their proposal that this would result in multivalent binding of higher affinity, which we also considered, is not supported by our findings.

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