Homogeneous Aggregation of the 14-kDa β-Galactoside Specific Vertebrate Lectin Complex with Asialofetuin in Mixed Systems†

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ABSTRACT: The galactose-specific 14-kDa family of animals lectins are an evolutionary conserved group of proteins that have been implicated in a wide variety of biological processes including cell proliferation, adhesion, and transformation. The present study demonstrates that the dimeric 14-kDa calf spleen lectin forms homogeneous aggregated cross-linked complexes with asialofetuin, a glycoprotein with multiple carbohydrate chains possessing terminal galactose residues, in the presence of other lectins with similar specificities and cross-linking activities. Several galactose-specific plant lectins also form homogeneous aggregated cross-linked complexes with ASF. These results demonstrate a new source of specificity for the 14-kDa family of vertebrate lectins, namely, the ability to selectively cross-link and aggregate glycoproteins in mixed systems. The results have important biological implications for the interactions of multivalent lectins and glycoconjugates, as well as the thermodynamic interactions of multivalent molecules in general.

Lectins are specific carbohydrate-binding proteins which are widely found in nature including in plants and vertebrate tissues (cf. Lis and Sharon, 1991). Binding of lectins to the carbohydrate moieties of glycoproteins and glycolipids on the surface of cells has been shown to be involved in a variety of biological recognition processes (cf. Brandley and Schnaar, 1986; Lis & Sharon, 1991). The cross-linking properties of multivalent lectins are also known to be important in their biological activities. For example, lectin-induced cross-linking of glycoconjugates on the surface of cells has been implicated in the mitogenic activities of plant lectins such as concanavalin A (Con A)[1] (cf. Hadden, 1988), in the signal transduction mechanisms of certain glycoprotein hormones (Sairam, 1989), in the arrest of bulk transport in ganglion cell axons (Edmonds & Koenig, 1990), and in the induction of mating reactions in fungi (Kooijman et al., 1989). Furthermore, lectin-induced cross-linking of transmembrane glycoproteins results in changes in their interactions with cytoskeletal proteins and alterations in the mobility and aggregation of other surface receptors (Carraway & Carraway, 1989; Edelman, 1976). Thus, lectin-mediated cross-linking of cell surface glycoconjugates is associated with a variety of biological signal transduction processes.

Multivalent plant lectins with a wide range of carbohydrate-binding specificities have been shown to bind and noncovalently cross-link branched chain oligosaccharides isolated from glycoproteins and glycolipids (cf. Bhattacharyya and Brewer, 1989; Bhattacharyya et al., 1988a, 1990). Studies have shown that these cross-linking interactions lead to the formation of unique homopolymeric cross-linked lattices between the carbohydrates and lectins, even in the presence of mixtures of the molecules (Bhattacharyya & Brewer, 1992; Bhattacharyya et al., 1988b, 1990). In addition, several cross-linked complexes have been shown to be crystalline and suitable for structural analysis by electron microscopy (Bhattacharyya et al., 1989, 1990) and X-ray diffraction analysis (Makowski, Sacchettini, and Brewer, unpublished results). In this regard, a crystalline cross-linked complex between the wheat germ agglutinin and a divalent sialoglycopeptide derived from glycophorin was recently reported (Wright, 1992).

Plant lectins have also been shown to form specific cross-linked complexes with glycoproteins. For example, Con A binds to the carbohydrate chains of the soybean agglutinin (SBA), which is a tetramer possessing a single Man9 oligomannose-type chain per monomer, and forms two types of cross-linked lattices with SBA, depending on the relative ratio of the molecules in solution (Khan et al., 1991). Evidence has also been presented that Con A forms specific cross-linked lattices with a variety of glycoproteins, even in the presence of mixtures of the glycoproteins (Mandal & Brewer, 1992b). These results thus suggested that a plant lectin can form homogeneous aggregates with specific glycoproteins via multivalent cross-linking interactions.

Certain animal lectins also possess carbohydrate-binding specificities and multisubunit structures (Drickamer, 1988; Hirabayashi & Kasai, 1993) similar to plant lectins and therefore have the potential for specific cross-linking interactions with glycoconjugates. An evolutionary conserved homologous family of soluble animal lectins that bind β-galactose residues includes the developmentally regulated class of dimeric proteins with 14-kDa subunits (Barondes, 1984; Hirabayashi & Kasai, 1993). The 14-kDa family of lectins has been found in a wide variety of mammalian tissues

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[1] Abbreviations: 14-kDa lectin, β-galactoside binding vertebrate lectin with subunit M_r of 14 000; EIL, lectin from the seeds of *Erythrina indica*; ECL, lectin from the seeds of *Erythrina cristagalli*; SBA, soybean agglutinin from the seeds of *Glycine max*; ConA, concanavalin A, lectin from jack bean; PNA, peanut agglutinin from the seeds of *Arachis hypogea*; ASF, asialofetuin; LacNAc, Galβ(1,4)GlcNAc; FPLC, fast-protein liquid chromatography; SDS-PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; PBS, 0.02 M sodium phosphate buffered with 0.15 M sodium chloride, pH 7.4; EDTA, ethylenediaminetetraacetic acid, disodium salt; HEPES, *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid; DTT, dithiothreitol. All sugars are in the D configuration with the exception of Fuc which is in the L configuration.

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including muscle, lung, and brain, and studies have suggested that they are involved in cellular functions including the regulation of cell proliferation, adhesion, and transformation (cf. Caron et al., 1990). In the present study, we show that the 14-kDa galactose-specific lectin from calf spleen forms homoaggregated cross-linked complex(es) with asialofetuin (ASF), a well-defined glycoprotein, in the presence of other lectins with similar specificities. The results have important implications for the biological properties of multivalent lectins and glycoproteins, as well as other multivalent ligand-receptor systems.

MATERIALS AND METHODS

EIL, ECL (Bhattacharyya et al., 1981), SBA (Bhattacharyya et al., 1988a), PNA (Lotan et al., 1975), and the 14-kDa lectin (Mandal & Brewer, 1992a) were purified as described earlier. Protein concentrations were determined spectrophotometrically at 280 nm using extinction coefficients ($A^{1\%,1cm}$) of 13.4 for EIL (Bhattacharyya et al., 1981), 15.9 for ECL, 7.7 for PNA (Lotan et al., 1975), and 12.8 for SBA (Lotan et al., 1974), respectively. Concentration of the 14-kDa lectin was determined by the Lowry method (Lowry et al., 1951). Monomer molecular masses of the lectins are 34 kDa for EIL, 28 kDa for ECL, and 30 kDa for PNA and SBA.

Fetuin (Sigma Chemicals) was desialylated and the product (ASF) purified by FPLC on a Superdex 75 column, as described (Spiro & Bhoyroo, 1974). The concentration of ASF was determined by the phenol–sulphuric method (Dubois et al., 1956) with Man and Gal as standards using 21 moles of hexose (a mixture of 9 Man and 12 Gal) per mole of protein (Nilsson et al., 1979; Spiro, 1960). The structures and purities of the oligosaccharides were confirmed by 500-MHz ^{1}H NMR.

Radiolabeling of Proteins. The *Erythrina* lectins and SBA were radiolabeled with [^{14}C]formaldehyde in 0.1 M Hepes buffer pH 7.2, containing 1 mM $CaCl_2$ and 1 mM $MnCl_2$, as previously described (Mandal & Brewer, 1992a). The 14-kDa lectin was radiolabeled with [^{14}C]formaldehyde in 20 mM PBS containing 2 mM DTT, at pH 7.2. ASF was radiolabeled in 0.1 M sodium phosphate buffer, pH 7.2, with [^{3}H]formaldehyde. PNA was labeled with [^{14}C]formaldehyde in 20 mM PBS containing 1 mM $CaCl_2$. The specific activities of the proteins are expressed as cpm/nmol.

Quantitative Precipitation Assay. Quantitative precipitation assays with the 14-kDa lectin and SBA in the presence of ASF were performed in 100 μL of 0.1 M HEPES buffer, pH 7.4, containing 2 mM DTT, 1 mM $CaCl_2$, and 1 mM $MnCl_2$ in the presence and absence of 0.9 M NaCl. Quantitative precipitation assays with the 14-kDa lectin and PNA in the presence of ASF were performed in 100 μL of 20 mM PBS buffer at pH 7.2 containing 2 mM DTT in the presence of 0.9 M NaCl. The mixtures were allowed to stand overnight at 22 °C. No significant difference in the amount of precipitates was observed as a function of salt concentration. Inhibition of formation and dissolution of the precipitates occurred in the presence of 0.1 M lactose but not with nonbinding sugars such as Glu and Fuc.

RESULTS AND DISCUSSION

ASF is a monomeric glycoprotein of M_r 48 000 (Spiro, 1960) which possesses three triantennary N-linked oligosaccharide chains with terminal *N*-acetyllactosamine (LacNAc) residues (74%) (Figure 1A) and a small amount of isomer (9%) with a Galβ(1,3) linkage in the outer Manα(1,3) arm, a biantennary chain with terminal LacNAc residues (17%)

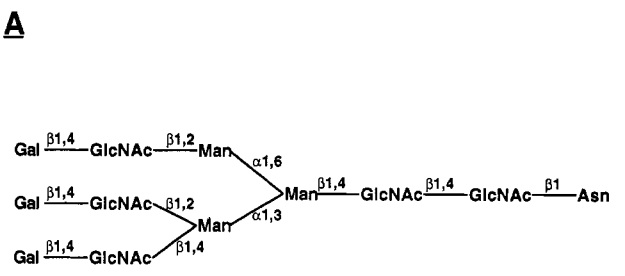

FIGURE 1: Structures of the (A) N-linked triantennary complex type glycopeptide and (B) O-linked glycopeptide of ASF.

Table 1: Stoichiometries of the Cross-Linked Complexes of ASF with the 14-kDa Calf Spleen Lectin in the Presence of SBA and PNA at 22 °C

lectin	no. of cross-linked complexes observed	stoichiometry, lectin:ASF
14-kDa lectin	2	9:1
		3:1
SBA	1[a]	3:1[a]
PNA	1	3:1

[a] Stoichiometries and cross-linked ratios from previous data (Mandal & Brewer, 1992a).

(Green et al., 1988) and three O-linked disaccharide chains (Figure 1B) (Nilsson et al., 1979). The 14-kDa galactose-specific lectin from calf spleen is a dimeric lectin of $M_r \sim$ 30 000 (Allen et al., 1987) with one carbohydrate-binding site per monomer (Mandal & Brewer, 1992a). We have recently demonstrated that the 14-kDa lectin forms two stoichiometric cross-linked complexes with ASF, depending on the relative ratio of the two proteins in solution (Mandal & Brewer, 1992a). At high ratios of the 14-kDa lectin to ASF in solution, a 9:1 lectin:glycoprotein cross-linked complex forms, and with increasing concentration of ASF, a 3:1 cross-linked complex forms. The 9:1 complex results from binding and cross-linking of the 14-kDa lectin to each arm of the three triantennary chains of ASF (Figure 4A), while the 3:1 complex results from one molecule of lectin binding to each triantennary chain (Figure 4B).

The strategy for determining whether homopolymeric cross-linked complexes occur for the 14-kDa animal lectin with ASF was to carry out quantitative precipitation profiles of the two in the presence of SBA or PNA. SBA is a Gal/GalNAc-specific tetrameric lectin (M_r of 120 000) which binds to the three N-linked carbohydrate chains of ASF and forms only a 3:1 cross-linked complex (due presumably to its larger size) (Figure 4C) (Mandal & Brewer, 1992a). PNA is a tetrameric lectin (M_r of 110 000) which preferentially binds to the three O-linked chains of ASF and also forms a 3:1 complex (Gupta et al., unpublished data) (Table 1). The experiments with SBA were carried out as follows. The 14-kDa lectin and SBA were radiolabeled with ^{14}C. Two parallel mixed-precipitation profiles with [^{3}H]ASF were determined using one labeled lectin and the other unlabeled in each experiment, which allowed the amount of both lectins precipitated with [^{3}H]ASF to be determined from the two

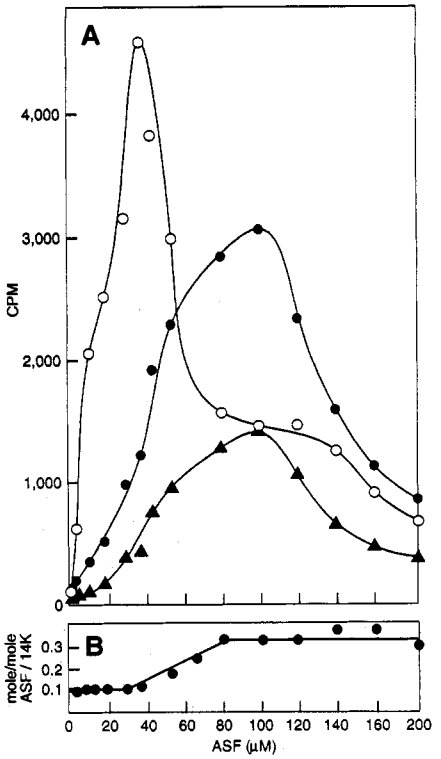

FIGURE 2: Quantitative precipitation profile of ASF in the presence of a mixture of the 14-kDa calf spleen lectin and SBA at 22 °C: (A) CPM profiles of [^{3}H]ASF (●), [^{14}C]SBA (▲), and [^{14}C]14-kDa lectin (○) in the precipitates; (B) mole ratio of ASF/14-kDa lectin (monomer) (●) in the precipitates. The concentrations of the 14-kDa lectin and SBA were 100 μM each (monomer). The specific activities of radiolabeled SBA, the 14-kDa lectin, and ASF were 0.88 × 10^3, 7.0 × 10^3, and 5.0 × 10^3 cpm/nmol, respectively. The lines through the data points are visual aids and not fits to the data. The estimated error in each point in part A is 5% and in part B 10%.

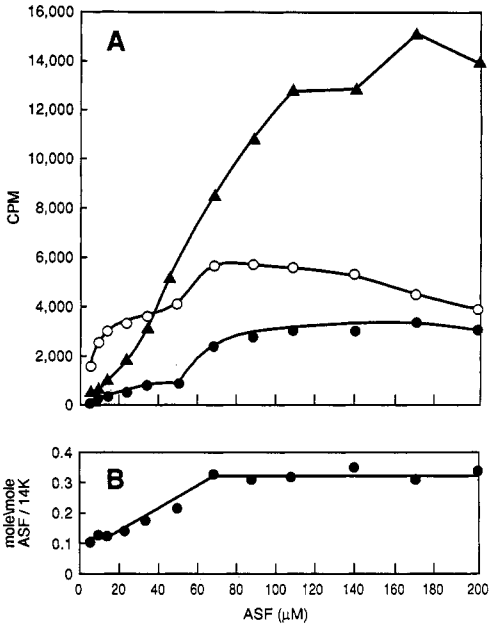

FIGURE 3: Quantitative precipitation profile of ASF in the presence of a mixture of the 14-kDa calf spleen lectin and PNA at 22 °C: (A) CPM profiles of [^{3}H]ASF (●), [^{14}]PNA (▲), and [^{14}C]14-kDa lectin (○) in the precipitates; (B) mole ratio of ASF/14-kDa lectin (monomer) (●) in the precipitates. The concentrations of the 14-kDa lectin and PNA were 100 and 130 μM, respectively (monomer). The specific activities of radiolabeled PNA, the 14-kDa lectin, and ASF were 3.6 × 10^3, 3.6 × 10^3, and 1.8 × 10^3 cpm/nmol, respectively. The lines through the data points are visual aids and not fits to the data. The estimated error in each point in part A is 5% and in part B 10%.

experiments. The amount of ASF precipitated with SBA was calculated from their respective specific activities, assuming that SBA forms only a 3:1 cross-linked complex. The difference in the amount of ASF precipitated by both lectins and the amount of ASF precipitated by SBA permits calculation of the ratio of the 14-kDa lectin/ASF cross-linked complex(es) in the mixture. If the same stoichiometric cross-linked complexes for the 14-kDa lectin with ASF are formed in the mixtures as found for the lectin and ASF alone, this is taken as evidence for the formation of homogeneous cross-linked complexes between ASF and the animal lectin, as well as between ASF and SBA.

Figure 2A shows the quantitative precipitation profile of ASF in the presence of 100 μM SBA and 100 μM 14-kDa lectin. Both SBA and the 14-kDa lectin form bell-shaped profiles with ASF (Mandal and Brewer, 1992a). However, the profile for the 14-kDa lectin shows a major peak near 40 μM ASF, and a shoulder near 110 μM ASF. SBA, on the other hand, shows a single peak near 100 μM ASF, which is close to that observed for ASF itself. Figure 2B shows the mole ratio of ASF/14-kDa lectin, assuming a 3:1 cross-linked complex for SBA with the glycoprotein. At concentrations of ASF up to a 30 μM, the 14-kDa lectin forms a 9:1 lectin/ASF cross-linked complex, and then with increasing concentrations of ASF, a 3:1 cross-linked complex forms (Table 1). These two complexes correspond to the peak and shoulder of the 14-kDa lectin in Figure 2A. Thus, the presence of both cross-linked complexes of the 14-kDa lectin with ASF in the mixture is the same as those for the animal lectin and ASF alone (Mandal & Brewer, 1992a).

Mixed quantitative precipitation experiments carried out with the 14-kDa lectin and ASF in the presence of PNA are shown in Figure 3A. Although the profiles for the 14-kDa lectin and ASF differ from those in Figure 2A, Figure 3B shows that the 14-kDa lectin forms an initial 9:1 cross-linked complex with ASF, and with increasing concentration of ASF, a 3:1 complex forms (assuming that PNA forms a 3:1 cross-linked complex with ASF) (Table 1). The presence of the 9:1 and 3:1 complexes with ASF is similar to that of 14-kDa lectin and ASF alone (Mandal & Brewer, 1992a).

Similar studies were carried out with two dimeric plant lectins, *Erythrina indica* (EIL) and *Erythrina cristagalli* (ECL), which have binding specificities similar to that of the 14-kDa lectin and which also form 9:1 and 3:1 cross-linked complexes with ASF as well as a lower ratio complex (Mandal & Brewer, 1992a). The precipitation profile of each lectin was determined with increasing concentrations of ASF in the presence of a constant concentration of SBA. Assuming that SBA forms a 3:1 cross-linked complex with ASF in these mixtures, both EIL and ECL also form 9:1 and then the lower ratio complexes with ASF (data not shown). Thus, both plant lectins, like the 14-kDa animal lectin, exhibited cross-linked lattice stoichiometries with ASF in mixtures with SBA that were the same as those formed with the individual lectins alone.

The data in this study thus provide evidence that the 14-kDa calf spleen lectin forms homogeneous cross-linked complexes with ASF, even in the presence of SBA and PNA which possess similar carbohydrate specificities and cross-linking activities. Conversely, SBA and PNA also must form homogeneous lattices with ASF, as must EIL and ECL. These findings are similar to those recently reported for Con A which indicated the formation of homogeneous cross-linked complexes between the plant lectin and five different glycoproteins

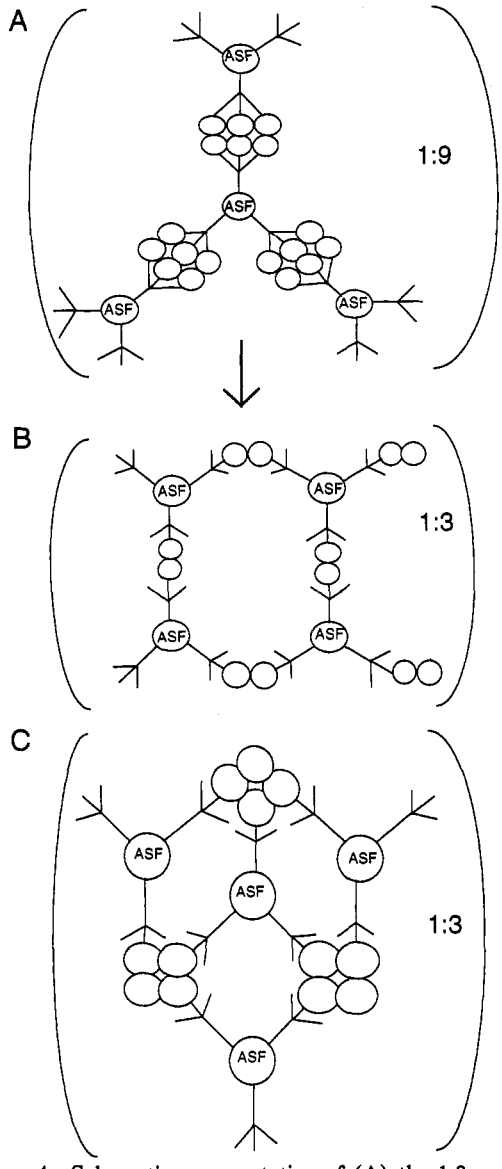

FIGURE 4: Schematic representation of (A) the 1:9 and (B) 1:3 ASF:14-kDa lectin cross-linked complexes (per monomer), and (C) the 1:3 ASF:SBA cross-linked complex. The circles with ASF, a pair of circles, and the four circles clustered together represent ASF, dimeric 14-kDa lectin, and tetrameric SBA, respectively. The connecting lines between ASF and lectin molecules are the triantennary complex type oligosaccharide chains of ASF (Figure 1A). The arrow between A and B indicates transformation of one cross-linked complex to another complex.

in binary mixtures of the glycoproteins (Mandal & Brewer, 1992b).

The present findings thus indicate a new source of specificity in multivalent glycoprotein–lectin interactions, namely, the formation of unique homogeneous cross-linked lattices even in the presence of mixtures of the molecules. These interactions appear to be important in lectin–glycoprotein receptor mediated aggregation events involving cellular recognition and signal transduction processes in both plants and animals. For example, the 14-kDa animal lectin (Sanford & Harris-Hooker, 1990) and many plant lectins including Con A, *Erythrina* lectins, SBA, and PNA (cf. Brown and Hunt, 1978; Sanford & Harris-Hooker, 1990) are mitogenic in certain cell systems, which appears to require aggregation of their respective glycoconjugate receptors. Our findings suggest that these lectins may bind to the carbohydrate moieties of glycoconjugate receptors (glycoproteins and glycolipids) and cross-link them into homoaggregates. For example, the insulin receptor in adipose cells which has been suggested to require aggregation for activation (cf. Fujita-Yamaguchi et al., 1989) is known to be directly activated by the binding of Con A or wheat germ agglutinin (Cuatrecasas & Tell, 1973). The immunomodulatory activities of lectins may also be related, as for example, Con A activation of human T lymphocytes which involves cross-linking of the T cell receptor and a closely associated membrane protein, CD3 (Liscastro et al., 1993). Alternately, signal transduction of aggregated receptors induced by lectin binding may occur via "mechanochemical transducer" mechanisms such as integrin-mediated activation pathways (cf. Pumigia and Feinstein, 1993). It is also important to note that most C-type (Ca^{2+} dependent) animal lectins possess multimeric structures (Drickamer & Taylor, 1993) and are therefore potentially capable of forming cross-linked complexes with specific multivalent carbohydrate ligands which may be important for their biological activities.

Lastly, since the driving force for the formation of homoaggregates of multivalent lectins with carbohydrates and glycoproteins is solely thermodynamic, other multivalent ligand–macromolecular systems must possess the same properties. The only requirement for the specificity of multivalent interaction described here is that one of the two interacting molecules must be greater than divalent so that two- or three-dimensional noncovalent cross-linked lattices form and crystalline-type packing constraints exist. Since clustering of receptors is a known requirement for signal transduction in many systems (cf. Carraway and Carraway, 1989), the present results suggest a common thermodynamic mechanism for selectively aggregating a dispersed population of multivalent receptors in biological systems.

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