Wheat-germ-agglutinin and *Ricinus communis*-agglutinin-binding sites of BHK cells compared with each other and with 140 kDa fibronectin receptors

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We compared the wheat-germ agglutinin (WGA) and *Ricinus communis* agglutinin (RCA) binding sites of baby-hamster kidney (BHK) cells. There were 1.01×10^8 WGA-binding sites per cell ($K_d = 0.027$ nM) and 6×10^6 RCA-binding sites per cell ($K_d = 0.014$ nM). Binding of WGA or RCA to BHK cells resulted in more than 75% of the cell-surface binding sites becoming associated with the cytoskeleton (i.e. resistant to extraction with detergent), although no more than 10% of these sites were associated with the cytoskeleton before addition of the lectins. After binding of WGA to the cells, the cell surface was cross-linked so extensively that it remained intact even after detergent extraction of the treated cells, and could be observed by electron microscopy. A similar cross-linking effect did not occur after binding of RCA to cells, which may be because there were so many more binding sites for WGA than for RCA. The composition of WGA- and RCA-binding molecules was analysed by lectin affinity chromatography of metabolically radiolabelled BHK cells. We found that in the WGA-binding-molecule preparations there were eight major polypeptides, ranging in molecular mass from 93 to 340 kDa, and that the RCA-binding molecules were a subpopulation of the WGA-binding molecules. A polyclonal antibody against the 140 kDa fibronectin (FN) receptors of Chinese-hamster ovary (CHO) cells immunoblotted a 145 kDa polypeptide component in both WGA- and RCA-binding-molecule preparations. The results indicated that the 145 kDa component was present in at least two FN-receptor complexes that differed in glycosylation, only one of which was able to bind to RCA affinity columns. The oligomeric nature of the FN-receptor complex, which contained three polypeptides with molecular masses of 120–145 kDa, was demonstrated by using anti-(CHO-cell FN receptor) antibodies to immunoprecipitate extracts prepared from radioiodinated BHK cells.

INTRODUCTION

Adhesive interactions between cells and their matrices are important in the regulation of many cell functions, including growth and differentiation (e.g. [1–3]). Consequently there has been considerable interest in the mechanisms of these interactions. Several years ago we decided to use lectins to probe the cell-surface binding sites involved in cell adhesion. At that time, several laboratories had described lectin-resistant cell variants that were adhesion-defective (reviewed in [4]), and it had been proposed that fibronectin (FN) receptors were also *Ricinus communis* agglutinin I (RCA) binding molecules [5]. In our studies, we found that substrata coated with RCA or with wheat-germ agglutinin (WGA) promoted the attachment and spreading of baby-hamster kidney (BHK) cells, but, if the cells were pretreated with these lectins, then WGA, but not RCA, inhibited FN-mediated adhesion [6]. Consequently, it was difficult to reconcile our lectin-inhibition studies with the previous work on cell-adhesion variants, and the relationship between WGA- and RCA-binding sites of BHK cells and the effects of these lectins on cell adhesion has yet to be clarified.

Recently, several laboratories characterized a 140 kDa FN-receptor complex of fibroblastic cells [7–10] that has

been partially cloned and called Integrin [11]. Since the polyclonal antibody to 140 kDa FN receptors of Chinese-hamster ovary (CHO) cells [12] also inhibited FN-mediated adhesion of BHK cells, we thought that this antibody might be useful for analysing the relationship between RCA- and WGA-binding sites of BHK cells and the 140 kDa FN receptors. Therefore, we carried out experiments first to compare WGA- and RCA-binding sites of BHK cells with each other, and second to learn whether these binding sites included 140 kDa FN receptors.

EXPERIMENTAL

Cells

BHK cells adapted for growth in suspension culture were given by Dr. A. Chappel (Center for Disease Control, Atlanta, GA, U.S.A.). The cells were grown in suspension culture as described previously [13]. Exponentially growing cells were harvested by centrifugation at 500 g for 4 min (Sorvall HL-4 rotor), washed, and resuspended in Dulbecco's phosphate-buffered saline (DPBS) (150 mM-NaCl, 3 mM-KCl, 1 mM-CaCl₂, 0.5 mM-MgCl₂, 6 mM-Na₂HPO₄, pH 7.2). In some experiments, cells were incubated for 45 min at 37 °C on

Abbreviations used: WGA, wheat-germ agglutinin; RCA, *Ricinus communis* agglutinin; BHK cells, baby-hamster kidney cells; FN, fibronectin; CHO cells, Chinese-hamster ovary cells; DPBS, Dulbecco's phosphate-buffered saline; NP-40, Nonidet P-40; TBS, Tris-buffered saline.

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35 mm-diam. tissue-culture dishes (Falcon Plastics) previously treated with DPBS containing 5 μ g of FN (New York Blood Center)/ml.

Materials

Polyclonal anti-(CHO-cell FN receptor) antibody (IgG fraction) [12] was generously given by Dr. Rudy Juliano and Dr. Patricia Brown (University of Texas Health Science Center, Houston). RCA₁₂₀ and WGA were obtained from Boehringer-Mannheim. RCA-coupled agarose (4 mg/ml) and WGA-coupled agarose (7 mg/ml) were obtained from Vector Laboratories. [³H]Formaldehyde (radioactivity 50 μ Ci/ μ mol), Na¹²⁵I (sp. radioactivity 583.7 MBq, 15.8 mCi/ μ g), [³⁵S]methionine (sp. radioactivity 1123 μ Ci/ μ mol) and En³Hance were purchased from New England Nuclear Co.

Protein determination

Protein concentration was determined by the fluorescamine method, by using the fluorescamine assay reagent according to the manufacturer's instructions (Roche Diagnostics). Bovine serum albumin were used as the standard.

Radiolabelling of lectins

[³H]WGA and [³H]RCA were prepared by reductive alkylation with [³H]formaldehyde and NaBH₄ as has been described previously for FN [13]. The specific radioactivity of the preparations was 3000–5000 c.p.m./ μ g (approx. 430 μ Ci/ μ mol for RCA and 130 μ Ci/ μ mol for WGA). For all radioactivity measurements, samples to be tested were mixed with 10 ml of Budget-Solve (RPI Corp.) and counted for radioactivity in a Nuclear-Chicago mark II scintillation spectrophotometer.

Binding of radiolabelled lectins to cells

Radiolabelled lectins at the concentrations indicated were added to 10⁶ BHK cells in 1 ml of DPBS. The incubations were carried out for 45 min at 4 °C with frequent gentle mixing. At the end of the incubations, the samples were diluted 10-fold with ice-cold phosphate-buffered saline (150 mM-NaCl/5 mM-Na₂HPO₄, pH 7.2), and the cells were collected by centrifugation for 5 min at 500 *g*. Subsequently, the cells were resuspended in 1 ml of phosphate saline and centrifuged for 5 min at 500 *g* through 2 ml of phosphate saline containing 5% bovine serum albumin (Sigma type V). The final cell pellets were resuspended in 1 ml of phosphate-buffered saline. Portions of the samples were used to determine cell number, to ensure that cells were not lost during the handling procedures. The remainders of the samples were used to determine cell-associated radioactivity. All experiments were carried out in duplicate.

Triton X-100 extraction of cells

To prepare cytoskeletal-associated proteins, suspended cells were mixed with 1 ml of Triton extraction buffer [14] (0.02 M-Tris, 0.15 M-NaCl, 1 mM-CaCl₂, 1 mM-MgCl₂, 0.5% Triton X-100, 0.2 mM-phenylmethanesulphonyl fluoride, pH 7.4), incubated at 4 °C for 20 min, and centrifuged at 12000 *g* for 15 min in a Beckman Microfuge (model 11). Supernatants (Triton-soluble fractions) were collected; pellets (Triton-insoluble fraction) were washed with 2 $\times$ 1.0 ml of Triton extraction buffer (samples were centrifuged for 15 min at 12000 *g*) and solubilized with 2% (w/v) SDS. Spread cells were

rinsed with DPBS and then incubated for 20 min at 4 °C with 1 ml of Triton extraction buffer. The Triton-soluble fraction was centrifuged at 12000 *g* for 15 min to remove the small amount of Triton-insoluble material that had come off the substratum. The Triton-insoluble fraction that remained on the substratum was rinsed three times with Triton extraction buffer and solubilized with 2% (w/v) SDS.

Electron microscopy

Transmission electron microscopy was performed as described previously [15]. Briefly, samples were fixed for 2 h at 22 °C with 2% (v/v) glutaraldehyde/1% paraformaldehyde/1% tannic acid in 0.1 M-sodium cacodylate (pH 7.4). Samples were post-fixed for 30 min at 22 °C with aq. 1% OsO₄ and stained *en bloc* with aq. 1% uranyl acetate for 30 min at 4 °C. After dehydration, specimens were embedded in Epon 812. Thin sections were observed and photographed with a Philips 300 electron microscope.

Isolation of WGA- and RCA-binding molecules

Lectin-binding molecules were isolated from Nonidet P-40 (NP-40) extracts of BHK cells by affinity chromatography. BHK cells (approx. 2.5 $\times$ 10⁸) were metabolically radiolabelled for 16 h with 20 μ Ci of [³⁵S]methionine/ml in methionine-deficient medium and then combined with 7.5 $\times$ 10⁸ non-radiolabelled cells. The cells were harvested by centrifugation for 10 min at 650 *g* (Sorvall GSA rotor) and washed twice with 100 ml of DPBS. The washed cells were resuspended in 20 ml of NP-40 extraction buffer (phosphate-buffered saline, 1% NP-40, 10% glycerol, 1 mM-EDTA, 2 mM-phenylmethanesulphonyl fluoride, 1 μ g of leupeptin/ml, 1 μ g of pepstatin/ml, pH 7.2), vortex-mixed for 10 s, and incubated for 30 min at 4 °C. After extraction, samples were centrifuged for 30 min at 12000 *g* (Sorvall SS-34 rotor). To remove non-specific binding material from the extract, supernatants were applied to a 2 ml Sepharose CL-4B column (Sigma Chemical Co.) previously equilibrated with column buffer (0.01 M-Tris/HCl, 0.5 M-NaCl, 0.5% NP-40, pH 7.0). The flow-through from the Sepharose column was loaded on to a 2 ml WGA- or RCA-coupled agarose column that was previously equilibrated with column buffer. After the unbound material was collected, the columns were washed with 200 ml of column buffer. WGA-binding molecules were eluted from WGA-agarose with 15 ml of 1 M-N-acetyl-D-glucosamine. RCA-binding molecules were eluted from RCA-agarose with 15 ml of 0.2 M-galactose. The binding-molecule preparations were dialysed exhaustively against 0.01 M-sodium phosphate, pH 7.0, and freeze-dried.

Electrophoresis and fluorography

Samples for electrophoresis were dissolved in reducing sample buffer (62.5 mM-Tris, 2% SDS, 10% glycerol, 0.01% Bromophenol Blue, 5% mercaptoethanol, pH 6.8). Electrophoresis was performed as previously described in 4–16%-acrylamide-gradient slab gels containing a gradient of 3–8 M-urea [16,17]. Gels for autoradiography and fluorography were prepared by using Acrylaide (FMC Corp.) as the cross-linker instead of NN'-methylenebisacrylamide and supported with GelBond-PAG film (FMC Corp.). After electrophoresis, gels were fixed and stained with Coomassie Blue. Gels to

be fluorographed were impregnated for 2 h at 22 °C with En³Hance (containing 2 % glycerol). Gel films were dried in a 60 °C oven and exposed to Kodak XAR-5 film on a Cronex intensifying screen (E. I. Dupont De Nemours and Co.).

Immunoblotting

Samples of WGA- and RCA-binding molecules prepared as described above were subjected to SDS/polyacrylamide-gel electrophoresis. Subsequently, proteins were transferred to nitrocellulose paper (0.45 µm pore size; Schleicher and Schuell). The paper was air-dried for 30 min, blocked for 1 h with 3 % (w/v) gelatin in Tris-buffered saline (TBS; 20 mM-Tris, 500 mM-NaCl, pH 7.5), washed with two changes of 0.05 % Tween-20 in TBS (TTBS), and incubated for 2 h at 22 °C with anti-(CHO FN receptor) antibody [12] or non-immune goat IgG (100 µg/ml) in 1 % gelatin and 0.05 % Tween-20 in TBS (antibody buffer). The paper was then washed with two changes of TTBS, followed by incubation for 1 h at 22 °C with rabbit anti-goat IgG (1:3000 dilution in antibody buffer; Cooper Biomedical). After the second-antibody incubation, the paper was again washed twice with TTBS and subsequently incubated for 1 h at 22 °C with peroxidase-conjugated Protein A (5 µg/ml; E-Y Laboratories). Finally, the paper was washed twice in TTBS, once in TBS, and developed in colour development solution (0.5 mg of diaminobenzidine/ml in TBS with 0.03 % H₂O₂).

Immunoprecipitation

Immunoprecipitation of ¹²⁵I-surface-radiolabelled BHK cells with anti-(CHO FN receptor) antibody was performed as described previously [12]. Briefly, rabbit anti-goat IgG (Cooper Biomedical)-conjugated Protein A-Sepharose 4B beads (Pharmacia Fine Chemicals) were pre-incubated with non-immune IgG or anti-(CHO FN receptor) antibodies. Cells (2×10^7 – 3×10^7) were surface-radiolabelled with 1 mCi of Na¹²⁵I by the Iodo-Bead surface-labelling technique [18] and then extracted with NP-40 extraction buffer (see above) for 30 min at 4 °C. The extracts were centrifuged at 12000 *g* in a Beckman Microfuge (model 11) for 15 min. Cell extracts were first incubated for 2 h at 4 °C with non-immune goat IgG beads and then incubated overnight at 4 °C with anti-(CHO FN receptor) antibody beads. Finally, the beads were washed three times with NP-40 extraction buffer, once with PBS containing 0.5 M-NaCl, and one more time with NP-40 extraction buffer. The immunoprecipitated materials associated with beads after the washing procedures were solubilized in reducing sample buffer for electrophoresis and autoradiography.

RESULTS

Lectin binding to BHK cells

The numbers of WGA- and RCA-binding sites on BHK cells were determined by using lectins that had been radiolabelled by reductive alkylation. Binding assays were carried out at 4 °C to minimize endocytosis of the bound lectins. We found that at 4 °C the lectins bound to the cells rapidly, and equilibrium was reached after 30–45 min. The amount of binding after 5 min was about 60 % of the maximal value (results not shown).

BHK cells were incubated at 4 °C for 45 min with increasing concentrations of [³H]WGA (Fig. 1) or

Fig. 1. Binding of [³H]WGA to BHK cells

BHK cells were incubated at 4 °C with [³H]WGA at the concentrations indicated, and the extent of binding to the cells was measured. Other details are in the Experimental section.

Fig. 2. Binding of [³H]RCA to BHK cells

BHK cells were incubated at 4 °C with [³H]RCA at the concentrations indicated, and the extent of binding to the cells was measured. Other details are in the Experimental section.

[³H]RCA (Fig. 2). Saturable binding was observed in both cases. The data in Figs. 1 and 2 were used to prepare double-reciprocal plots and subjected to least-squares regression analysis (Figs. 3 and 4). Maximal lectin binding was calculated from the reciprocal of the ordinate intercept, and dissociation constants were calculated from the negative reciprocal of the abscissa intercept corrected to molar value by taking into consideration the molecular mass and incubation volume. We determined that there were 1.01×10^8 WGA-binding sites and 6.0×10^6 RCA-binding sites per cell, and the dissociation constants of these sites were 0.027 nM and 0.014 nM respectively. Therefore, RCA bound to the cells with a slightly higher affinity than WGA, but there were about 17 times more binding sites for WGA than for RCA. It should be noted, however, that complex glycoprotein molecules, such as those found on cell surfaces, are likely to have more than one lectin-binding site per molecule. Also, it should be pointed out that the curves in Figs. 1 and 2 did not entirely reach a plateau, which indicates that, at the higher lectin concentrations,

Fig. 3. Double-reciprocal plot of WGA-binding data

The binding data in Fig. 1 were replotted by the double-reciprocal method and subjected to least-squares regression analysis. Maximal lectin binding was calculated from the reciprocal of the ordinate intercept, and the dissociation constant was calculated from the negative reciprocal of the abscissa intercept, corrected to a molar value by taking into consideration the molecular mass and incubation volume.

Fig. 4. Double-reciprocal plot of RCA-binding data

The binding data in Fig. 2 were treated as described in Fig. 3 legend.

there may have been a low extent of non-specific binding or binding of lectins to a class of low-affinity receptors.

In control experiments (results not shown) we found that the radiolabelling procedure did not appear to change the cell-binding activity of the lectins. For instance, mixtures of radiolabelled and non-radiolabelled WGA with calculated specific radioactivities ranging from 64 to 446 c.p.m./μg showed similar cell-binding reactivity. Specificity of radiolabelled-lectin binding to BHK cells was shown by the greater than 95% inhibition of radiolabelled-lectin binding if the cells were pretreated with non-radiolabelled lectins. Most of the RCA-binding sites were D-galactose residues, since binding of [³H]RCA

to the cells was inhibited by 85–90% by 0.1 M-D-galactose. Others have shown that WGA binding to cells involves complex interactions with N-acetyl-D-glucosamine and sialic acid carbohydrate groups, and free sugars by themselves were reported to be poor inhibitors of WGA binding [19,20]. Consistent with these findings, we observed that binding of [³H]WGA to the cells was inhibited by only about 50% by 0.1 M-N-acetyl-D-glucosamine. Indeed, to obtain complete inhibition of WGA binding to BHK-cell extracts it was necessary to add both 4 mg of fetuin/ml and 0.5 M-N-acetyl-D-glucosamine.

Coupling of lectin-binding sites to the BHK-cell cytoskeleton

In addition to determining the numbers and affinities of WGA- and RCA-binding sites, we also determined how well these binding sites could couple to the BHK-cell cytoskeleton. BHK cells were incubated in suspension

Table 1. Coupling of lectin-binding sites to the cell cytoskeleton

Values in parentheses are percentages of total (insoluble + soluble) lectin binding.

Conditions	Triton fraction	$10^{-7} \times$ No. of WGA-binding sites/cell	$10^{-7} \times$ No. of RCA-binding sites/cell
Suspended cells treated with lectins before extraction*	Insoluble	5.51 ± 0.07 (79.7%)	0.28 ± 0.02 (75.7%)
	Soluble	1.40 ± 0.07	0.09 ± 0.02
	Total	6.91	0.37
Spread cells treated with lectins before extraction†	Insoluble	5.47 ± 0.13 (76.3%)	0.15 ± 0.03 (45.5%)
	Soluble	1.70 ± 0.13	0.18 ± 0.03
	Total	7.17	0.33
Spread cells treated with lectins after extraction‡	Insoluble	0.73 ± 0.08 (10.2%)	0.04 ± 0.02 (12.1%)

* BHK cells in suspension were incubated for 45 min at 4 °C with [³H]WGA (60 μg/ml) or [³H]RCA (50 μg/ml) and then subjected to Triton extraction. Radioactivity associated with the insoluble and soluble fractions was measured, and number of lectin-binding sites/cell was calculated.

† BHK cells spread on FN-coated substrata were treated with radiolabelled lectins and then subjected to Triton extraction.

‡ BHK cells spread on FN-coated substrata were extracted with Triton and then incubated with radiolabelled lectins. Other details are in the Experimental section.

Fig. 5. Ultrastructural appearance of detergent-extracted cells

(a) Triton-insoluble fraction of BHK cells showing nuclear remnants (N) and other debris (magnification $\times 10\,500$). (b) High magnification ($\times 114\,000$) of membrane-like material encircled in (a). (c) Triton-insoluble fraction of BHK cells previously treated with WGA (Table 1) showing nuclear remnants (N) and stabilized cell surface (magnification $\times 12\,000$). (d) High magnification ($\times 114\,000$) of cell-surface region encircled in (c). Other details are in the Experimental section.

with radiolabelled WGA and RCA, washed, and cytoskeletal preparations were obtained by extracting the cells with 0.5% Triton X-100 buffer. About 75% of the radioactivity stayed with the Triton-insoluble fraction (Table 1), indicating that most of the WGA- and RCA-binding sites were coupled to the cytoskeleton under

these conditions. The total amounts of WGA and RCA bound to the cells, which were calculated by adding the Triton-insoluble and -soluble amounts, were similar to results found in the other binding assays (Figs. 1–4).

Analogous experiments were performed with BHK cells that were attached and spread on FN-coated

substrata. The total number of lectin-binding sites was similar on spread cells and suspended cells (Table 1). That is, most lectin-binding sites still were accessible even after cell spreading on FN, which is consistent with previous studies using lectin-coated beads [6]. About 75% of the WGA-binding sites and 46% of the RCA-binding sites of spread cells were found in the Triton-insoluble fraction (i.e. cytoskeletal-associated).

In the above experiments, the lectins were added before the samples were subjected to extraction with Triton buffer. Although this was necessary with suspended cells, for technical reasons, with spread cells it was also possible to extract the cells first with Triton buffer and then to add the lectins. The results of this experiment (Table 1) indicated that only about 10% of the lectin-binding sites stayed associated with the cytoskeleton in the absence of the lectins. Therefore, binding of the lectins to the cells at 4 °C resulted in a shift in the Triton-solubility of the lectin-binding sites.

In the experiments with suspended BHK cells described above, we noticed the presence of what appeared to be intact cell ghosts in the pellets of cells that had been treated with WGA, but not in the pellets from control cells or cells that had been treated with RCA. Examination of these samples by electron microscopy showed that, after detergent extraction, control cell pellets contained nuclear remnants (Fig. 5a) and other cell debris, including some small membrane fragments (Figs. 5a and 5b). In marked contrast, cells treated with WGA before detergent extraction were observed to have relatively intact cell-surface remnant (Fig. 5c) that was much thicker than the original plasma membrane (Fig. 5d).

Isolation and characterization of lectin-binding sites

The chemical composition of lectin-binding molecules was determined by lectin affinity chromatography of NP-40 extracts prepared from BHK cells that had been metabolically radiolabelled with [³⁵S]methionine. Cell extracts were passed through a Sepharose CL-4B column (to remove components in the cell extracts that might bind to Sepharose non-specifically), after which the extracts were subjected to affinity chromatography on WGA- or RCA-coupled agarose columns. Similar results were obtained in several different preparations, and typical fractionation data are shown in Table 2.

The NP-40-soluble extract contained about 66% of the radioactivity in the starting cell preparation. Most of this material passed through the lectin affinity columns. There was, however, a small but reproducible portion of the detergent-soluble proteins that bound to the lectin columns and could be eluted with the appropriate sugars. On the basis of the radioactivity recovered in the eluted samples, WGA- and RCA-binding molecules amounted to approx. 1.5% and 0.15% of the radioactivity associated with the detergent-soluble proteins. This result indicated that there were about 10 times more WGA-binding molecules than RCA-binding molecules.

The composition of the WGA- and RCA-binding-molecule preparations were characterized by gel electrophoresis and fluorographic analysis. Many polypeptides were observed in the NP-40 cell extracts (Fig. 6, lane a), most of which did not bind to the WGA or RCA columns (lanes b and d). The WGA-binding-molecule preparations (lane c) contained about eight major components, ranging in molecular mass from 93 to

Table 2. Preparation of WGA- and RCA-binding molecules from BHK cells

Metabolically radiolabelled BHK cells were extracted at 4 °C with NP-40 extraction buffer, and the soluble extract was passed through a Sepharose CL-4B column before affinity chromatography. WGA-binding molecules were eluted from a WGA-agarose column with 15 ml of 1 M-*N*-acetyl-D-glucosamine. RCA binding molecules were eluted from RCA-agarose with 0.2 M-galactose. Other details are in the Experimental section.

Fraction	Radioactivity in fractions (c.p.m.)	
	WGA column	RCA column
Starting material	2.82×10^9	
NP-40-soluble extract	1.86×10^9	
Native Sepharose unbound fraction	1.07×10^9	
Affinity-column load	5.35×10^8	5.35×10^8
Affinity-column unbound fraction	4.70×10^8	5.26×10^8
Eluted lectin-binding molecules	7.90×10^6	7.87×10^5

Fig. 6. Fluorography of WGA- and RCA-binding molecules isolated from BHK cells

Samples from the lectin-binding-molecule isolation procedure (Table 2) were solubilized in reducing sample buffer and analysed electrophoretically. Lanes: (a), affinity-column load; (b), WGA-column-unbound; (c) WGA-binding molecules; (d) RCA-column-unbound; (e), RCA-binding molecules; (f), RCA-column-unbound, with WGA-binding molecules as column load; (g) RCA-/WGA-binding molecules. The major components of the WGA-binding molecules (lane c) had molecular masses ranging from 93 to 340 kDa (arrowheads on the right). The RCA-binding components appeared identical, regardless of whether they were isolated from whole-cell extracts (lane e) or from WGA-binding-molecular preparations (lane g). Other details are in the Experimental section.

340 kDa, and the RCA-binding-molecule preparations contained about six major components, ranging in molecular mass from 110 to 340 kDa (lane *e*). Since there appeared to be several bands in common, experiments were carried out in which RCA-binding molecules were prepared from WGA-binding-molecule preparations. From the recovery of radioactivity, 10.7% of the WGA-binding molecules also were RCA-binding molecules, about the same amount that was obtained when RCA-binding molecules were isolated directly from NP-40-soluble proteins. The profile of RCA-binding molecules prepared in this manner (lane *g*) was identical with the profile of RCA-binding molecules prepared from cell extracts (lane *e*). In addition, unique WGA-binding molecules that did not bind to RCA columns were observed (lane *f*). It could therefore be concluded that the RCA-binding molecules were a subpopulation of the WGA-binding molecules.

Analysis of 140 kDa fibronectin receptors in lectin-binding molecules

To determine the presence of 140 kDa FN receptors in the lectin-binding-molecule preparations, immunoblotting experiments were performed with a polyclonal antibody to the 140 kDa FN receptors of CHO cells. At a concentration of 1 mg/ml, this antibody inhibited BHK-cell spreading by over 75% (results not shown). Immunoblots of the WGA-binding preparations detected primarily a 145 kDa component, and, to a lesser extent, a lower-molecular-mass component (Fig. 7, lane *b*).

Fig. 7. Immunoblotting of WGA-, RCA- and RCA/WGA-binding molecules with polyclonal anti-(CHO FN receptor) antibody

WGA- and RCA-binding molecules (20 μ g/lane) were subjected to SDS/polyacrylamide-gel electrophoresis. Subsequently, the polypeptides were transferred on to a nitrocellulose paper and immunoblotted with non-immune IgG (lane *a*) or anti-(CHO FN receptor) IgG (lanes *b*–*e*). Lanes: (*b*), WGA-binding molecules; (*c*), RCA-binding molecules; (*d*), WGA-binding molecules that did not bind to RCA columns; (*e*), RCA/WGA-binding molecules. Other details are in the Experimental section.

Non-immune goat IgG did not react with any components in the preparations (lane *a*). The antibody also detected the 145 kDa component in RCA-binding molecules prepared from whole-cell extracts (lane *c*) and in RCA-binding molecules prepared from WGA-binding molecules (lane *e*). The WGA-binding components that were not bound by RCA columns contained the lower-molecular-mass component of the FN-receptor complex, but also contained the 145 kDa component (lane *d*). The presence of this 145 kDa component in the unbound fraction could not be accounted for by column capacity, since running the unbound material through a regenerated RCA column did not result in additional binding of the 145 kDa component (results not shown). These results indicated that there were at least two glycosylation states of the FN-receptor complex, only one of which could bind to the RCA columns.

To analyse the composition of the FN-receptor complex, anti-(CHO-cell FN receptor) antibody was also used to immunoprecipitate NP-40 extracts of 125 I-surface-radiolabelled BHK cells. From the diverse set of iodinated components in the crude extract (Fig. 8, lane *a*), the anti-(CHO FN receptor) antibodies immunoprecipitated a complex of three polypeptides with molecular masses 120–145 kDa (lane *b*) that were not immunoprecipitated by non-immune goat IgG (lane *c*).

Fig. 8. Immunoprecipitation of cell-surface proteins by polyclonal anti-(CHO FN receptor) antibody

125 I-surface-radiolabelled cells were solubilized in NP-40 extraction buffer, and the soluble extracts were subjected to immunoprecipitation with the anti-(CHO FN receptor) antibody. Lanes: (*a*), total 125 I-radiolabelled cell-surface proteins (20×10^3 c.p.m.); (*b*), proteins immunoprecipitated by anti-(CHO FN receptor) IgG (5×10^3 c.p.m.); (*c*), proteins immunoprecipitated by non-immune goat IgG (5×10^3 c.p.m.). Other details are in the Experimental section.

DISCUSSION

The purpose of the present studies was to compare WGA- and RCA-binding sites of BHK cells and determine their relationship to 140 kDa FN receptors. Binding studies carried out with radiolabelled lectins showed that there were 1.01×10^8 WGA-binding sites per cell ($K_d = 0.027$ nM) and 6.0×10^6 RCA-binding sites per cell ($K_d = 0.014$ nM). Therefore, BHK cells had about 17 times more cell-surface WGA-binding sites than RCA-binding sites, but the affinity of RCA for the cells was slightly higher than that of WGA. These data are consistent with previous studies in which iodinated lectins were used to study the number of RCA and WGA receptors on the 21-C13 BHK cell line [21]. When the WGA and RCA molecules were isolated from NP-40 extracts of metabolically radiolabelled BHK cells, we found a 10:1 ratio of WGA to RCA molecules, which is slightly less than the difference in the number of cell-surface sites detected in lectin-binding studies. It should be noted, however, that the lectin-binding studies are specific for cell-surface sites, whereas our lectin affinity-column procedure would detect both cell-surface and intracellular sites. Also, there may have been low-affinity WGA- and RCA-binding sites on the cell surface, but analysis of these sites was not performed.

Studies on BHK cells attached and spread on FN-coated substrata showed that about 10% of the binding sites were coupled to the cell cytoskeleton, on the basis of detergent-extraction studies. When the cells were treated with lectins before extraction, more than 75% of the binding sites became cytoskeleton-associated. Similarly, more than 75% of the binding sites of suspended BHK cells were linked to the cytoskeleton after binding of the lectins. Others have shown previously that cell-surface components of platelets and *Amoeba* cross-linked by concanavalin A can become associated with the cell cytoskeleton [22,23].

An intriguing observation made in connection with the detergent-extraction studies was the ability of WGA-treated cells to retain surface structure after detergent extraction. The thickness of this structure, 30–50 nm, might be accounted for by the bound WGA molecules. Indeed, WGA seems to be acting as a biological 'fixative' in this situation. The inability of RCA to stabilize a similar structure might be accounted for by the 17-fold fewer cell-surface RCA-binding sites than WGA-binding sites. Whether the cell-surface structure stabilized by WGA is related to the 'plasma membrane skeleton' described by others [24–26] is unknown.

Molecular analysis of WGA-binding molecules from metabolically radiolabelled cells showed that there were about eight major polypeptides, ranging in molecular mass from 93 to 340 kDa. RCA-binding molecules contained fewer components, all of which appeared to correspond to components found in the WGA preparations. That is, the composition of RCA-binding molecules was identical regardless of whether they were prepared from whole-cell extracts or from WGA-binding-molecule preparations. It could be concluded, therefore, that the RCA-binding molecules were a subpopulation of the WGA-binding molecules.

We analysed FN receptors within the lectin-binding-molecule preparations by immunoblotting with anti-(CHO FN receptor) antibody. Both the WGA- and RCA-binding molecules of BHK cells contained a 145 kDa

component recognized by the anti-(CHO FN receptor) antibody. This component appeared to be a part of two different FN-receptor complexes that differed in glycosylation, only one of which bound to RCA affinity columns. Although others have presented evidence suggesting that 140 kDa FN receptors are WGA-binding sites [8,27], our findings are the first to show that 140 kDa FN receptors are RCA-binding sites. Our observation is consistent with the studies in which it was reported that RCA-deficient cell variants were defective in their adhesion to FN substrata [5]. Moreover, our observation of a subset of 145 kDa FN receptors that were able to bind to WGA, but not to RCA, may help to explain why WGA is a better inhibitor of FN-mediated cell adhesion than is RCA [6].

The anti-(CHO FN receptor) antibody immunoprecipitated a polypeptide complex (120–145 kDa) from extracts of BHK cells that had been surface-radiolabelled. It seems likely, therefore, that the 140 kDa FN receptors of BHK cells are a cell-surface oligomeric complex of three polypeptides, which is consistent with previous studies on BHK cells [28]. Recently, evidence has been presented indicating that adhesion-receptor complexes are related to the VLA cell-surface proteins of lymphocytes, which share common lower-molecular-mass subunits and have unique higher-molecular-mass subunits [29]. Antibodies directed against the common subunit of these receptor complexes would be expected to inhibit cell adhesion promoted by several different ligands, whereas antibodies directed against the unique subunits would be expected to be very specific. Consistent with this notion, our immunoblotting experiments showed that anti-(CHO FN receptor) antibody binds primarily to the unique (i.e. higher molecular mass) subunit of the BHK FN-receptor complex, and it has been shown previously that this antibody inhibits adhesion promoted by FN, but not by other ligands [9,12].

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