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CO-OPERATIVE BINDING OF CONCANAVALIN A TO A GLYCOPROTEIN IN LIPID BILAYERS

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Lectin-binding curves are reported for a concanavalin A receptor glycoprotein in lipid bilayers and intact cells. The results are consistent with previous studies of the structurally dissimilar transmembrane glycoprotein, glycophorin. High-affinity lectin binding to model membranes was influenced by the presence of apparently unrelated macromolecules, which we suggest is an example of receptor modulation by local interactions. Furthermore, high-affinity binding to the model membranes displayed characteristics, including positive cooperativity, similar to those seen with intact cells.

Introduction

In recent years, numerous studies have focussed on the interactions of lectins with eucaryotic cells, especially with an eye to possible implications for mechanisms of growth control. Thus, for instance, lectin structural requirements for mitogenicity have been considered in some detail in the case of concanavalin A [1]. The physical response of associated cellular machinery to lectin binding has also been considered, although the picture is as yet very sketchy. An interesting early observation was that local interaction with concanavalin A receptors had a cooperative effect upon the cellular structures involved in cell and receptor movement [2]. There have been a number of studies which quantitated lectin binding using radiolabelled lectins [3–11], and positive cooperativity is a feature common to all such work. The implication has sometimes been drawn that a complex machinery may be the source of this cooperativity. Although studies of intact cells are clearly irreplaceable, there are certain aspects of this area of research that are well worth examining in model membranes – an approach which can permit less ambiguous interpretation of specific details. We have used membrane glycoproteins as lectin receptors after assembling them into lipid bilayers. In our hands, these structures most accurately mimic the (high-affinity) binding properties of cells when handled in the presence of serum albumin or dextran – macromolecules known to coat membranes with an adsorbed surface layer [12–15]. The explanation of this observation is not clear, although eucaryotic cells also possess a substantial layer of surface material – the glycocalyx.

To our knowledge, the earliest model system experiments which specifically address the origin of positive cooperativity in lectin binding are those of Jennissen [16,17]. He clearly demonstrated, using as a model the binding of phosphorylase *b* to alkylated Sepharose beads, the importance of receptor arrangement and local density in controlling multivalent binding affinity – and hence the potential importance of these factors to cooperativity in lectin binding to cells. In our own laboratory, during previous work with lipid bilayers bearing the glycoprotein, glycophorin, we found binding of wheat germ agglutinin to be positive cooperative and influenced by the presence of a

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surface layer of adsorbed albumin or dextran [18,19]. Glycophorin is a transmembrane protein of mol. wt. 30000 which is 60% carbohydrate [20]. It bears 15 identical short O-linked oligosaccharide chains with wheat germ agglutinin-binding potential [21]. The concanavalin A receptor glycoprotein of human erythrocytes is a 95000 molecular weight species which is a subpopulation of band 3 [22,23]. This glycoprotein bears 3–8% carbohydrate as a single N-linked segment [24,25]. Hence, glycophorin and band 3 represent two structurally very different receptors: yet, we demonstrate here that their lectin-binding curves share common features.

Isolation of band 3 glycoproteins and their reassembly into lipid bilayers have been reported by various workers [26–29]. We previously used the affinity chromatography method of Findlay [22] to isolate the concanavalin A-binding subpopulation for reassembly into bilayers by detergent dialysis [27]. However, in the present work we have combined such an approach with a technique which produces large, sealed liposomes: isolated concanavalin A receptor with some associated lipid was dissolved in 2-chloroethanol with additional lipid, and then solvent was evaporated to produce a lipid/glycoprotein film which could be subsequently hydrated gently. The resultant structures lend themselves well to differential centrifugation assays of lectin binding as originally applied to intact cells.

Materials and Methods

Materials

The concanavalin A receptor of human erythrocytes was isolated from outdated bank blood by a modification of the method of Findlay [22] as described previously [27]. Lipids were obtained from Sigma and were pure as judged by thin-layer chromatography on silica gel plates (Silica Gel GF 254 from Stahl). 2-Chloroethanol was from Eastman Kodak and unlabelled lectins were from Sigma. Concanavalin A receptor was ^3H labelled by a modification of the method of Moore and Critchton [30] and was typically 76% trichloroacetic acid precipitable with a specific activity of $3.5 \cdot 10^5$ cpm/mg protein. $NaB[^3H]H_4$ was obtained from New England Nuclear. ^3H-labelled concanavalin A was also obtained from New England Nuclear and was 75% trichloroacetic acid precipitable with a specific activity of $1.8 \cdot 10^6$ cpm/μg of protein. Bovine serum albumin was either ultrapure globulin free, or the 96–99% fraction V purchased from Sigma (both of which gave the same results). Dextran T-500 was obtained from Pharmacia.

Liposome preparation

To the desired amount of concanavalin A receptor in 50 mM dodecyltrimethylammonium bromide (as eluted from an affinity column [27]) was added a 0.5 mol ratio of phospholipid. This solution was dialyzed at 0°C against 5 mM Hepes buffer containing 0.025% azide for 3–4 days, and the precipitate collected by centrifugation for 10 min at $9770 \times g$. This precipitate was dissolved in 2–6 ml of 2-chloroethanol, and to the solution were added enough additional phospholipid and cholesterol (in a small volume of 2-chloroethanol) to make the final mol ratio 1.5 : 4 cholesterol/phospholipid and the lipid/protein weight ratio 1:1. The solution was shaken vigorously and transferred to a 500 ml round-bottom flask for solvent removal by rotary evaporation at 34°C. The resultant film was further dried overnight in a vacuum desiccator. Subsequent hydration with physiological saline at 37°C produced liposomes. Glass beads could be added to gently roll lipid from the walls. The subpopulation of liposomes large enough to sediment when centrifuged at $850 \times g$ for 5 min was used in these experiments.

Binding assay

The basic procedures used for measuring lectin binding to liposomes and cells have been described elsewhere [18,19]. Incubation time was 25 min for model membranes and 80 min for intact cells.

Results

The original affinity column procedure of Findlay [22] for isolating the concanavalin A-binding subpopulation of band 3 employed the detergent, Triton; but it is readily adapted to use of the dialyzable and ultraviolet-transparent species, dodecyltrimethylammonium bromide [27,28]. The

result is a relatively pure (detergent-containing) solution of the receptor, to which one may add various quantities of lipid. Subsequent removal of the detergent has been shown to lead to assembly of a lipid-protein complex with demonstrable lectin binding and anion transport function [27,28]. In the work described here, only small quantities of phospholipid were added back to the detergent solution for the purpose of producing a stable precipitable form of the concanavalin A receptor. For the actual binding studies it was desirable to have large, stable, multilamellar liposomes. The best method for preparing such liposomes seems to be hydration of lipid films previously produced by evaporation of organic solvent from a lipid solution [31]. Since both lipid and concanavalin A receptor were found to dissolve in 2-chloroethanol, this approach was applicable to our system. Low-speed differential centrifugation isolated a population of large, multilamellar liposomes which could be handled like intact cells.

Table I illustrates typical data obtained with radiolabelled concanavalin A receptor and lipids in an attempt to characterize the extent of lipid/protein association, and the stability of this association to repeated washing procedures. Receptor-bearing liposomes were made as described in Materials and Methods, but with the inclusion of tracer quantities of radiolabelled material. The liposomes were then subjected to repeated differential centrifugation with subsequent supernatant removal. Sample recovery in the pellet was monitored at each stage. Clearly, there is a substantial loss of structures not sedimentable at low g forces during the first washing, and this loss apparently is greater for those (less dense) lipid structures possessing relatively less protein, as expected. It is perhaps appropriate to emphasize at this point that variability of liposome size and receptor distribution, both within a given preparation and from one preparation to another, is an important limitation of these experiments. Note, however, that the liposomes are relatively stable to subsequent washings, even after many hours, and insensitive to the presence or absence of adsorbed macromolecules. Fig. 1 illustrates the appearance in the fluorescence microscope of receptor-bearing liposomes used in our experiments.

Fig. 2 typifies the lectin-binding behavior of model membranes bearing the concanavalin A receptor glycoprotein. Such membranes, when coated with a layer of serum albumin, accurately mimic

TABLE I

LIPOSOME STABILITY TO EXHAUSTIVE WASHING PROCEDURES

Receptor-bearing liposomes of dimyristoylphosphatidylcholine/cholesterol were prepared as described in Materials and Methods, but with [^{3}H]concanavalin A receptor and [^{14}C]phosphatidylcholine. Liposomes were washed in Ca^{2+}- and Mg^{2+}-containing phosphate-buffered saline by differential centrifugation (850× g for 5 min). BSA, bovine serum albumin.

	% concanavalin A receptor remaining after washing		% lipid remaining after washing	
	+BSA	−BSA	+BSA	−BSA
Sample prior to washing	100	100	100	100
1st wash	57	57	44	43
2nd wash	60	62	50	45
3rd wash	56	66	45	45
4th wash	55	58	37	39
After 6 h (5th wash)	45	50	40	34

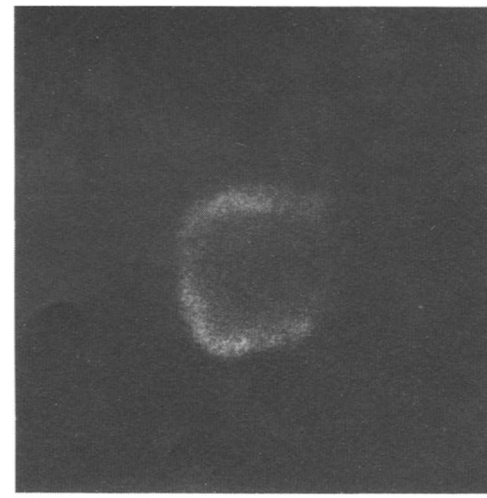

Fig. 1. Fluorescence micrograph of an albumin-coated, receptor-bearing liposome stained with rhodamine-labelled concanavalin A and extensively washed by differential centrifugation. The lipid was dimyristoylphosphatidylcholine/cholesterol. Photographed at 23°C on a Zeiss fluorescent microscope (×1600).

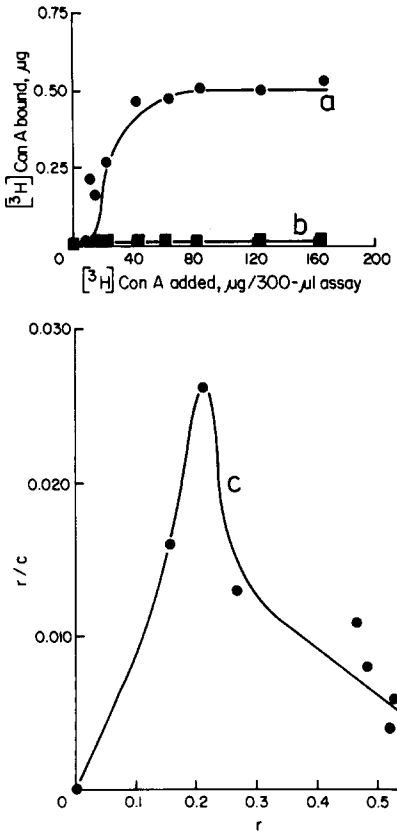

Fig. 2. High-affinity ^{3}H-labelled concanavalin A binding to liposomes of dimyristoylphosphatidylcholine/cholesterol bearing the concanavalin A receptor glycoprotein from human erythrocytes. Curve a is for liposomes exposed to serum albumin, and curve c is a Scatchard plot of the same data. The liposomes in curve b were not treated with serum albumin. r is the number of micrograms of bound lectin per assay tube (final volume 300 μl); c is the number of micrograms of free lectin. The calculated average number of high-affinity sites per liposome from curves a and c is $6 \cdot 10^5$ (see footnote, p. 198). Temperature of assay 23°C. Con A, concanavalin A.

the positive cooperative binding curves reported for intact cells exposed to various lectins [3–11]. The positive cooperative feature is emphasized by Scatchard treatment of the data (Fig. 2c). Note that in the absence of a surface coat of adsorbed albumin (Fig. 2b), the lectin binding is unstable to repeated washing procedures. Lectin binding was reversible at any stage by the inhibitory sugar, α-methyl-D-mannoside. Fig. 3 demonstrates that the striking dependence of high-affinity lectin binding on the presence of a surface coat of serum

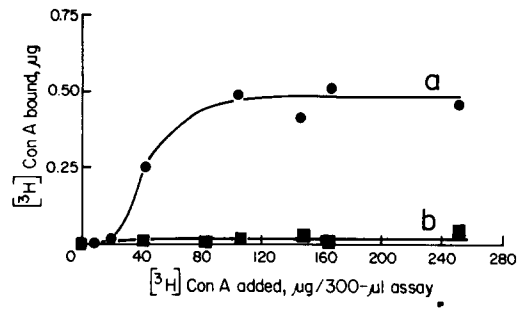

Fig. 3. High-affinity ^{3}H-labelled concanavalin A binding to receptor-bearing liposomes of dimyristoylphosphatidylcholine/cholesterol: (a) coated with Dextran T-500, and (b) uncoated. The average number of lectin-binding sites per liposome was $2 \cdot 10^6$ (see footnote, p. 198). Temperature of assay 23°C.

albumin is not peculiar to that material: dextran has a similar effect. Yet dextran (a high molecular weight neutral polysaccharide) and serum albumin have little in common other than their propensity for binding to lipid membranes.

The dimyristoylphosphatidylcholine/cholesterol lipid bilayers used for most of this work are fluid enough to permit gross glycoprotein redistribution on the time scale (25 min) of the experiments (Refs. 32 and 33 and personal observations). However, Fig. 4 displays data obtained with the more rigid lipid matrix, dipalmitoylphosphatidylcholine/cholesterol, in which rapid glycoprotein translational diffusion is greatly reduced. Fig. 4 is basically similar to Figs. 2 and 3, suggesting that

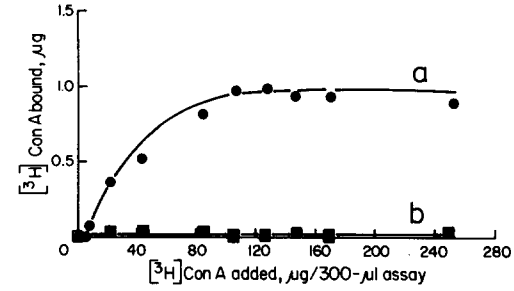

Fig. 4. High-affinity ^{3}H-labelled concanavalin A binding to receptor-bearing liposomes of dipalmitoylphosphatidylcholine/cholesterol: (a) coated with serum albumin, and (b) uncoated. The average number of lectin-binding sites per liposome was $1 \cdot 10^6$ (see footnote, p. 198). Temperature of assay 23°C.

gross lateral receptor redistribution is not an essential feature of the experiments described here. This is in agreement with our previous observations on the wheat germ agglutinin/glycophorin system [18,19].

Since the concanavalin A-binding population of band 3 is the only substantial receptor for that lectin in human erythrocytes, it seems reasonable to compare concanavalin A-binding curves for red blood cells with those obtained using our model membranes. Several such curves are shown in Fig. 5a and b. The same data appear in Scatchard analysis in Fig. 5c and d. In fact, these curves are reminiscent of those already described in Figs. 2–4

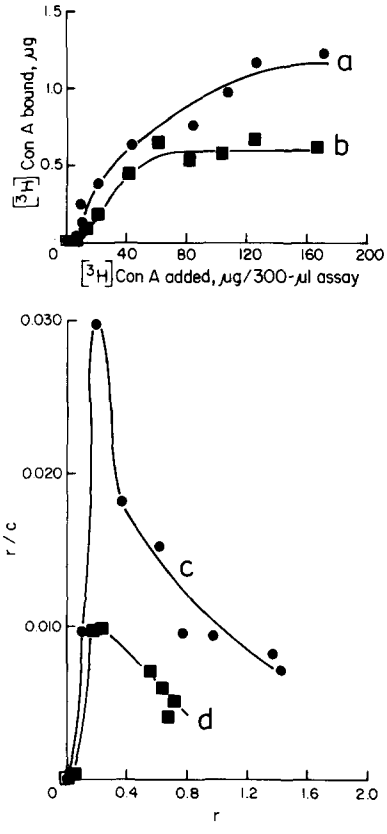

Fig. 5. High-affinity ^{3}H-labelled concanavalin A binding to human erythrocytes: (a) in the presence of serum albumin, and (b) in the absence of serum albumin. Curves c and d are obtained by Scatchard treatment of the data in curves a and b, respectively. r is the number of micrograms of bound concanavalin A per assay tube (final volume 300 μl); c is the number of micrograms of unbound concanavalin A. The numbers of lectin-binding sites per erythrocyte were calculated to be $4 \cdot 10^5$ (curves a and c) and $1 \cdot 10^5$ (curves b and d).

for lectin binding to model membranes. They clearly manifest a (positive) cooperative nature associated with a population of $1-4 \cdot 10^5$ sites per cell having a lectin affinity of about 10^6 M^{-1}. Previous authors have recorded sigmoidal lectin-binding curves [36] and downward-curved Scatchard plots [11] for red blood cells exposed to concanavalin A. Our values for number of sites and binding affinity are also in very good agreement with literature values from the same laboratories [11,34]. However, it has not been reported that lectin binding to cell membranes requires the presence of exogenous macromolecules such as albumin or dextran. Indeed, as Fig. 5b illustrates, we do not find such material to be necessary for high-affinity lectin binding to erythrocytes, although measurably more sites exist on erythrocytes in the presence of a layer of exogenously added serum albumin (Fig. 5a). In our work fresh human erythrocytes were drawn in citrate/saline and washed four times. In fact, though such washing may fail to remove as much as 50% of the original serum proteins bound by cells (Brooks, D., personal communication). Furthermore, cell membranes bear a substantial surface layer of material in the form of glycolipid and glycoprotein headgroups as well as peripheral proteins. Hence, it may not be surprising that the concanavalin A-binding curves obtained for intact erythrocytes are more or less independent of exogenously added material.

It must be stressed that the approx. 10^6 binding sites per liposome* described in this work are high-affinity sites. That is, although bound lectin molecules are readily displaced by inhibitory sugar, in the absence of such competition they remain bound in the face of extensive washing procedures lasting many minutes. However, Table II illustrates the fact that each liposome possesses much larger numbers of lower affinity sites. Such sites are only apparent in binding assays which do

* Average numbers of high-affinity binding sites per liposome or cell were calculated from Scatchard plot intercepts. In the case of liposomes, this number represents an average over a population differing greatly in size (e.g., 3–50 μm). K_a for lectin binding to the concanavalin A receptor in liposomes and intact cells (from the slope of the Scatchard plot descending limb) was in the range 10^5-10^6 M^{-1}.

TABLE II

HEMAGGLUTINATION INHIBITION USING STOCK LECTIN SOLUTIONS PREADSORBED WITH LIPOSOMES

Aliquots (50 μl) of the liposome suspensions used in our experiments ($2 \cdot 10^7$ liposomes per ml) were incubated with 150 μl of solutions of known concanavalin A concentration. These samples were centrifuged at $850 \times g$ prior to use of 50 μl of the supernatant material in a hemagglutination-inhibition assay. This approach avoided adding liposomes directly to microtiter wells. End points were recorded as the highest lectin concentration at which agglutination failed to occur. Inclusion of 3 mg albumin per ml did not alter the end points. Knowing the number of liposomes added to each stock solution of concanavalin A, one may calculate the amount of lectin bound per liposome (and thus removed from the stock solution). Liposomes with receptor bound $1.9 \cdot 10^7$ molecules of concanavalin A per liposome in the above experiment. This represents a conservative estimate of the number of available binding sites, but provides no information about the affinity of such sites

Sample	(μg) Concanavalin A originally added to stock solution corresponding to hemagglutination end point
No liposomes	0.03
Liposomes without receptor	0.47
Liposomes with concanavalin A receptor	3.75
Liposomes with concanavalin A receptor + 0.1 M α-methyl-D-mannoside [a]	0.03

[a] When the stock lectin solutions contained 0.1 M inhibitory sugar and were dialyzed subsequent to removal of liposomes, the hemagglutination test could be used to check specificity of binding. There is no binding to liposomes in the presence of inhibitory sugar.

not involve extensive washing, i.e., lectins bound to them have a dissociation rate which is fast on a time scale of minutes (as in fact do most noncovalent associations in solution). The numbers of these low-affinity sites can only be estimated from hemagglutination inhibition assays, but they do not appear to be strongly influenced by the presence or absence of albumin or dextran. From Table II it is apparent that there are more than 10^7 low-affinity sites per liposome, and also that these sites are largely 'specific' by the criterion of inhibitability by α-methyl-D-mannoside. Their counterpart on cell membranes is well known [35,36]. However, the significance of such low-affinity sites is unclear. Certainly, appreciable numbers of them are not related to saccharide receptors. For instance, various authors have pointed out that concanavalin A binds with low affinity to plastic, lipid, and glass – and this binding is sugar inhibitable (Refs. 35 and 36 and references therein). Table II demonstrates that, although liposomes of lipid alone bind lectin, considerably more low-affinity sites appear when the liposomes bear receptor glycoproteins. In our system, high-affinity binding sites only appear in liposomes bearing the appropriate receptor glycoprotein. Typically, it is the high-affinity binding sites which are considered to be instrumental in specific exogenous initiation of a cellular metabolic response, while the low-affinity sites are mentioned in connection with cell agglutinability [35]. The concanavalin A affinity for monosaccharides is only about 10^3 M^{-1} [1]. Lectins binding to a membrane surface with this affinity would be expected to be measured in agglutination assays or in assays which do not involve repeated washing. It seems reasonable to assume that the much higher affinity (10^6 M^{-1}) seen for a subpopulation of concanavalin A receptors in cell membranes arises from some form of polyvalent attachment [1].

Discussion

An important question that arises with regard to this work is, why should a surface layer of adsorbed macromolecules influence high-affinity lectin binding to a receptor glycoprotein? There is a considerable body of data in the literature recording the fact that molecules such as dextran and serum albumin adhere to cell membranes and lipid model membranes [12–15]. We have seen no resultant structural change at the level of light or electron microscopy in our bilayer systems. One might suggest that the important effect of the adsorbed layer is to make accessible oligosaccharide chains that were otherwise cryptic – perhaps by 'unclumping' tangled or collapsed headgroups. Our own measurements with spin-labelled glycoprotein headgroups [37], however, indicate that, if anything, a surface coat of adsorbed material reduces oligosaccharide free-

dom of motion. In any case, it seems likely from the hemagglutination studies that there is no lack of lectin-binding sites – only a lack of sites that can participate in high-affinity lectin attachment. One of several alternative possibilities is that the effect of serum albumin/dextran is to stabilize the lectin itself. If so, the requirement for stabilization of the lectin is only manifest at the membrane surface, since the same lectin preparations bind to intact cells in the absence of exogenous macromolecules. Our tentative explanation is a third possibility: that the function of the layer of adsorbed material is to influence somehow the opportunity for stable polydentate liganding. For instance, the adsorbed surface layer would be expected to reduce oscillatory motions of glycoprotein headgroups and/or of the lectin itself, which might improve the potential for long-term multidentate binding. Fig. 6 is included in an attempt to clarify these concepts: the features shown were taken from an article by one of us (C.W.M.G. in Ref, 38). The fact that adsorbed macromolecules

Fig. 6. Scale drawing of the concanavalin A receptor glycoprotein as it is thought to exist in the erythrocyte membrane. Most of the features of this figure have been described in detail by one of us (C.G.) previously [38]. Components shown and their ratio and arrangement correspond to literature descriptions of red cell membrane architecture (see references to coded insert). Where known, structural details were derived from CPK space-filling molecular models, while other species are shown only as globes of appropriate dimensions. The left-hand outer surface of the membrane section shown has been drawn with adsorbed serum albumin (oblong, 67000 molecular weight [44]) and IgG (T-shaped, 160000 molecular weight [45]). B3, band 3 [23,46] (One of the four shown has been blackened). G, glycophorin [46,47]. A, ankyrin [48]. AP and D, peripheral proteins such as alkaline phosphatase and glucose-6-phosphate dehydrogenase, respectively [46]. G1, glycolipid (ganglioside).

are capable of influencing the dynamic behavior of other surface structures has been demonstrated by Wolf et al. [39,40] and by McConnell and coworkers (personal communication) (see also Ref. 37).

If it is true that nonspecific macromolecular interactions at a model membrane/water interface can influence the nature of specific binding events, as seems to be the case in this study; then similar effects may well occur at cell surfaces. There is certainly no lack of opportunity for receptor interaction with local structures at the cell surface: oligosaccharide headgroups, peripheral proteins and adsorbed material contribute to a layer hundreds of angstroms thick. It has been reported, for instance, that insulin-binding affinity is a function of receptor mobility in human lymphocytes [41].

Positive cooperativity in binding events arises classically from induced exposure of new sites, or from induced affinity increases in preexisting sites. In most cells there exists the potential for active participation in this process via down regulation of receptors (negative cooperativity), generation of new receptors, or cytoskeletal rearrangement of existing receptors. None of these possibilities exist in our model membranes; however, the receptors are capable of lectin-dependent spontaneous redistribution and reorientation. In this regard, one would anticipate both receptor clustering (equivalent to patching in cells) and headgroup immobilization, subsequent to lectin binding. As pointed out in Results, our model membrane binding curves do not seem to depend strongly upon gross receptor redistribution over large distances. This also seems to be the case for intact mature human erythrocytes [34,42], although local rearrangements certainly must occur, if only involving headgroup cross-linking and reorientation (see Fig. 6). That creation of a higher local density of receptors (through clustering or conformational transition) may lead to positive cooperativity in lectin binding has been suggested by others [17,43]. This phenomenon was particularly clearly demonstrated in the model studies of Jennissen [16]. Another possible source of positive cooperativity in the model membranes described here would be lectin-induced exposure of new binding sites. This is much more difficult to envisage, however, than favorable rearrangement of existing ones. In any case, it seems clear that at least some membrane receptor glycoproteins are capable of cooperative behavior independent of the involvement of other cellular machinery.

Acknowledgements

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