Characterization of Monomeric Forms of Galectin-1 Generated by Site-Directed Mutagenesis†

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ABSTRACT: Galectin-1 is a β-galactoside-binding protein secreted by animal cells, and it exists in a monomer−dimer equilibrium ($K_d \approx 7$ μM). The function(s) of galectin-1 is(are) not yet defined, but dimerization and divalency are presumably important. Crystal structures of the mammalian galectin-1 dimer predict N- and C-terminal interactions at the subunit interface. To examine the mechanism of dimer formation and possibly generate active monomeric galectin-1, mutations were made in the N- and C-termini of recombinant hamster galectin-1. N-Gal-1 contains disruptions of three hydrophobic amino acids at the N-terminus; V5D-Gal-1 contains a single mutation of Val5 to Asp; N/C-Gal-1 contains multiple changes in hydrophobic amino acids at both the N- and C-termini. All mutants behave as monomers in size-exclusion HPLC and native gel electrophoresis. N-Gal-1 and V5D-Gal-1 bind weakly to lactosyl-Sepharose, but N/C-Gal-1 is nonfunctional. In equilibrium dialysis, N-Gal-1 and V5D-Gal-1 bind *N*-acetyllactosamine with a $K_d \approx 90$ μM, which is similar to that of native lectin. At high concentrations, V5D-Gal-1 and N-Gal-1 dimerize and can be covalently cross-linked with disuccinimidyl suberate. The K_d values of the monomer−dimer equilibrium for V5D-Gal-1 and N-Gal-1 are estimated to be ≈ 60 μM and ≈ 250 μM, respectively. The cross-linked dimers of V5D and N-Gal-1 were isolated and were similar to native lectin in both hemagglutinating activity and high-affinity binding to lactosyl-Sepharose. Thus, specific mutations in galectin-1 can alter monomer−dimer equilibrium without affecting carbohydrate-binding activity. The availability of active monomers and functional covalent dimers of galectin-1 should aid in future studies aimed at understanding the biological function(s) of the lectin and the role of divalency.

The galectins are a family of related β-galactoside-binding proteins also referred to as S-type or S-Lac lectins (Barondes et al., 1994a,b). Galectins exhibit an affinity for β-galactosides and share significant sequence similarity in the carbohydrate-binding domain (CRD) (Hirabayashi & Kasai, 1991). Eight members of the galectin family have now been identified (Barondes, 1994b; Oda et al., 1993; Madsen et al., 1995; Gitt et al., 1995a,b; Hadari et al., 1995). Galectins have been implicated in a variety of functions that include growth regulation (Wells & Mallucci, 1991), cell adhesion (Zhou & Cummings, 1993; Cooper at al., 1991), migration (Hughes, 1992), neoplastic transformation (Raz et al., 1990), immune responses (Cherayil et al., 1990; Liu, 1993), and T-cell maturation (Perillo et al., 1995). However, the widespread expression of multiple members of the galectin family and presumed overlaps in carbohydrate-binding specificities have made it difficult to establish the *in vivo* functions of individual members of this class of proteins (Poirier & Robertson, 1993).

Galectin-1, also known as galaptin, L-14, and BHL, is probably the best characterized member of the galectin family and was the first galectin to be identified (Teichberg et al., 1975; de Waard et al., 1976; Nowak et al., 1976). It is expressed by fibroblasts, endothelial cells, smooth and skeletal muscles, and many other cell types (Couraud et al., 1989). Galectin-1 is a homodimer with a subunit molecular mass of 14.5 KDa, but it exist in a reversible monomer−dimer equilibrium with a K_d of ≈ 7 μM (Cho & Cummings, 1995a,b). X-ray crystallographic analyses of bovine galectin-1 and human galectin-2 in complex with haptenic sugars have recently been published (Lobsanov et al., 1993; Liao et al., 1994; Bourne et al., 1994a). These studies revealed that galectin-1 exists as an extended β-sandwich in a jelly-roll topology typical of legume lectins (Liao et al., 1994). The crystal structure of the dimeric lectin bound to *N*-acetyllactosamine reveals that the subunit interface between the monomers contains interactions through N- and C-terminal residues of each subunit (Liao et al., 1994).

Many of the suggested functions of galectin-1 are believed to be mediated by the divalent protein, although the evidence does not rule out contributions by monomeric species (Cooper et al., 1991; Zhou & Cummings, 1993; Barondes et al., 1994b; Woynarowska et al., 1994; Lotan et al., 1994; Baum et al., 1995a,b). To better understand the functional differences between monomers and dimers and to define the residues critical for dimer formation, we have mutated residues within the N- and C-termini of hamster galectin-1. Our results demonstrate that mutations of residues within the extreme N-terminus of galectin-1 interfere with dimer formation and the monomeric proteins can dimerize only at high concentrations. The monomeric forms of the lectin are functional and display normal affinity to *N*-acetyllactosamine. The mutated lectins can be covalently cross-linked at high concentrations, and the covalently cross-linked dimers display normal hemagglutinating activity and binding to immobilized

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ligands. These studies demonstrate that a few N-terminal residues are critical for dimer formation and that a functional monomeric form of the galectin-1 can be generated.

EXPERIMENTAL PROCEDURES

Materials. Bovine serum albumin (fraction V), lactosyl-Sepharose, β-mercaptoethanol, phenylmethanesulfonyl fluoride, pepstatin, aprotinin, leupeptin, anti-rabbit IgG mouse antibody, and ethanolamine were obtained from Sigma Chemical Co. Molecular mass markers (14−200 kDa) for SDS−polyacrylamide gel electrophoresis were purchased from GIBCO. The Western blot kit containing 5-bromo-4-chloro-3′-indolylphosphate *p*-toluidine salt and nitro-blue tetrazolium chloride was obtained from BioRad Laboratories. Lactose, acetone, ethylenediaminetetraacetic acid (EDTA-disodium salt), and sodium bicarbonate were purchased from Baker Chemical Co. BCA reagents for protein determination and disuccinimidyl suberate (DSS)[1] were obtained from Pierce. UDP-[6-^{3}H]Gal (48.8 Ci/mmol) was obtained from DuPont-NEN. Purified antibodies to galectin-1 were prepared as described previously (Cho & Cummings, 1995a).

Mutation, Expression, and Purification of Monomeric Galectin-1. The CHO galectin-1 cDNA (Cho & Cummings, 1995a) was modified using PCR primer-directed mutagenesis that introduced amino acid changes at either N-terminal or C-terminal contact sites presumed to be important for dimerization. A *Bam*HI site was placed in the 5′ primer, and a *Hin*dIII site was placed in the 3′ primer (5′ primer for both N-Gal-1 and N/C-Gal-1: 5′-GGGGGATCCGCCTCTG-GTCAGGACTCAAGCAAC; 5′ primer for V5D-Gal-1: 5′-GGGGGATCCGCCTGTGGTCTGGACGCAAGCAAC; 3′ primer for N-Gal-1 and V5D-Gal-1: 5′-GGGAAGCTTT-CACTCAAAGGCCACGCA; 3′ primer for N/C-Gal-1: 5′-GGGAAGCTTTCACTCAAAGGCCTCGCACTTAGTCTT-GTAGTCACCGTC). These primers were synthesized by Operon Technologies, Inc. (Alameda, CA). Separate constructs were prepared containing the 5′-mutated galectin-1 and 3′ and 5′ double-mutated galectin-1 cDNA and ligated into the *Bam*HI site and the *Hin*dIII site of pQAE9 (Qiagen). The mutated sequences were confirmed by bidirectional DNA sequencing, using the automated sequencing facility at Oklahoma State University (Stillwater, OK).

E. coli strain M15 (Qiagen) was transformed with these plasmids to express 5′-mutated galectin-1 (N-Gal-1 and V5D-Gal-1) and the 5′ and 3′ doubly-mutated galectin-1 (N/C-Gal-1) at high levels. Mutated galectins were purified from sonicated *E. coli* cell extracts by affinity chromatography using a lactosyl-Sepharose column (0.7 × 15 cm). The column was preequilibrated with SPB-azide (6.7 mM KH_2PO_4, 150 mM NaCl, pH 7.4, containing 14 mM 2-mercaptoethanol and 0.02% NaN_3) and washed with the same buffer. Bound material was eluted by SPB-azide containing 0.1 M lactose. Each fraction was monitored by the absorbance at 280 nm and analyzed by SDS−PAGE (Laemmli, 1970). The fractions containing galectin were pooled and dialyzed against SPB-azide and concentrated using Centriprep10 (from Amicon). Protein concentration was determined by BCA assay and Bio-Rad protein assay.

[1] Abbreviations: PBS, phosphate-buffered saline; SPB, PBS containing 14 mM β-mercaptoethanol; DSS, disuccinimidyl suberate; Gal-1, galectin-1; PAGE, polyacrylamide gel electrophoresis; HPLC, high-performance liquid chromatography.

Separation of Monomer and Dimer of Galectin-1 on Size-Exclusion HPLC and Hemagglutination Assay. Separation of monomeric and dimeric forms of C2S-Gal-1 and mutated galectins was accomplished by size-exclusion HPLC, as described by Cho and Cummings (1995a), using a TSK-GEL SW 2000 column (Beckman) (7.5 mm × 30 cm) on a Beckman System Gold HPLC. Assays for hemagglutination using rabbit erythrocytes are described in Cho and Cummings (1995a).

Density Gradient Sedimentation and Native Gel Electrophoresis. C2S-Gal-1 and N-Gal-1 (60 μM) in SPB-azide were sedimented in an L7 ultracentrifuge (Beckman) at a rotor speed of 25 000 rpm at 4 °C for 9 h. Samples were analyzed in preformed sucrose gradients (5−12%), and 200 μL fractions were collected after centrifugation. The amount of galectin in each fraction was determined by ELISA. A 96 microtiter plate was coated with 20 μL of each fraction, blocked with 5% BSA solution in TPBS (PBS containing 0.05% Tween 20) for 2 h at 4 °C, and washed 3 times with TPBS. Anti-galectin-1 polyclonal antibody (Cho & Cummings, 1995a) was incubated for 1 h and washed 3 times with the same buffer, and alkaline phosphatase-conjugated anti-rabbit IgG mouse antibody was added for color detection. Native gel electrophoresis was performed in 7−15% polyacrylamide gradient gels in reducing conditions, as described by Laemmli (1970), in the absence of SDS.

Equilibrium Dialysis. Equilibrium dialysis was performed in an equilibrium dialysis apparatus from Pharmacia. The radiolabeled disaccharide ligand [^{3}H]Galβ1 → 4GlcNAc was synthesized by incubation of bovine β1,4-galactosyltransferase with GlcNAc and UDP-[^{3}H]Gal, as described previously (Rivera-Marrero & Cummings, 1990). In a typical experiment, [^{3}H]Galβ1 → 4GlcNAc (18 000 cpm) was mixed with different amounts (0.025 mM up to 13 mM) of the unlabeled disaccharide Galβ1 → 4GlcNAc in dialysis cells. Galectin-1 and mutated forms of galectin-1 were added to opposite cells at a concentration of 0.75 mg/mL. In control cells, the lectins were omitted. Dialysis cells were incubated for 24 h at 4 °C on rotators. The radioactivity in each cell containing the lectin was determined by liquid scintillation counting. Specific binding was determined by subtracting the amount of radioactivity in control wells lacking protein from that obtained in the experimental wells containing lectin. The equilibrium constant for binding was determined by Scatchard analysis (Scatchard et al., 1934). To determine the stability of lectins during the equilibrium dialysis, one pair of cells contained lectin without *N*-acetyllactosamine, and at the end of dialysis, the sample was analyzed for activity by chromatography on lactosyl-Sepharose. The run-through, retarded and bound protein, was measured by a Bio-Rad protein assay. The protein in the run-through fractions was taken as the percent of inactive lectin and the remainder was considered active lectin.

Preparation of Cross-Linked Dimers. Disuccinimidyl suberate (DSS) was used to cross-link N-Gal-1 or V5D-Gal-1 according to the manufacture's instructions. Cross-linked products were separated by size-exclusion HPLC, and 0.3 mL fractions were collected for further characterization. The activity of cross-linked products was measured by affinity chromatography on a column of lactosyl-Sepharose (2 mL). The fractions from the column were analyzed by SDS−

	N-terminus (1 ... 10) ... C-terminus (130 ... 134)
Gal-1	M-A-C-G-L-V-A-S-N-L-N-L-K..//..A-D-G-D-F-K-I-K-C-V-A-F-E
C2S-Gal-1	M-A-**S**-G-L-V-A-S-N-L-N-L-K..//..A-D-G-D-F-K-I-K-C-V-A-F-E
V5D-Gal-1	M-A-C-G-L-**D**-A-S-N-L-N-L-K..//..A-D-G-D-F-K-I-K-C-V-A-F-E
N-Gal-1	M-A-**S**-G-**Q**-**D**-**S**-S-N-L-N-L-K..//..A-D-G-D-F-K-I-K-C-V-A-F-E
N/C-Gal-1	M-A-**S**-G-**Q**-**D**-**S**-S-N-L-N-L-K..//..A-D-G-D-**Y**-K-**T**-K-C-**Q**-A-F-E

FIGURE 1: N- and C-terminal sequences of native hamster galectin-1 and galectin-1 mutants constructed in this study. Altered amino acids are indicated in boldface lettering.

PAGE (Laemmli, 1970). The cross-linked dimer of V5D-Gal-1 was tested for its stability by storage at 4 °C in the presence of β-mercaptoethanol. After 1 month, $\sim^2/_3$ of the protein retained the ability to bind to lactosyl-Sepharose, and elution required 0.1 M lactose. This is similar to the stability observed for native Gal-1.

RESULTS

Mutagenesis of Galectin-1. Our previous studies showed that galectin-1 exists in a reversible monomer−dimer equilibrium ($K_d \approx 7\ \mu M$) (Cho & Cummings, 1995a). The crystal structures of galectin-1 and -2 (Liao et al., 1994; Lobsanov et al., 1993) suggest that dimer formation is based on hydrophobic interactions between residues within the N- and C-termini at the subunit interface. Using the reported X-ray crystallographic data on bovine galectin-1, we searched for atoms with interatomic distances within 4.0 Å across the dimer interface. Interestingly, the possible hydrophobic interactions between amino acid side chains occur mainly between amino acids on the first β-strand (residues 4−7) in the N-terminus and amino acids on the last β-strand (residues 126−132) in the C-terminus. For example, dimer formation appears to largely result from interactions between Val5 of monomer A and Ala6 of monomer B; Ser7 of monomer A and Val5 of monomer B; Ile128 of monomer A and Phe133 of monomer B; and Phe133 of monomer A and Ile128 of monomer B. We reasoned that mutating these candidate amino acid residues to hydrophilic amino acid residues may impair dimer formation. The hamster galectin-1 is highly homologous in sequence with bovine and human galectin-1 and is identical to them at the N- and C-termini positions indicated above, which are hypothesized to be critical for subunit interactions. Specific mutations were created in the N- and C-termini of recombinant hamster galectin-1, as indicated in Figure 1.

The native form of Gal-1 requires either reducing agents or carbohydrate ligands to maintain activity. For convenience, we also utilized the C2S-Gal-1 mutant, which behaves identically to Gal-1 in terms of carbohydrate-binding activity and in monomer−dimer equilibrium; however, it is stable in the absence of either reducing agents or carbohydrate ligands (Hirabayashi & Kasai, 1991; Cho & Cummings, 1995a). The V5D-Gal-1 mutant contains a single change of V5 to D, which is a presumed main point of contact between subunits. In the N-Gal-1 mutant, three amino acids potentially involved in β-strand interactions are changed; L4 to Q, V5 to D, and A6 to S. Because some C-terminal amino acids also appear to be involved in dimer formation, another mutant termed N/C-Gal-1 was constructed that contained changes in both N- and C-terminal residues. Each of the recombinant proteins was expressed normally by *E. coli* and had the predicted molecular weight.

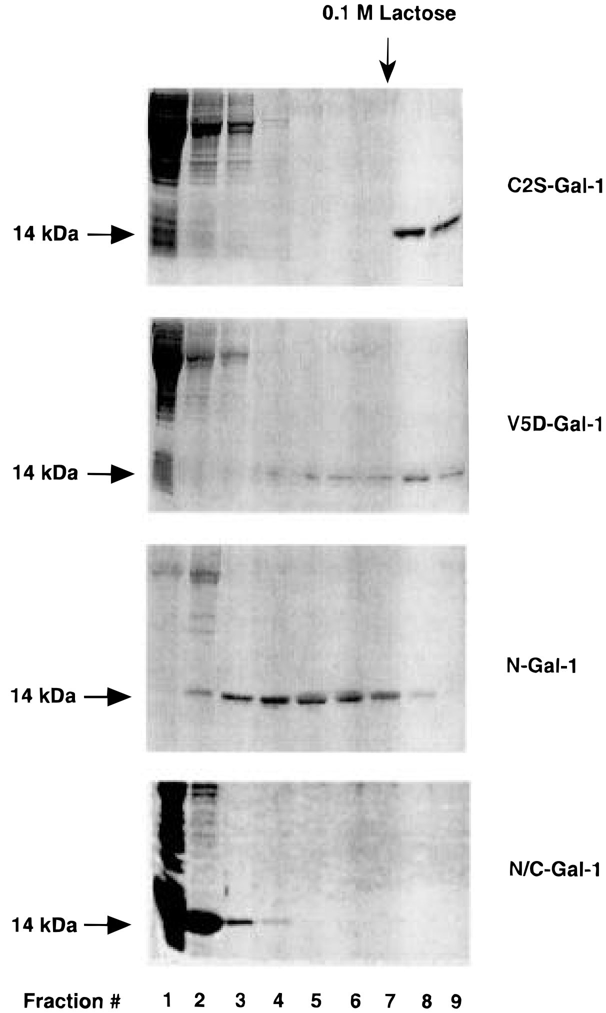

FIGURE 2: SDS−PAGE of galectins after chromatography on lactosyl-Sepharose. *E. coli* extracts expressing high levels of galectins were applied to columns of lactosyl-Sepharose, and bound lectins were eluted by addition of lactose, as described under Experimental Procedures. Each fraction was analyzed by SDS−PAGE and Coomassie blue staining. The arrow in each panel indicates the position of migration of lysozyme, 14.3 kDa.

Galectin-1 Mutants Display Differential Affinity for Lactosyl-Sepharose. E. coli extracts expressing each mutant galectin-1 were loaded onto a column of lactosyl-Sepharose. The unbound fractions and bound fractions eluted by 0.1 M lactose solution were collected and analyzed on SDS−PAGE followed by Coomassie staining (Figure 2). C2S-Gal-1 quantitatively binds to the column and is eluted with 0.1 M lactose, whereas both V5D-Gal-1 and N-Gal-1 are retarded in their elution. Based on the elution pattern in Figure 2, however, V5D-Gal-1 binds slightly better to lactosyl-Sepharose than N-Gal-1. The N/C-Gal-1 displays little affinity for lactosyl-Sepharose, and in all studies, it appears to be inactive. It was not explored any further. These results demonstrate that mutations in V5D-Gal-1 and N-Gal-1 alter the affinity of the lectins for lactosyl-Sepharose.

N-Gal-1 and V5D-Gal-1 Bind N-Acetyllactosamine Similarly to Native Lectin. Although the ability of the mutated proteins to bind lactosyl-Sepharose is impaired, it seemed surprising that a single point mutation far from the known carbohydrate-binding domain, as in V5D-Gal-1, could so drastically affect carbohydrate-binding activity (Liao et al., 1994). We reasoned that the apparent reduced affinity of

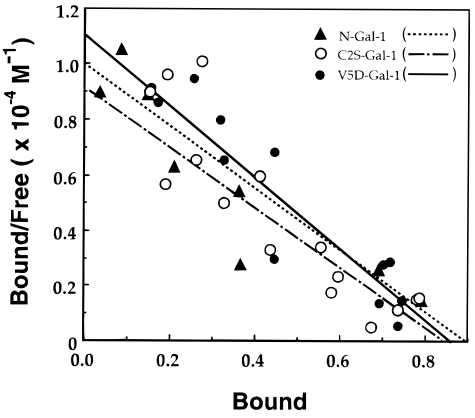

FIGURE 3: Scatchard plot of the results of equilibrium dialysis of galectins binding to *N*-acetyllactosamine. Equilibrium dialysis was carried out as described under Experimental Procedures using [^{3}H]-*N*-acetyllactosamine. Bound, moles of *N*-acetyllactosamine bound per mole of galectin-1. Free, concentration of free *N*-acetyllactosamine.

N-Gal-1 and V5D-Gal-1 to lactosyl-Sepharose could result from their inability to dimerize. V5D-Gal-1 might appear to bind more strongly than N-Gal-1 to lactosyl-Sepharose because it can dimerize more efficiently than N-Gal-1.

In one approach to test this hypothesis, we measured the binding of C2S-Gal-1, V5D-Gal-1, and N-Gal-1 to *N*-acetyllactosamine in equilibrium dialysis for 24 h at 4 °C. A Scatchard plot of the results is shown in Figure 3. These data demonstrate that each lectin binds *N*-acetyllactosamine with a similar affinity: C2S-Gal-1, $K_d \approx 77$ μM; V5D-Gal-1, $K_d \approx 91$ μM; and N-Gal-1, $K_d \approx 90$ μM with approximately one binding site per monomer. In these experiments, we noted that V5D-Gal-1 is slightly less stable than C2S-Gal-1 and N-Gal-1 over a 24 h period and a small fraction of the protein was found to be inactive in binding to lactosyl-Sepharose (data not shown). This small difference in activity is responsible for the slight difference between V5D-Gal and the other lectins in the number of binding sites. These results demonstrate that mutations in the extreme N-terminus of galectin-1 do not significantly alter its affinity for *N*-acetyllactosamine. Other experiments to check the binding activity of the mutated proteins are described below.

Both N-Gal-1 and V5D-Gal-1 Are Monomers. To determine whether the mutated proteins are monomeric as predicted, we examined the proteins by three independent methods: (i) sucrose density gradient sedimentation; (ii) native gel electrophoresis; and (iii) high-performance size-exclusion chromatography. Purified N-Gal-1, V5D-Gal-1, and C2S-Gal-1 were serially diluted, and the diluted samples were kept at 4 °C for 20 h to allow equilibration (Cho & Cummings, 1995a). In native gel electrophoresis, N-Gal-1 migrates as a single band at high concentration (70 μM) (Figure 4A), which is also true for V5D-Gal-1 (data not shown). In contrast, at 70 μM, C2S-Gal-1 migrates as one species, whereas at 8 μM it occurs as two species (Figure 4A). The faster migrating species of C2S-Gal-1 observed at 8 μM, which comigrates with N-Gal-1, represents the monomeric protein (Figure 4A). Thus, by native gel electrophoresis, both V5D-Gal-1 and N-Gal-1 behave as monomeric proteins. The proteins were also analyzed by density gradient sedimentation in 5−12% sucrose at an initial lectin concentration of 60 μM. A different distribution of the proteins was observed, with N-Gal-1 being sedimented

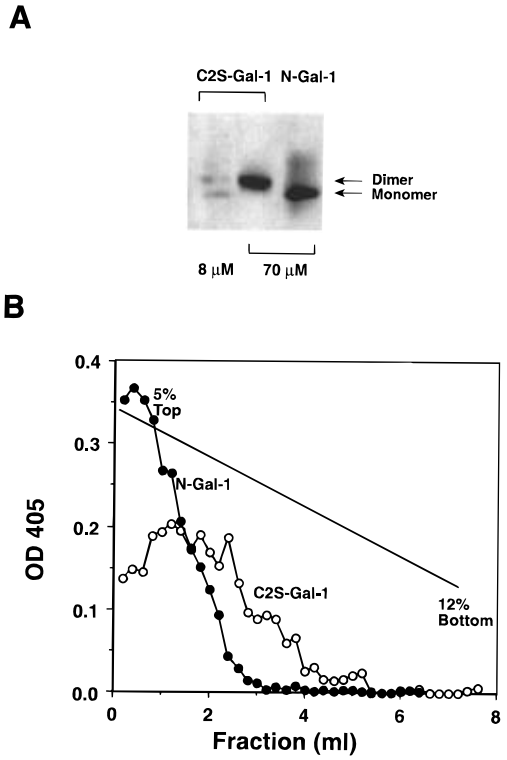

FIGURE 4: N-Gal-1 exists as a monomer at high concentrations. (A) C2S-Gal-1 (8 and 70 μM) and N-Gal-1 (70 μM) were analyzed by native gel electrophoresis. (B) N-Gal-1 and C2S-Gal-1 were equilibrated at 60 μM, and 50 μL of each solution was analyzed by sucrose density gradient sedimentation on 5−12% sucrose gradients. Distribution of galectin-1 was detected by ELISA as described under Experimental Procedures. N-Gal-1 (●); C2S-Gal-1 (○).

slower than C2S-Gal-1 (Figure 4B). This is consistent with the occurrence of N-Gal-1 as a monomer and C2S-Gal-1 as a mixture of monomer−dimer.

We also examined the forms of the proteins by size-exclusion HPLC. In this analysis, C2S-Gal-1 is a dimer at high concentrations (80 μM) and is a mixture of monomer−dimer at a lower concentration (8 μM) (Figure 5), consistent with our previous report (Cho & Cummings, 1995a). In contrast, V5D-Gal-1 and N-Gal-1 behave mainly as monomeric species at both high and low concentrations (Figure 5). Taken together, the results of native gel electrophoresis, density gradient sedimentation, and size-exclusion HPLC demonstrate that V5D-Gal-1 and N-Gal-1 occur as monomeric species.

It should be noted that these three analytical approaches are valid only if the equilibration rate of monomer−dimer is relatively slow with regard to the time it takes to analyze their forms; i.e., preformed dimers that might occur at high concentrations dissociate to monomers relatively slowly. For C2S-Gal-1, the dissociation rate of dimer to monomer was found to be $t_{1/2} \approx 3$ h (Cho & Cummings, 1995a). Size-exclusion HPLC, the most rapid of the three techniques, takes approximately 15 min. As shown below, V5D-Gal-1 is able to form a dimer at lower concentrations than N-Gal-1, but the dimers of the V5D-Gal-1 appear to rapidly dissociate upon dilution.

Hemagglutinating Activity of Native Galectin-1 and the Mutated Forms of Galectin-1. One sensitive measure of multivalency for galectins is hemagglutination, since each subunit has a single carbohydrate recognition domain (CRD)

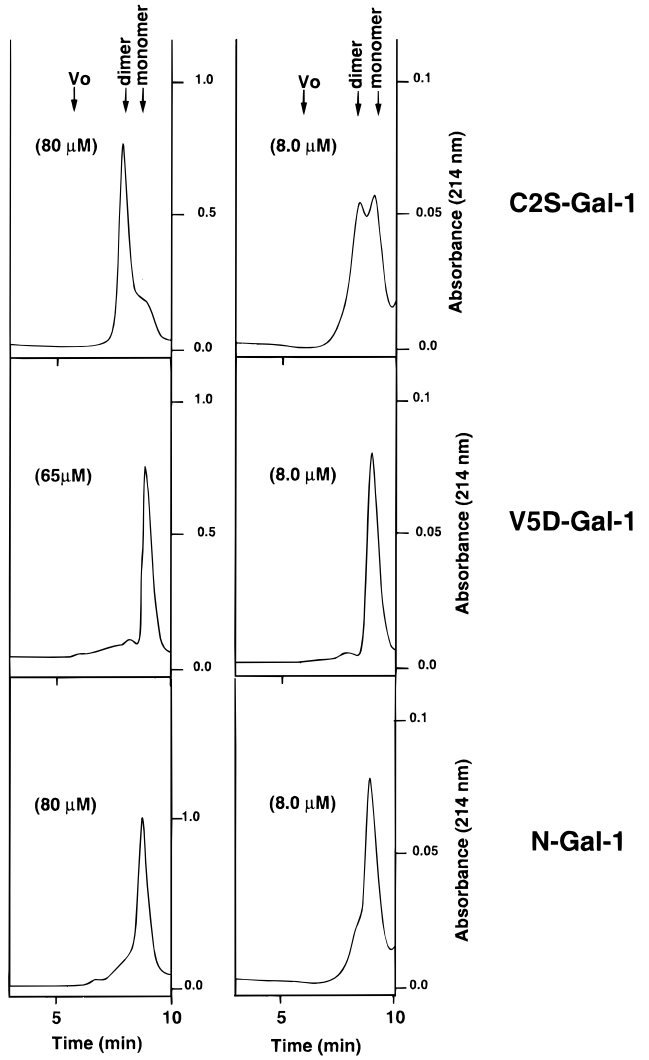

FIGURE 5: Monomer and dimer forms of galectin-1 in solution. Solutions of C2S-Gal-1, N-Gal-1, and V5D-Gal-1 were prepared at the concentrations indicated and allowed to sit at 4 °C for 20 h to equilibrate. From each sample, 50 μL was passed through a SW 2000 size-exclusion column, and elution was monitored by A_{214} using System Gold HPLC.

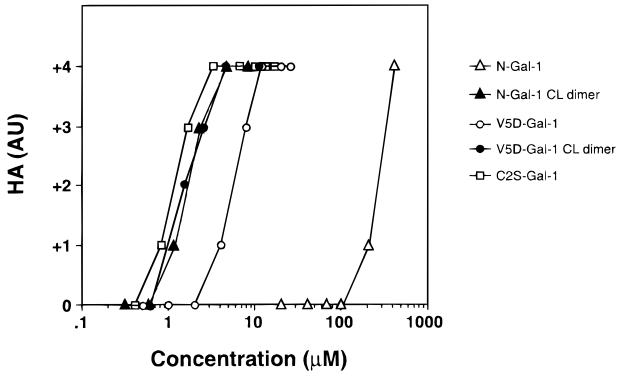

FIGURE 6: Hemagglutinating activity of each of the galectins. The hemagglutination activities of varying concentrations of galectins were monitored as described under Experimental Procedures. Each concentration of lectin was prepared by serial dilution and allowed to equilibrate at 4 °C for 20 h, before assaying hemagglutinating activity. The hemagglutinating activity of the lectin is shown as 0−4+, with 4+ indicating maximal activity and 0+ indicating no activity. CL indicates cross-linked dimers, prepared by treatment with disuccinimidyl suberate (DSS), as described in the text.

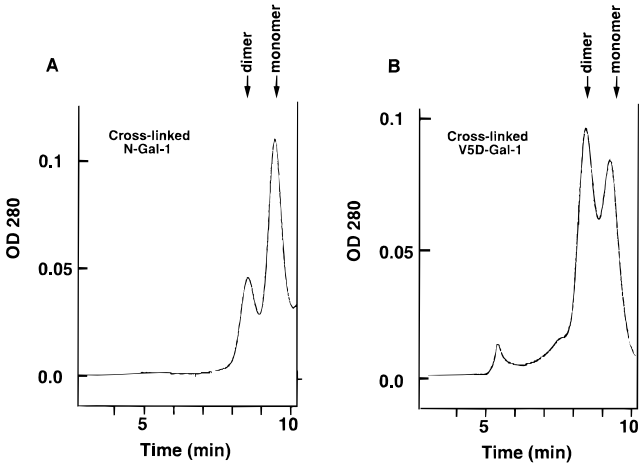

FIGURE 7: N-Gal-1 and V5D-Gal-1 can be covalently cross-linked with disuccinimidyl suberate (DSS). Serially diluted and equilibrated samples of (A) N-Gal-1 and (B) V5D-Gal-1 were treated with DSS (3 mM) and analyzed by size-exclusion HPLC.

and individual monomers are monovalent. We measured, therefore, the hemagglutinating activity of both N-Gal-1 and V5D-Gal-1 in comparison to C2S-Gal-1. The hemagglutinating activity of C2S-Gal-1 can be detected at a +1 level at a lectin concentration of ~0.8 μM (Figure 6). N-Gal-1 has extremely poor hemagglutinating activity, and only at a concentration of ~200 μM is +1 agglutination noted. V5D-Gal-1 has detectable hemagglutinating activity at ~4 μM (Figure 6). These results suggest that both N-Gal-1 and V5D-Gal-1 are impaired in their ability to dimerize. Although they can dimerize at high concentrations, they appear to differ in their ability to form stable dimers active as hemagglutinins.

Cross-Linking Experiments Demonstrate That both N-Gal-1 and V5D-Gal-1 Can Dimerize. To directly determine whether the mutated proteins can dimerize at high concentrations, the proteins were treated with the homobifunctional cross-linker disuccinimidyl suberate (DSS). V5D-Gal-1 and N-Gal-1 were serially diluted and incubated at 4 °C overnight to allow equilibration, and then incubated with DSS for 2 h on ice. The treated samples were analyzed by size-exclusion HPLC. Both V5D-Gal-1 and N-Gal-1 can be cross-linked with DSS at high lectin concentrations, and the cross-linked lectins behave as dimeric forms with an apparent M_r ≈29K (Figure 7). The cross-linked dimers were also analyzed by SDS−PAGE, as discussed below, and the proteins migrated as species with apparent M_r ≈29K. The yield of dimeric protein for V5D-Gal-1 was higher than for N-Gal-1, indicating that the former is a more stable dimer than the latter. The degree of cross-linking as a function of lectin concentration at equilibrium was determined, and the results are shown in Figure 8. Approximately 50% of the V5D-Gal-1 could be cross-linked at a concentration at ~65 μM. As an independent test of the efficiency of cross-linking of dimers by DSS, we performed the same experiment with C2S-Gal-1 at concentrations above its dissociation K_d of 7 μM. A majority of the C2S-Gal-1 was cross-linked at all higher concentrations (data not shown), confirming that DSS efficiently cross-links dimers of the lectin. Because DSS is efficient at cross-linking dimeric species, it is possible to use the data in Figure 8 to estimate the K_d of the monomer−dimer equilibrium for the mutant proteins. It was estimated that the K_d values of monomer−dimer formation for V5D-Gal-1 and N-Gal-1 are ≈60 μM and ≈250 μM, respectively.

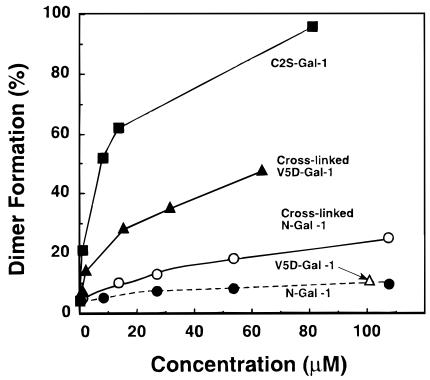

FIGURE 8: Monomer−dimer forms of C2S-Gal-1, V5D-Gal-1, and N-Gal-1 with or without chemical cross-linking. The amounts of dimer formed at different concentrations of C2S-Gal-1, V5D-Gal-1, and N-Gal-1 were estimated following size-exclusion HPLC, as shown in Figure 5. At the indicated concentrations, V5D-Gal-1 and N-Gal-1 were treated with DSS, and the amount of cross-linked dimer recovered was determined, as shown in Figure 7. For each sample following HPLC, the peak areas were integrated, and the concentration dependency of dimer formation was plotted.

Cross-Linked Dimers of N-Gal-1 and V5D-Gal-1 Have Similar Activity to C2S-Gal-1 Native Dimers. To determine whether the cross-linked dimers of the mutated proteins retained carbohydrate-binding activity, they were isolated following size-exclusion HPLC and directly tested for hemagglutinating activity. The cross-linked dimeric forms of both V5D-Gal-1 and N-Gal-1 exhibit hemagglutinating activity similar to that of C2S-Gal-1 (Figure 6). These results demonstrate that the cross-linked dimers of the mutated proteins are active in binding cells and there is little difference in their activity compared to the non-cross-linked C2S-Gal-1.

Our inability to observe dimeric forms of V5D-Gal-1 by size-exclusion HPLC in the absence of cross-linking (Figure 5) led us to speculate that the V5D-Gal-1 dimers might weakly associate, and dissociate rapidly upon dilution. To test this possibility, we measured the hemagglutinating activity of C2S-Gal-1 and V5D-Gal-1 at a concentration of 4 μM and 8 μM, respectively, and obtained a +4 agglutination, as expected from the results in Figure 6. We then diluted the proteins by 4-fold and measured the hemagglutinating activity within 3 min. The C2S-Gal-1 retained +4 hemagglutinating activity, but the V5D-Gal-1 was reduced to +1. Although indirect, these results are consistent with the proposition that V5D-Gal-1 dimers dissociate rapidly (<3 min). This rapid dissociation probably accounts for our inability to observe dimeric species of the protein in size-exclusion HPLC. More complex techniques, such as stop-flow kinetics or surface plasmon resonance, would have to be performed to precisely define the dissociation rates of V5D-Gal-1.

We also tested the behavior of the cross-linked dimeric forms of V5D-Gal-1 and N-Gal-1 upon chromatography on a column of lactosyl-Sepharose. V5D-Gal-1 was treated with DSS, as described above, and the treated sample was analyzed by size-exclusion HPLC (Figure 9A). A fraction of the cross-linked dimeric form of V5D-Gal-1 was removed (pool 1), and the remainder of the material containing both monomers and dimers was pooled (pool 2). The pool 1 material containing the cross-linked dimeric V5D-Gal-1 bound tightly to lactosyl-Sepharose, and elution required 0.1 M lactose (Figure 9B). In contrast, the monomeric material

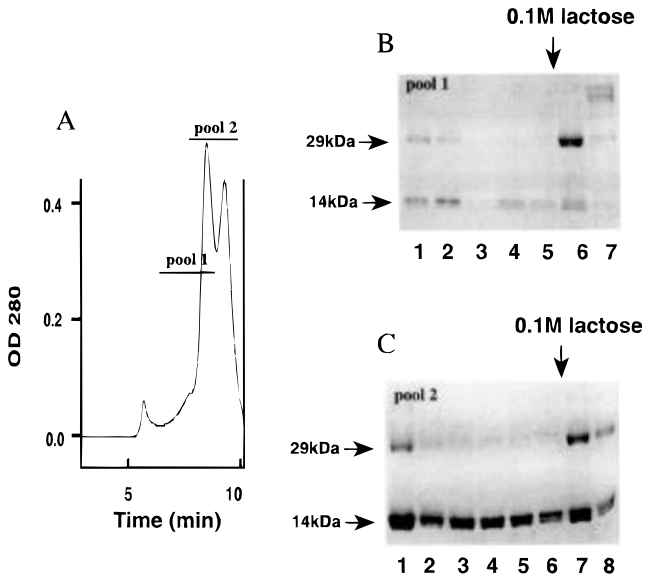

FIGURE 9: Covalently cross-linked dimers of V5D-Gal-1 bind tightly to lactosyl-Sepharose. (A) Reaction mixtures of V5D-Gal-1 cross-linked with DSS were analyzed by size-exclusion HPLC. Portions of fractions enriched for the cross-linked dimer were pooled (pool 1), and portions of fractions representing both cross-linked dimers and non-cross-linked monomers were pooled (pool 2). Both pool 1 samples (B) and pool 2 samples (C) were applied to a column of lactosyl-Sepharose. Each fraction was analyzed by SDS−PAGE followed by Coomassie staining. Covalently cross-linking of the lectins with DSS decreased their staining intensity with Coomassie compared to untreated proteins.

in pool 2 was largely retarded in its elution from the column, while the cross-dimeric material in this pool 2 sample was bound and eluted from the column by 0.1 M lactose (Figure 9C). Similar results results were obtained for preparations of cross-linked N-Gal-1 (data not shown). These results demonstrate that the cross-linked dimeric proteins have high-affinity binding to lactosyl-Sepharose. As noted under Experimental Procedures, these cross-linked dimers are highly stable at 4 °C. More importantly, the cross-linked dimers have both hemagglutinating activity and elution behavior on lactosyl-Sepharose similar to the non-cross-linked native lectin C2S-Gal-1. Furthermore, these results demonstrate that the weak binding of the non-cross-linked forms of V5D-Gal-1 and N-Gal-1 to lactosyl-Sepharose (Figure 2) results from weak interactions of the monomeric proteins to the support, rather than an effect of the mutations on carbohydrate-binding activity. This conclusion is consistent with the results of equilibrium dialysis, which indicate that the mutated V5D-Gal-1 and N-Gal-1 bind *N*-acetyllactosamine with an affinity similar to C2S-Gal-1.

DISCUSSION

These studies reveal that amino acids within the extreme N-terminus of galectin-1 are critical for dimer formation and they do not contribute to carbohydrate-binding activity. Interestingly, this monomer−dimer equilibrium can be dramatically affected by even a single mutation of V5 to D. More extensive mutations in this region, as in the N-Gal-1 mutant, drastically disrupt the ability to form a dimer, and dimers can only be efficiently formed at high concentrations (>100 μM). At concentrations below 100 μM, the N-Gal-1 mutant exhibits no detectable hemagglutinating activity. The importance of the N-terminal residues in dimer formation is

consistent with the reported crystal structure for bovine galectin-1 (Liao et al., 1994), which indicates that the dimer forms a 22-strand antiparallel β-sandwich, with the N- and C-termini of each monomer at the dimer interface. The integrity of the dimer is maintained by β-sheet interaction across the monomers and by the formation of a hydrophobic core common to both. Our results demonstrate that the introduction of hydrophilic amino acid residues to this hydrophobic core (as in N-Gal-1 and V5D-Gal-1) destabilizes the dimer and favors equilibrium toward the monomer.

The N- and C-termini of each monomer face each other at the dimer interface, which is far from the carbohydrate-binding site (Liao et al., 1994). It was thus not expected that N/C-Gal-1, which contains mutations within both the extreme N- and C-termini of the protein, is nonfunctional, while N-Gal-1 and V5D-Gal-1 retain normal activity. These results suggest that the C-terminal domain may be critical in some way to folding or conformation of the protein. This possibility is consistent with the results of Abbott and Feizi (1991), who reported that a deletion of 10 amino acids from the C-terminus of galectin-1 completely abrogated its ability to bind lactosyl-Sepharose, whereas a recombinant lectin containing a deletion of 8 amino acids from the N-terminus retained a weak ability to bind the support.

Site-directed mutagenesis has been used previously to successfully disrupt the oligomerization of proteins, by mutating amino acid residues in the subunit interface of oligomeric proteins, *e.g.*, malate dehydrogenase (Breiter et al., 1994), lactate dehydrogenase (Jackson et al., 1992), and aldolase (Beernink & Tolan, 1994). Chemical modifications, but not mutagenesis procedures, have been used to produce monomeric and monovalent forms of some plant lectins, such as concanavalin A (Fraser et al., 1976) and *Maackia amurensis* leukoagglutinin (Kaku et al., 1993). Most site-directed mutagenesis studies of galectins have focused on defining residues critical for carbohydrate-binding activity (Hirabayashi & Kasai, 1991, 1994; Abbott & Feizi, 1991). Since many animal lectins are oligomeric and multivalent, our findings may be pertinent to future studies aimed at preparing monomeric forms of other animal lectins to test the role of monovalency in their functions.

Our results may also bear some relationship to recent findings about galectin-5. This protein contains a single carbohydrate-binding domain and was found to behave as a monomer on size-exclusion chromatography (Gitt at al., 1995a). However, despite its apparent monomeric state, galectin-5 exhibits hemagglutination activity at a concentration only 3 times higher than that of galectin-1 (Gitt at al., 1995a). Galectin-5 is different from galectin-1 in that galectin-5 has 14 additional amino acid residues on its N-terminus. Our results suggest the possibility that the additional residues at the N-terminus of galectin-5 may interfere with the stability of the dimeric species; the protein may weakly dimerize in solution, and this weak dimer may be responsible for the observed hemagglutinating activity. This dimeric species, like V5D-Gal-1, may dissociate rapidly to monomers upon dilution. Cross-linking experiments of the type we have performed on galectin-1 would test this hypothesis.

The current ideas about the function(s) of galectin-1 generally postulate that multivalency, and hence dimerization, is important (Zhou et al., 1993; Bourne et al., 1994b; Akimoto et al., 1995; Puche & Key, 1995; van den Brule et al., 1995; Baum et al., 1995a,b). A recent study has demonstrated that human galectin-1 can cause apoptosis of immature T-cells; this effect is observed at lectin concentrations above 7 μM, implying that the galectin-1 dimer is perhaps more potent than the monomer (Perillo et al., 1995). The availability of monomeric forms of galectin-1 will now allow a direct test of this prediction. Unfortunately, the concentration of galectin-1 *in vivo* is not known, making it unclear as to whether monomers or dimers or both forms are important biologically.

In a recent attempt to discern more about the function(s) of galectin-1, homozygous mutant mice were generated that lacked galectin-1 (Poirier & Robertson, 1993). These mice develop normally and do not have major phenotypic abnormalities. It is possible that other members of the galectin family might compensate for the absence of galectin-1 and/or that the developmental abnormalities might involve specific tissues not assessed in the initial study, such as abnormalities in T-cell maturation. The growing list of galectins, such as the recent discoveries of galectins-5 and -6 (Gitt et al., 1995a,b), galectin-7 (Madsen et al., 1995), and galectin-8 (Hadari et al., 1995), strongly supports the possibility of compensatory effects between galectins.

We anticipate that monomeric galectin-1 may be a useful reagent for studies of lectin function and activity *in vivo* and *in vitro*. The monomeric galectin-1 could be used to identify properties or effects of the lectin independent of its multivalency and cross-linking activity. The ability to stably cross-link galectin-1 and produce a covalent dimeric form of the protein should also aid in vitro studies of lectin function and the potential differential activities of monomers and dimers. In addition, expression of the monomeric protein by animal cells could alter their interactions with extracellular matrices. It might be useful to consider expressing a monomeric, mutated form of galectin-1 as a transgene in mouse under selected tissue-specific promoters to aid in discerning the *in vivo* function of this widely-expressed protein. Presumably, the monomeric form of galectin-1, if overexpressed, might act as a rogue lectin, in which it binds to glycans also recognized by other members of the galectin family and thereby effectively blunts their functions that might arise through multivalency. Such future studies will be necessary to identify the role of multivalency in galectin function.

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REFERENCES

Abbott, M. W., & Feizi, T. (1991) *J. Biol. Chem. 266*, 5552−5557.

Akimoto, Y., Hirabayashi, J., Kasai, K., & Kirano, H. (1995) *Cell Tissue Res. 280*, 1−10.

Barondes, S. H., Castronovo, V., Cooper, D. N. W., Cummings, R. D., Drickamer, K., Feizi, T., Gitt, M. A., Hirayabashi, J., Hughes, C., Kasai, K., Leffler, H., Liu, F.-T., Lotan, R., Mercurio, A. M., Monsgny, M., Pillai, S., Poirier, F., Raz, A., Rigby, P. W. J., Rini, J. M., & Wang, J. L. (1994a) *Cell 76*, 597−598.

Barondes, S. H., Cooper, D. N., Gitt, M. A., & Leffler, H. (1994b) *J. Biol. Chem. 269*, 20807−20810.

Baum, L. G., Pang, M., Perillo, N. L., Wu, T., Delegeane, A., Uittenbogaart, C. H., Fukuda, M., & Seilhamer, J. J. (1995a) *J. Exp. Med. 181*, 877−887.

Baum, L. G., Seilhamer, J. J., Pang, M., Levine, W. B., Beynon, D., & Berliner, J. A. (1995b) *Glycoconj. J. 12*, 63−68.
Beernink, P. T., & Tolan, D. R. (1994) *Protein Sci. 3*, 1383−1391.
Bourne, Y., Bolgiano, B., Liao, D. I., Strecker, G., Cantau, P., Herzberg, O., Feizi, T., & Cambillau, C. (1994a) *Nat. Struct. Biol. 1*, 863−870.
Bourne, Y., Bolgiano, B., Nesa, M. P., Penfold, P., Johnson, D., Feizi, T., & Cambillau, C. (1994b) *J. Mol. Biol. 235*, 787−789.
Breiter, D. R., Resnik, E., & Banaszak, L. J. (1994) *Protein Sci. 3*, 2023−2032.
Cherayil, B. J., Chaitovitz, S., Wong, C., & Pillai, S. (1990) *Proc. Natl. Acad. Sci. USA 87*, 7324−7328.
Cho, M., & Cummings, R. D. (1995a) *J. Biol. Chem. 270*, 5198−5206.
Cho, M., & Cummings, R. D. (1995b) *J. Biol. Chem. 270*, 5207−5212.
Cooper, D. N. W., Massa, S. M., & Barondes, S. H. (1991) *J. Cell Biol. 115*, 1437−1448.
Courand, P., Casentini, Borocz, D., Bringman, T. S., Griffith, J., McGrogan, M., & Nedwin, G. E. (1989) *J. Biol. Chem. 264*, 1310−1316.
de Waard, A., Hickman, S., & Kornfeld, S. (1976) *J. Biol. Chem. 251*, 7581−7577.
Fraser, A. R., Hemperly, J. J., Wang, J. L., & Edelman, G. M. (1976) *Proc. Natl. Acad. Sci. U.S.A. 73*, 790−794.
Gitt, M. A., Massa, S. M., Leffler, H., & Barondes, S. H. (1992) *J. Biol. Chem. 267*, 10601−10606.
Gitt, M. A., Wiser, M. F., Leffler, H., Herrmann, J., Xia, Y., Massa, S. M., Cooper, D. N. W., Lusis, A. J., & Barondes, S. H. (1995a) *J. Biol. Chem. 270*, 5032−5038.
Gitt, M. A., Colnot, C., Xia, Y. R., Atchison, R. E., Lusis, A. J., Poirier, F., Barondes, S. H., & Leffler, H. (1995b) *Glycoconj. J. 12*, 548.
Hadari, Y. R., Paz, K., Dekel, R., Mestrovic, T., Accili, D., & Zick, Y. (1995) *J. Biol. Chem. 270*, 3447−3453.
Hirabayashi, J., & Kasai, K. (1991) *J. Biol. Chem. 266*, 23648−23653.
Hirabayashi, J., & Kasai, K. (1994) *Glycoconj. J. 11*, 437−442.
Jackson, R. M., Gelpi, J., Cortes, A., Emery, D., Wilks, H., Moreton, K., Halsall, D., Sleigh, R., Behan-Martin, M., Jones, G., Clarke, A., & Holbrook, J. (1992) *Biochemistry 31*, 8307−8314.
Kaku, H., Mori, Y., Goldstein, I. J., & Shibuya, N. (1993) *J. Biol. Chem. 268*, 13237−13241.
Laemmli, U. K. (1970) *Nature 227*, 680−685.
Liao, D.-I., Kapadia, G., Ahmed, H., Vasta, G. R., & Herzberg, O. (1994) *Proc. Natl. Acad. Sci. U.S.A. 91*, 1428−1432.
Liu, F. T. (1993) *Immunol. Today 14*, 486−490.
Lobsanov, Y. D., Gitt, M. A., Leffler, H., Barondes, S. H., & Rini, J. M. (1993) *J. Biol. Chem. 268*, 27034−27038.
Lotan, R., Belloni, P. N., Tressler, R. J., Lotan, D., Xu, X. C., & Nicolson, G. L. (1994) *Glycoconj. J. 11*, 462−468.
Madson, P., Rasmussen, H. H., Flint, T., Gromov, P., Kruse, T. A., Monore, B., Vorum, H., & Celis, J. E. (1995) *J. Biol. Chem. 270*, 5833−5829.
Nowak, T. P., Haywood, P. L., & Barondes, S. H. (1976) *Biochem. Biophys. Res. Commun. 68*, 650−657.
Oda, Y., Heemann, J., Gitt, M. A., Turck, C. W., Burlingame, A. L., Barondes, S. H., & Leffler, H. (1993) *J. Biol. Chem. 268*, 5929−5939.
Perillo, N. L., Pace, K. E., Seilhamer, J. J., & Baum, L. G. (1995) *Nature 378*, 736−739.
Poirier, F., & Robertson, E. J. (1993) *Development 119*, 1229−1236.
Puche, A. C., & Key, B. (1995) *J. Comp. Neurol. 357*, 513−523.
Raz, A., Zhu, D. G., Hogan, V., Shah, N., Raz, T., Karkash, R., Pazerini, G., & Carmi, P. (1990) *Int. J. Cancer 46*, 871−877.
Rivera-Marrero, C. A., & Cummings, R. D. (1990) *Mol. Biochem. Parasitol. 43*, 59−68.
Scatchard, G., Coleman, J. S., & Shen, A. L. (1934) *J. Am. Chem. Soc. 56*, 658.
Teichberg, V. I., Silman, I., Beitsh, D. D., & Resheff, G. (1975) *Proc. Natl. Acad. Sci. U.S.A. 72*, 1383−1387.
van den Brule, F. A., Buicu, C., Baldet, M., Sobel, M. E., Cooper, D. N., Marschal, P., & Castronovo, V. (1995) *Biochem. Biophys. Res. Commun. 209*, 760−767.
Wells, V., & Mallucci, L. (1991) *Cell 64*, 91−97.
Woynarowska, B., Skrincosky, D. M., Haag, A., Sharma, M., Matta, K., & Bernacki, R. J. (1994) *J. Biol. Chem. 269*, 22797−22803.
Zhou, Q., & Cummings, R. D. (1990) *Arch. Biochem. Biophys. 281*, 27−35.
Zhou, Q., & Cummings, R. D. (1993) *Arch. Biochem. Biophys. 300*, 6−17.

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