BINDING OF [^{125}I]WHEAT GERM AGGLUTININ TO CHINESE HAMSTER OVARY CELLS UNDER CONDITIONS WHICH AFFECT THE MOBILITY OF MEMBRANE COMPONENTS

PAMELA STANLEY and JEREMY P. CARVER

From the Department of Medical Genetics, University of Toronto, Toronto, Ontario, Canada M5A, 1S8. Dr. Stanley's present address is the Department of Cell Biology, Albert Einstein College of Medicine, Bronx, New York 10461

ABSTRACT

The binding of [^{125}I]wheat germ agglutinin ([^{125}I]WGA) of high specific activity to Chinese hamster ovary (CHO) cells has been examined over a millionfold range of WGA concentrations and correlated with the phenomena of agglutination and capping by WGA. Analysis of the binding data by the method of Scatchard gives a complex curve indicative of positive cooperativity amongst high-affinity binding sites. Binding assays performed under conditions which inhibit capping and/or agglutination, such as low temperature or glutaraldehyde fixation, give similarly complex binding curves. Thus, the gross mobility of WGA receptors in the membrane does not appear to be responsible for the cooperative binding of WGA to CHO cells.

KEY WORDS Scatchard plots · wheat germ agglutinin · membrane mobility

The interaction of wheat germ agglutinin (WGA) with cell surfaces has been used extensively to follow structural changes in the plasma membranes of animal cells (see references 10 and 11). However, the interpretation of WGA-binding characteristics for intact cells is complex. Scatchard analysis of the binding data for Chinese hamster ovary (CHO) cells over a millionfold range of WGA concentrations gives a curve indicative of a number of different WGA-binding sites and of positive cooperativity amongst the high-affinity sites (17, 18). These results are in general agreement with those of Cuatrecasas (6) who examined the binding of [^{125}I]WGA to fat cells and suggest that simple descriptions of WGA binding to cells reported previously are due to the relatively small range of WGA concentrations investigated (1, 4).

The binding of WGA at the plasma membrane might be expected to be complicated, for a variety of reasons. First, sugars which are known to interact with WGA (7) are distributed amongst carbohydrate side chains of different sugar sequences which, in turn, may be distributed amongst many different surface glycoprotein and glycolipid molecules (see references 8 and 15). Second, cells incubated with WGA become agglutinated and also exhibit "capping" of WGA receptors (see references 10 and 11). Both of these phenomena might be expected to contribute to interactions between different WGA-binding sites and/or to generate new binding sites.

To define the various classes of WGA-binding sites on the cell membrane, it is important to determine the extent to which the mobility of membrane macromolecules contributes to the binding characteristics. Therefore, we have examined WGA binding to CHO cells under conditions which inhibit agglutination between cells and/or

capping of WGA receptors. In this paper, we present evidence that the major binding characteristics displayed by WGA for CHO cells are a reflection of the number and types of distinct WGA-binding sites and do not appear to arise from the gross mobility of membrane components.

MATERIALS AND METHODS

Materials

WGA and bovine serum albumin (BSA) were obtained from Sigma Chemical Co. (St. Louis, Mo.); fluorescinated WGA (fl-WGA) from Miles Laboratories Inc., Miles Research Products (Elkhart, Ind.); ^{125}I from Amersham (England); alpha medium from Kansas City Biological Co. (Kansas City, Mo.); fetal calf serum (FCS) from Flow Laboratories, Inc. (Rockville, Md.); Colcemid from Grand Island Biological Co. (Grand Island, N. Y.); and colchicine from Sigma Chemical Co. $(GlcNAc)_3$ was a gift from Nicole Lacelle (University of Toronto, Toronto, Ontario, Canada); Traysylol (5) and phenylmethylsulfonylfluoride (PMSF) were gifts from Mark Pearson (University of Toronto). All other chemicals were reagent grade.

Methods

CELL LINES: The CHO auxotrophic clones Pro$^-$5 (proline-requiring) and Gat$^-$2 (glycine,adenosine,thymidine-requiring) (16). Cells were cultured at 37°C in suspension in alpha medium containing 10% FCS as previously described (16, 18).

PREPARATION OF [^{125}I]WGA: WGA was dissolved in phosphate-buffered saline (PBS), pH 7.2, at a concentration of 2–4 mg/ml determined from $E^{1\%}_{280\ nm}$ of 15.0 (9) and stored at 4°C. As described previously, 30 μg of WGA was iodinated via the chloramine-T method in the presence of dimethylsulfoxide to a sp act of ~12–15 μCi/μg (17, 18).

BINDING ASSAY: Binding assays were performed as described previously in detail (17, 18). Briefly, cells from exponentially growing cultures were washed three times with PBS at 4°C, counted, diluted, and incubated at room temperature for 1 h in an assay mixture containing PBS, 2% BSA, unlabeled WGA, [^{125}I]WGA (~3,000–50,000 cpm) and 5×10^5 cells in a final volume of 0.2 ml. Final WGA concentrations ranged from 10^3 to 10^9 pg/ml with each concentration being performed in duplicate. To ensure that [^{125}I]WGA and unlabeled WGA possessed identical binding properties, tubes containing 10^4, 10^5, 10^6, 10^7, and 10^8 pg/ml final WGA concentrations were assayed at two different concentrations of [^{125}I]WGA. Unbound [^{125}I]WGA was removed by filtration on GF/C glass fiber filters presoaked in 10% BSA for 2 h at room temperature. Reaction tubes before the addition of cells, washed filters, and washed tubes were counted on a Nuclear Chicago Autogamma counter (efficiency 41%; Nuclear-Chicago Corp., Des Plaines, Ill.). The results were analyzed by the method of Scatchard (14) where r is the amount of WGA bound per cell (determined from the cpm bound per filter, the specific activity of the [^{125}I]WGA, and the number of cells per assay tube) and A is the amount of free WGA (determined from the total cpm per assay tube, the cpm remaining per washed assay tube [which were assumed not to be available for the binding reaction], the cpm bound per filter and the specific activity of the [^{125}I]WGA). All of the WGA binding was taken to be specific because, over the entire concentration range, binding was at least 97% inhibited by 2 mM $(GlcNAc)_3$. Also, binding to the filters was found to be 1–2% of the total cpm present over the entire WGA concentration range and therefore considered negligible.

ANOMALOUS EFFECTS ON THE BINDING RESULTS: During the course of these studies, it became clear that the age of the [^{125}I]WGA preparation had a significant effect on the binding results. Not only were less cpm bound with older [^{125}I]WGA preparations but also the characteristic shape of the binding curve was altered. It was shown that low molecular weight, ^{125}I-labeled material accumulated during storage at 4°C and that the released material did not bind to CHO cells. It seemed likely, therefore, that it consisted of either free ^{125}I or inactive [^{125}I]WGA peptides. However, attempts to correct the binding data based on both of these models did not give rise to predicted values. Therefore, it seems probable that the [^{125}I]WGA was itself partially inactivated and that an appropriate correction of the data would be complicated. These results indicate that it is preferable to use freshly prepared [^{125}I]WGA when quantitative comparisons are to be made. In some of the experiments reported here, where only qualitative comparisons were to be made, older [^{125}I]WGA preparations have been used.

A second anomalous result concerns the finding that the Scatchard plots were altered when the number of cells used per assay tube was reduced. The predicted result in this situation (assuming simple equilibrium between cooperative sites) would be a shift in the calculated points for a given WGA concentration to higher r values with a concomitant change in the r/A values so that the points fell on a different part of the same curve. Instead, an entirely different curve was obtained when different numbers of cells were used. Various explanations of this phenomenon were investigated: (*a*) FCS (10% vol/vol) was shown not to be an inhibitor of the binding reaction; (*b*) binding was not stimulated by the presence of the protease inhibitors Traysylol (50 KIU/ml) or PMSF (10^{-4} M); (*c*) the supernate from cells incubated under the conditions of the binding assay was shown to possess negligible inhibitory activity; and (*d*) it was shown by dilution that the filtration method used in the binding assay was accurate from 2.5×10^3 to 10^6 cells per assay tube. Therefore, it would appear that while the cause of this cell number

affect remains unknown, binding comparisons between cell lines should always be performed using an identical number of cells per assay tube.

GLUTARALDEHYDE FIXATION: Glutaraldehyde fixation of cells was performed similarly to the method described by Bornens et al. (3). Cells washed twice with cold PBS were resuspended at about 10^7 cells/ml in 0.05% glutaraldehyde in PBS (in contrast to the 0.5% glutaraldehyde used in reference 3) and incubated at 4°C for 15 min. After centrifugation, the cell pellet was resuspended in 10 ml of 0.1 M lysine in PBS, recentrifuged, and subsequently diluted with PBS for the binding assay.

AGGLUTINATION: Agglutination was scored at each WGA concentration used in the binding assay by examining a 5-μl aliquot from each assay tube by phase-contrast microscopy. Samples were read "blind" and in random order to facilitate objective scoring of the degree of agglutination, which was taken from 0 (no cells in clumps) to 4+ (essentially all cells in large clumps).

CAPPING: Capping experiments were performed as described by Aubin et al. (2). Briefly, cells growing as monolayers on glass cover slips were washed with cold PBS and incubated at 4°C with different concentrations of fl-WGA for 30 min. Unbound fl-WGA was subsequently removed by washing with warm PBS, and the cells were incubated at 37°C. After 30 min, the number of cells per field exhibiting fluorescent "caps" was scored using a fluorescence microscope and expressed as a percentage of the total cells per field.

RESULTS

Scatchard plots for the binding of [^{125}I]WGA to two CHO auxotrophic clones are shown in Fig. 1. In Fig. 1 *A* the binding data are plotted using the conventional linear scale for r as described previously (17, 18). The first part of the curve shows highly positively cooperative binding occurring at low WGA concentrations while the latter part of the curve is typical of either negatively cooperative binding or the existence of several classes of sites with different binding affinities. We have previously shown, by expanding the r scale at low r values, that a plateau occurs in the binding curve at around 1–2 fg WGA bound per cell (18). However, this plateau is masked when the binding data for a millionfold range of WGA concentrations are plotted against r on a linear scale. A more adequate representation of all the binding data may be obtained on one graph by plotting against $\log_{10}[r]$ as shown in Fig. 1 *B*. This method clearly demonstrates that there are two maxima occurring in the positively cooperative region of the Scatchard, although it deemphasizes the data obtained at high r values. However, the log plot provides a much better overall description of the binding data.

Agglutination between cells and capping of

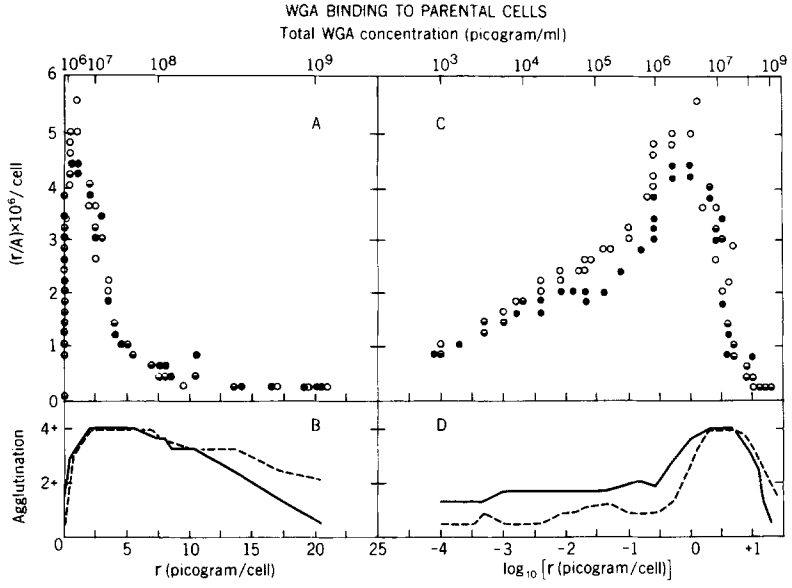

FIGURE 1 Binding of [^{125}I]WGA (~13 μCi/μg; stored 3 days at 4°C) to parental CHO cells Pro$^-$5 (○) and Gat$^-$2 (●). The data are plotted according to the method of Scatchard using the conventional linear scale for r (A) or plotting $\log_{10}[r]$ (B). Agglutination titers for Pro$^-$5 (—) and Gat$^-$2 (– –) cells were determined at each WGA concentration and are also plotted against r (C) or $\log_{10}[r]$ (D). The upper nonlinear scales give the total amount of WGA present per reaction tube.

membrane macromolecules by WGA are well-documented (see references 10 and 11). To determine the WGA-binding parameters affected by these phenomena, it was necessary, first, to determine how agglutination and capping correlate with the WGA-binding curve and secondly to examime WGA binding under conditions which inhibit agglutination and/or capping. The correlation between WGA-induced agglutination and the binding of WGA to CHO cells is shown in Fig. 1. Although there is some variability at low r values, agglutination between cells does not increase significantly above that observed for cells in PBS alone until ~0.5 pg of WGA is bound per cell (Fig. 1 *D*). Agglutination reaches a maximum at a WGA concentration of $\sim 10^7$ pg/ml (~1–10 pg of WGA bound per cell) and subsequently declines at higher WGA concentrations. Maximum agglutination does not appear to correlate directly with the peak in positively cooperative binding but rather with the binding which occurs at higher WGA concentrations (Fig. 1 *D*).

The correlation between capping by fl-WGA and WGA binding gave similar results. At concentrations of fl-WGA below 10^6 pg/ml, cell-associated fluorescence was not strong enough to clearly observe WGA binding or capping. Therefore, capping was investigated only over the concentration range 10^6–10^8 pg/ml of fl-WGA. At 10^6 pg/ml of fl-WGA, 50% CHO cells exhibited caps after 30 min at 37°; at 8×10^6 pg/ml of fl-WGA, 99% of the cells were capped; and at 10^8 pg/ml of fl-WGA, the number of capped cells was reduced again to 50%. Thus, capping of WGA receptors in CHO cells shows a dependence on WGA concentration similar to that observed for agglutination. Both phenomena are inhibited at high WGA concentrations.

To define conditions under which either agglutination and/or capping are inhibited, the effects on these phenomena of temperature and brief glutaraldehyde fixation were examined. Capping of CHO cells by WGA was found to be maximal at 37°C and not to occur at 4°C. In fact, at 4°C, fl-WGA is seen as a ring around the cell and neither patches nor caps are observed. Glutaraldehyde fixation also inhibited cap and patch formation (at 10^7 pg/ml of WGA). Cells under these assay conditions become brightly fluorescent over the entire surface. The effects of these assay conditions on agglutination are shown in Fig. 2. No significant effect was observed on agglutination when the assays were performed at 37° or

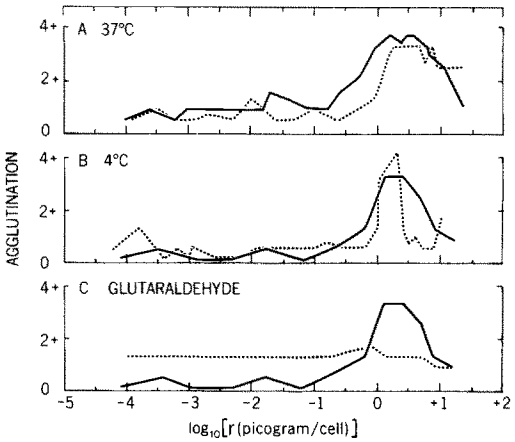

FIGURE 2 Agglutination titers of Pro$^-$5 cells incubated with WGA (10^3 to 10^9 pg/ml) at (A) room temperature (—) and 37°C (- -); (B) room temperature (—) and 4°C (- -); (C) room temperature, untreated control (—) and room temperature, 0.05% glutaraldehyde-treated (- -). The data are plotted against $\log_{10}[r]$ where r is the amount of [^{125}I]WGA bound per cell at each WGA concentration examined.

4°C. However, agglutination was abolished by prior fixation of the cells with glutaraldehyde.

Having established conditions under which (*a*) both agglutination and capping occur (37°C), (*b*) both agglutination and capping are inhibited (glutaraldehyde fixation), and (*c*) agglutination occurs while capping is inhibited (4°C), it was possible to determine whether these phenomena contributed significantly to the complex binding parameters described in Fig. 1. The effects of temperature and glutaraldehyde on the binding of [^{125}I]WGA to CHO cells are shown in Fig. 3. At 37°C, where both agglutination and capping occur, no significant alteration in the Scatchard plot was observed, compared with that obtained with cells incubated at room temperature. Also, at 4°C, where capping is inhibited but agglutination occurs, the shape of the Scatchard plot was essentially identical to that obtained at room temperature. Thus, none of the major binding parameters are altered under conditions where capping of WGA receptors is inhibited. Finally, when both agglutination and capping were inhibited by glutaraldehyde fixation, the major features of the binding curve were preserved although increased WGA binding occurred over a small range of WGA concentrations (Fig. 3). Thus, it would appear that neither agglutination nor capping is responsible for the positively cooperative binding of [^{125}I]WGA to CHO

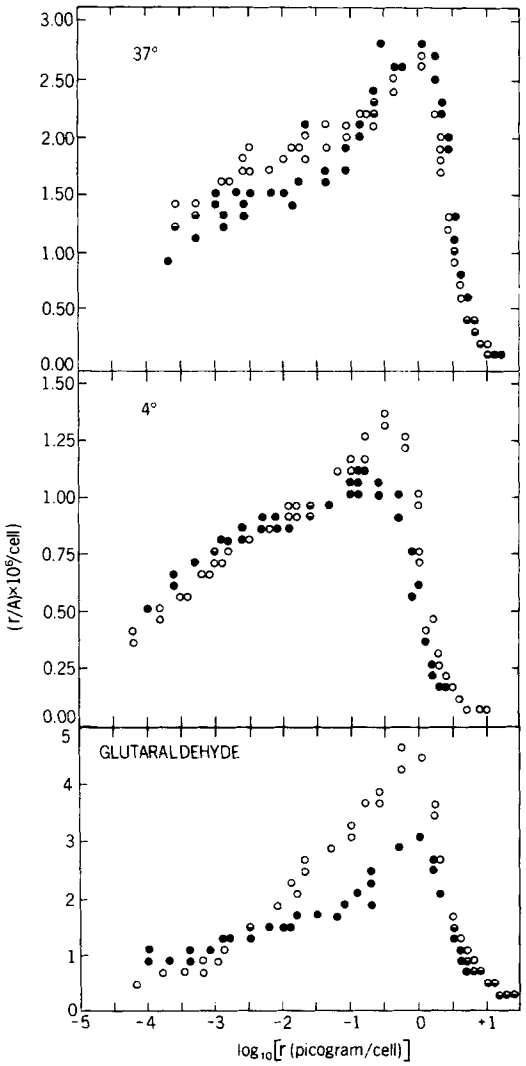

FIGURE 3 The effect of temperature and glutaraldehyde on the binding of WGA to CHO cells. (A) Binding of [^{125}I]WGA (~5 μCi/μg; stored 8 days at 4°C) to Pro$^-$5 cells incubated at 37°C (●) or room temperature (○); (B) binding of [^{125}I]WGA (~3.2 μCi/μg; stored 30 days at 4°C) to Pro$^-$5 cells incubated at 4°C (●) or room temperature (○); (C) binding of [^{125}I]WGA (~14 μCi/μg; stored 1 day at 4°C) to untreated (●) and glutaraldehyde-treated (○) Pro$^-$5 cells at room temperature.

cells. Secondly, the inhibition of gross receptor mobility does not appear to affect the latter part of the Scatchard plot—the region which corresponds to WGA concentrations which give rise to agglutination and capping.

DISCUSSION

The complex binding of WGA to CHO cells has previously been reported from this laboratory (17, 18). The binding curve is characterized by extensive positive cooperativity (concave downward Scatchard plot) at low degrees of saturation and concave upward behavior at high degrees of saturation. In the present study, we have investigated two phenomena in particular which might contribute to the cooperative binding behavior: WGA-induced agglutination between cells and/or capping of WGA surface receptors. We have shown that, at 4°C, when capping is inhibited but agglutination still occurs, the binding curve is not significantly altered, suggesting that none of the major binding characteristics arise from cap formation. Similarly, the inhibition of both cap formation and agglutination between cells in glutaraldehyde-fixed cells did not result in changing the major features of the binding curve. Clearly, glutaraldehyde fixation does alter the binding curve over a certain WGA concentration range (Fig. 3). However, this alteration does not appear to correspond to a loss in positive cooperativity. Thus, it would appear that the positive cooperativity exhibited by WGA in binding to CHO cells does not arise from the gross aggregation of membrane components which result in agglutination and capping by WGA. However, the positive cooperativity may arise from more localized clustering of WGA receptors not observable by these assays and not inhibited by low temperature or glutaraldehyde fixation.

Other factors which may account for certain of the WGA-binding characteristics are heterogeneity amongst the lectin population and/or amongst the cell population. Isolectins of WGA have been reported (13), and the preparation of WGA used in these studies was a mixture of these isolectins. However, we have shown that purified isolectins WGA-I and WGA-II exhibit essentially identical binding to CHO cells compared with the WGA preparation used here (Lacelle et al., manuscript in preparation). Heterogeneity amongst the cell population might be expected to arise due to the cells being in different stages of the cell cycle. WGA has been shown to bind differentially to cells containing metaphase chromosomes if they are not derived by colchicine blockade (12). However, since mitotic cells represent only 2% of the cells in exponential growth (P. Stanley, unpublished observation), the major characteristics of the binding curve probably reflect the binding properties of the majority of the cells in the population.

We conclude that the cooperativity in the binding data presented in Fig. 1 is not generated from interactions between WGA-binding sites which require gross receptor aggregation. Thus, it is conceivable that the positive cooperativity observed may be an intrinsic property of the WGA-receptor interaction for particular classes of binding sites. This possibility is currently being explored via mathematical analysis of the binding properties of parental CHO cells and Wga^R mutants which have lost certain WGA-binding sites (18).

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