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Interaction of human erythrocyte Band 3 with *Ricinus communis* agglutinin and other lectins

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Agglutination and competition studies suggest that human erythrocyte Band 3 can interact with both mannose/glucose- and galactose-specific lectins. Purified Band 3 reconstituted into lipid vesicles binds concanavalin A, but the nonspecific binding component, measured in the presence of α-methylmannoside, is very high. This glycoprotein also carries binding sites for the galactose-specific lectin *Ricinus communis* agglutinin. Binding was inhibited poorly by lactose, but much more effectively by desialylated fetuin glycopeptides, suggesting that the lectin recognizes a complex oligosaccharide sequence on Band 3. The glycoprotein bears two separate classes of binding sites for *R. communis* agglutinin. High-affinity binding sites exist which show strong positive cooperativity and correspond in number to the outward-facing Band 3 molecules. A low-affinity binding mode is abolished by 40% ethyleneglycol, suggesting the involvement of hydrophobic lectin-glycoprotein interactions. Studies on binding of *R. communis* agglutinin to human erythrocytes indicate positively cooperative binding to $7 \cdot 10^5$ very-high-affinity sites per cell, and lectin binding is completely inhibitable by lactose. Based on its binding characteristics in vesicles, it seems likely that Band 3 forms the major receptor for this lectin in human erythrocytes. Properties such as positive cooperativity thus appear to be a common feature of the interaction of Band 3 with a variety of lectins of different specificity, both in erythrocytes and lipid bilayers.

Introduction

Cell surface glycoproteins are known to play an important role in the transmission of signals across the cell membrane, and in the regulation of many important cellular functions such as cell growth, differentiation and intercellular recognition [1,2]. Plant lectins have emerged as uniquely useful tools in the study of cell surface glycoconjugates [3,4] and have been used both to quantify the number and affinity of binding sites (via radiolabel tags) and to study the three-dimensional arrangement of glycoprotein receptors at the cell surface (via fluorescent or electron-dense tags). The characteristics of lectin binding to glycoprotein receptors, particularly features such as positive cooperativity, may be important in determining cellular responses [5–7]. A serious drawback in lectin-binding studies on intact cells is that in most cases little is known about the molecular nature and complex carbohydrate structure of cell surface lectin receptors. Different lectins may bind to the same or different

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Abbreviations: PC, phosphatidylcholine; PS, phosphatidylserine; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; RCA 120, *Ricinus communis* agglutinin (120 kDa); RCA 60, *Ricinus communis* lectin (60 kDa).

groups of glycoproteins (or glycolipids) and whether the binding characteristics of a given receptor vary depending on the particular lectin bound is not clear. It has thus been very difficult to relate the molecular properties of a lectin receptor glycoprotein to its binding characteristics as seen in cell surface labelling studies. These problems have resulted in only limited information being produced from lectin-binding studies on intact cells.

We, and others, have demonstrated that model systems which employ well-characterized reconstituted glycoproteins can effectively mimic many of the lectin-binding characteristics associated with the receptors in intact cells [8–12]. Thus, reconstituted lipid bilayer systems, which are well-defined and easily manipulated by the experimenter, may provide important information on the relationship between molecular behavior and macroscopic binding characteristics. Previous studies in our laboratory have focussed on the concanavalin A receptor fraction of Band 3, the major protein of the human erythrocyte membrane. This 95 kDa protein is the carrier responsible for anion transport across the red cell membrane and has been widely used in recent years as a model transmembrane glycoprotein. The polypeptide backbone has been shown to contain at least five membrane-spanning segments [13] and the structure of the single complex *N*-linked oligosaccharide chain has been partially elucidated [14,15]. Some 20% of the Band 3 population is known to carry effective binding sites for the lectin concanavalin A [16,17], and we have previously explored the interaction of this Band 3 subpopulation with succinyl-concanavalin A in lipid bilayers [9].

The possibility of other lectins binding to this glycoprotein has not been investigated and we feel that this model system provides a unique opportunity to answer some of the current questions in lectin-glycoprotein interactions. Our objectives include mapping the multiple lectin-binding specificities of human erythrocyte Band 3, and comparing the binding characteristics of lectins with different saccharide specificity to this particular receptor. Our results demonstrate that, in addition to D-mannose/D-glucose-specific lectins such as concanavalin A, Band 3 is also capable of binding D-galactose-specific lectins such as RCA 120, and may form the major receptor for this lectin in the intact erythrocyte. We also show that lectins can interact with Band 3 in a variety of different ways, but properties such as positive cooperativity appear to be a common feature in the interaction of lectins with this glycoprotein.

Materials and Methods

Egg PC and PS (bovine brain) were obtained from Sigma and were used without further purification (purity > 98–99%). Both phospholipids were pure as judged by thin-layer chromatography on Silica gel G. Fetuin from fetal calf serum (Sigma) was desialylated by acid hydrolysis and the glycopeptides prepared using exhaustive papain digestion [18,19]. The protein concentration of fetuin glycopeptide solutions was determined using a Biuret assay [20]. The galactose content of the fetuin glycopeptide solutions was calculated from the measured protein concentrations and the carbohydrate and protein composition data given in Refs. 18 and 19. D-Galactose, *N*-acetyl-D-galactosamine, lactose and α-methyl-D-mannoside were obtained from Sigma.

Human erythrocyte ghosts were prepared from outdated blood bank red cells by the method of Dodge et al. [21]. The concanavalin A receptor fraction of band 3 was isolated from human erythrocyte ghosts by affinity chromatography on Con A-Sepharose 4B (Pharmacia) as previously described [9].

Reconstitution of Band 3 glycoprotein. The human erythrocyte Band 3 glycoprotein was reassembled into large unilamellar phospholipid vesicles using the detergent-dialysis technique of Chicken and Sharom [9]. The glycoprotein in 25 mM dodecyltrimethylammonium bromide solution was added to the appropriate phospholipid mixture solubilized in 200 mM of the same detergent, all in 5 mM Hepes-buffered saline (pH 7.4). The lipid/protein mixture was dialyzed exhaustively against 5 mM Hepes-buffered saline (pH 7.4) for 48 h at 4°C. The reconstituted vesicles were harvested by ultracentrifugation and resuspended in Hepes-buffered saline (pH 7.4) at a phospholipid concentration of 5–10 mg/ml. Phospholipid recoveries were quantitated by inclusion of tracer quantities (0.025 μCi/mg phospholipid) of di[1-

[^{14}C]palmitoyl-L-α-phosphatidylcholine (Amersham, specific activity 80–120 mCi/mmol) in the phospholipid mixture, followed by liquid scintillation counting of the final vesicle suspension. Protein content of the vesicles was determined by the method of Peterson [22]. Reconstituted vesicles were stored frozen in small aliquots and those used for binding studies were thawed only once.

Lectins. Phaseolus vulgaris hemagglutinin, wheat germ agglutinin, soybean agglutinin, concanavalin A, pea lectin, lentil lectin and RCA 60 were obtained from Sigma. RCA 120 and peanut agglutinin were purchased from Beohringer-Mannheim. Succinyl-concanavalin A was supplied by Vector Laboratories. Concanavalin A, succinyl-concanavalin A and RCA 120 were radioiodinated using Na^{125}I (carrier-free, Amersham), and 1,3,4,6-tetrachloro-3α,6α-diphenylglycoluril ('Iodogen', Pierce) as previously described [9]. Unreacted ^{125}I was removed from concanavalin A by the method of Phillips et al. [23], and from succinyl-concanavalin A and RCA 120 by two successive gel filtration steps on Bio-Gel P-10 (Bio-Rad). Final specific activities of ^{125}I-labelled lectins were in the range $9 \cdot 10^4$–$3 \cdot 10^5$ cpm/μg.

Agglutination of reconstituted vesicles by lectins. Agglutination tests were carried out on plain glass microscope slides at room temperature, using the general procedure outlined earlier [8]. Reconstituted lipid vesicles containing approx. 50 μg of lipid (2:1, w/w lipid/protein) were incubated with either 100 μg/ml of the appropriate lectin in phosphate-buffered saline (pH 7.4), or in buffer alone as a control. The slide was incubated at room temperature for several minutes with occasional agitation. Vesicles containing Band 3 were agglutinated rapidly (less than 5 min) by the lectin if the test was positive.

Binding of lectins to reconstituted Band 3. Binding of ^{125}I-labelled lectins to human erythrocyte Band 3 reassembled into large unilamellar vesicles was quantitated by rapid filtration on fibre-glass filters. Reconstituted vesicles containing 160 μg of phospholipid (lipid/protein ratio 4:1, w/w) were incubated in phosphate-buffered saline (pH 7.4) with the appropriate concentration of ^{125}I-labelled lectin for 1 h at room temperature. Vesicles with bound lectin were harvested by vacuum filtration on 13 mm fiber-glass filters as previously described [9]. All binding assays were carried out in duplicate and the required concentration of the appropriate sugar inhibitor was included where necessary. Lectin binding to vesicles was corrected for the amount bound to the filters, which was at most a few percent of the total lectin bound.

Binding of lectins to human erythrocytes. Binding of ^{125}I-labelled lectins to intact human erythrocytes was quantitated using a modification of the procedure of Okada [24]. Human erythrocytes were washed three times with isotonic phosphate buffer (pH 7.4) and resuspended in phosphate-buffered saline (pH 7.4) at a concentration of $(8–10) \cdot 10^8$ cells/ml. 50 μl of cell suspension (approx. $5 \cdot 10^7$ cells) was incubated with the appropriate concentration of ^{125}I-labelled lectin in a total volume of 250 μl. Following 1 h incubation at room temperature, cell-bound and free lectin were separated by differential centrifugation through a dibutylphthalate cushion. Tube bottoms were sliced-off and the red cell pellet was counted in a Beckman 5500 gamma counter. Nonspecific binding was measured in the presence of 0.1 M of the appropriate sugar inhibitor.

Results and Discussion

Agglutination and competition studies

The interaction of lectins with reconstituted Band 3 vesicles can be assessed from a qualitative point of view using a simple agglutination slide test, as described in earlier work on reconstituted glycoproteins [8]. This type of test is based on the fact that large lipid vesicles bearing glycoprotein receptors can be visibly agglutinated by lectins or antibodies of appropriate specificity, much like erythrocytes in blood-typing tests. Table I shows that Band 3-bearing vesicles are agglutinable by lentil and pea lectins, which have a similar sugar specificity to concanavalin A. RCA 120 also causes dramatic agglutination, demonstrating that the concanavalin A receptor fraction of Band 3 is able to bind galactose-specific lectins. Agglutination was reversible by the appropriate sugar inhibitor in each case. Band 3 thus appears to bear receptor sites for both mannose/glucose-specific and galactose-specific lectins. The lectins from soybean, wheat germ and *P. vulgaris* do not produce vesicle

TABLE I

AGGLUTINATION OF VESICLES CONTAINING RECONSTITUTED BAND 3 BY VARIOUS LECTINS

Reconstituted vesicles containing Band 3 (lipid/protein ratio = 2:1, lipid used was egg PC) were tested with 100 μg/ml of each lectin in phosphate-buffered saline (pH 7.4), using a simple slide test. Agglutination was reversible by addition of the appropriate sugar inhibitor.

Lectin	Sugar specificity	Valence	Agglutination [b]
Concanavalin A	α-D-Man/α-D-Glc	4	+
Succinyl-concanavalin A	α-D-Man/α-D-Glc	2	−
Lentil	α-D-Man/α-D-Glc	2	+
Pea	α-D-Man/α-D-Glc	2	+
RCA 120	D-Gal	2 [a]	+
RCA 60	D-Gal/D-GalNAc	1 [a]	−
Peanut	β-D-Gal/β-D-Gal-(1-3)-D-GalNAc	4	+
Soybean	D-GalNAc	4	−
Wheat germ agglutinin	β-D-GlcNAc/Neu*N*Ac	4	−
P. vulgaris	complex [c]	4	−

[a] Some controversy exists as to the valence of these lectins, with a recent report indicating that RCA 120 and RCA 60 have four and two sugar-binding sites, respectively [25].

[b] + indicates agglutinable, − indicates nonagglutinable.

[c] See Ref. 26.

agglutination, indicating that Band 3 does not carry receptor sites for these lectins. Divalent succinyl-concanavalin A and monovalent RCA 60 do not cause agglutination of Band 3 vesicles, demonstrating the importance of lectin valence in forming stable vesicle-vesicle crosslinks.

Studies on competition between lectins should be a useful means of obtaining information on binding specificities and association constants, and to establish the identity of receptor populations. To date, however, only a few studies have examined competition between lectins for shared binding sites on the membrane surface, and these have been concerned with mapping receptor populations in intact cells [27,28]. We have previously shown that reconstituted Band 3 vesicles bind succinyl-concanavalin A with high affinity ($K_a = 2 \cdot 10^6\ M^{-1}$) and we have fully characterized this binding [9], thus providing a useful model system for competition studies. As shown in Fig. 1, several lectins inhibit binding of ^{125}I-labelled succinyl-concanavalin A to Band 3 in phospholipid bilayers. Concanavalin A is the most potent inhibitor, followed by succinyl-concanavalin A itself, while lectins such as pea and peanut are much poorer competitors for succinyl-concanavalin A binding sites on Band 3. Lineweaver-Burk (dou-

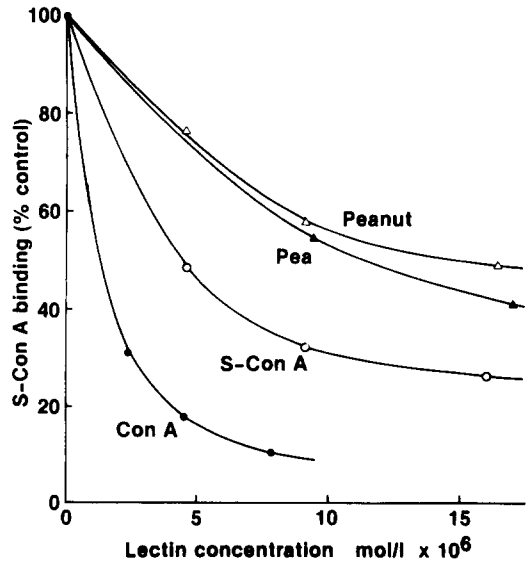

Fig. 1. Inhibition of ^{125}I-labelled succinyl-concanavalin A (S-Con A) binding to reconstituted Band 3 by increasing concentrations of other lectins. Vesicles containing 15 μg of Band 3 (4:1, w/w lipid/protein ratio) were incubated with 50 μg/ml ^{125}I-labelled succinyl-concanavalin A and varying concentrations of unlabelled lectins at 22°C. ●———●, concanavalin A (Con A); ○———○, succinyl-concanavalin A; ▲———▲, pea lectin; △———△, peanut agglutinin.

ble-reciprocal) or Woolf plots may be used to estimate association constants for binding of the various lectins, using concentrations in the range demonstrated to give a linear response on both Scatchard and double-reciprocal plots (previously shown for concanavalin A, see Ref. 9) and the results are shown in Table II. Clearly, the Band 3 subpopulation under study provides binding sites with a variety of affinities, even for lectins with similar saccharide specificities.

Competition studies such as these can be impossible to interpret when the competing lectin is a glycoprotein which cross-reacts with the first lectin. For example, in our hands the addition of either unlabelled soybean agglutinin or RCA 120 to reconstituted vesicles leads to dramatically enhanced binding (3–9-fold) of ^{125}I-labelled succinyl-concanavalin A to the receptor, presumably due to binding of succinyl-concanavalin A to mannose residues on these lectins (see Ref. 4 for complete data on lectin structural features). A similar phenomenon has been observed in lectin competition studies using neuroblastoma cells [27] and places obvious restrictions on lectin pairs suitable for this type of experiment. Inhibition of succinyl-concanavalin A binding to reconstituted Band 3 vesicles by lentil lectin and wheat germ agglutinin could not be assessed due to a nonspecific interaction of the mixed lectins with the fiber-glass filters used in the binding assay.

Interaction of concanavalin A with Band 3 in lipid bilayers

Previous work in our laboratory has focussed on binding of succinyl-concanavalin A to reconstituted Band 3, since initial investigation showed that concanavalin A itself had a very high 'nonspecific' binding component. Its succinylated derivative, however, gave nonspecific binding (measured in the presence of 0.1 M α-methylmannoside) of less than 10% of the total lectin bound [9]. Binding of concanavalin A to reconstituted vesicles is only about 25% inhibitable by 0.1 M α-methylmannoside and is not readily saturable at high lectin concentrations (Fig. 2A). Direct measurement of concanavalin A binding to reconstituted Band 3 is thus complicated by these factors, as well as the additional problem that the curve shown in Fig. 2A represents lectin binding to both phospholipids and glycoprotein receptors. Concanavalin A is known to bind to phospholipid bilayers [29,30] as well as to glass and plastic surfaces [31]. Because of these complicating factors, the association constant for concanavalin A binding to Band 3 in vesicles can only be determined indirectly, by competition studies, as described above.

Higher concentrations of sugar inhibitor result in only slightly increased levels of inhibition, reaching a maximum of less than 50% at 0.5 M sugar (Fig. 2B). This high level of 'nonspecific' binding is not seen for binding of concanavalin A to

TABLE II

EFFECTS OF VARIOUS LECTINS ON SUCCINYL-CONCANAVALIN A BINDING TO RECONSTITUTED BAND 3

Binding of ^{125}I-labelled succinyl-concanavalin A to reconstituted vesicles containing Band 3 (lipid/protein ratio (w/w) = 4:1, lipid composition = 4:1, w/w egg PC/PS) was measured in the presence of 150–400 μg/ml of various unlabelled lectins. Association constants for binding of these lectins to Band 3 were estimated from Lineweaver-Burk (double-reciprocal) and Woolf plots of the inhibition data in the lectin concentration range 100–500 μg/ml where a linear response was previously demonstrated (see Ref. 9).

Lectin	Sugar specificity	Binding [a]	Association constant K_a (M^{-1})
Concanavalin A	α-D-Man/α-D-Glc	↓	$5 \cdot 10^6$
Succinyl-concanavalin A	α-D-Man/α-D-Glc	↓	$2 \cdot 10^6$
Pea	α-D-Man/α-D-Glc	↓	$5 \cdot 10^5$
Peanut	β-D-Gal/β-D-Gal-(1–3)-D-GalNAc	↓	$2.5 \cdot 10^5$
RCA 120	D-Gal	↑ [b]	–
Soybean	D-GalNAc	↑ [b]	–

[a] ↓ indicates binding of succinyl-concanavalin A was reduced by the presence of the other lectin, ↑ indicates binding was increased.
[b] These lectins are mannose-containing glycoproteins and themselves bind to succinyl-concanavalin A.

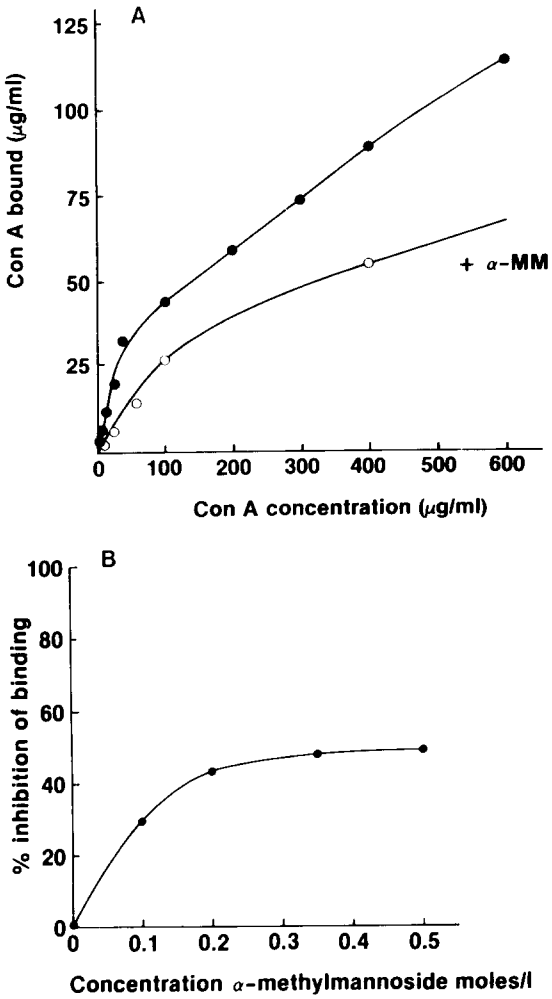

Fig. 2. Binding of ^{125}I-labelled concanavalin A (Con A) to reconstituted Band 3 vesicles (4:1, w/w lipid/protein ratio). (A) Binding curve measured in the absence (●———●) and presence (○———○) of 0.1 M α-methyl-D-mannoside. (B) Inhibition of concanavalin A binding by increasing concentrations of the sugar inhibitor α-methyl-D-mannoside. Lectin concentration was 450 μg/ml and binding was measured at 22°C.

lymphocyte plasma membrane glycoproteins reconstituted in the same fashion (Ref. 10 and Sharom, F.J., unpublished data) and thus seems to be dependent on the molecular characteristics of the membrane protein itself. It has recently been suggested that hydrophobic interactions may play an important role in the binding of concanavalin A to large complex glycoproteins [32]. It seems likely that the nonspecific binding seen here may be hydrophobic in nature, and perhaps occurs through interactions of the hydrophobic binding site of concanavalin A with nonpolar domains in the peptide backbone of the receptor protein. This high nonspecific contribution to binding is not seen in studies of concanavalin A binding to intact human erythrocytes, where more than 95% of the bound lectin is displaced in the presence of 0.1 M α-methylmannoside (our personal observations). This suggests that certain Band 3 peptide domains may be 'protected' from concanavalin A in the intact erythrocyte, perhaps via self-association (dimerization) or interaction with other membrane glycoproteins such as glycophorin. Since Band 3 is reconstituted symmetrically in unilamellar vesicles, it is also possible that the lectin is capable of interacting with cytoplasmic portions of the glycoprotein which are normally cryptic in the intact cell.

Interaction of RCA 120 with Band 3 in lipid bilayers

Both the agglutination and competition studies described above indicate that the concanavalin A receptor subfraction of Band 3 is capable of interacting with galactose-specific lectins such as RCA 120 and peanut agglutinin. Partial sequencing of the carbohydrate headgroup of Band 3 has revealed that it possesses several terminal galactose residues which could potentially be available for lectin binding at the cell surface [14,15]. Characterization of the interaction between the glycoprotein and a galactose-specific lectin was thus carried out, to allow comparison with the well-studied interaction with concanavalin A and its derivatives. Binding of ^{125}I-labelled RCA 120 to reconstituted Band 3 vesicles was quantitated using a rapid filtration assay, and at a lectin concentration of 160 μg/ml, 0.1 M lactose gives very poor inhibition of binding (less than 30%). Indeed, as shown in Fig. 3A, lactose concentrations as high as 0.5 M show only 75% inhibition of binding for RCA 120. Macroscopic agglutination of Band-3-containing vesicles by RCA 120 (Table I) was, however, reversible by lactose, suggesting that only the 'specific' portion of binding leads to agglutination, while nonspecific binding does not. Desialylated fetuin glycopeptide, a known inhibitor of RCA 120 binding to human erythrocyte receptors [33], is a much more potent inhibitor

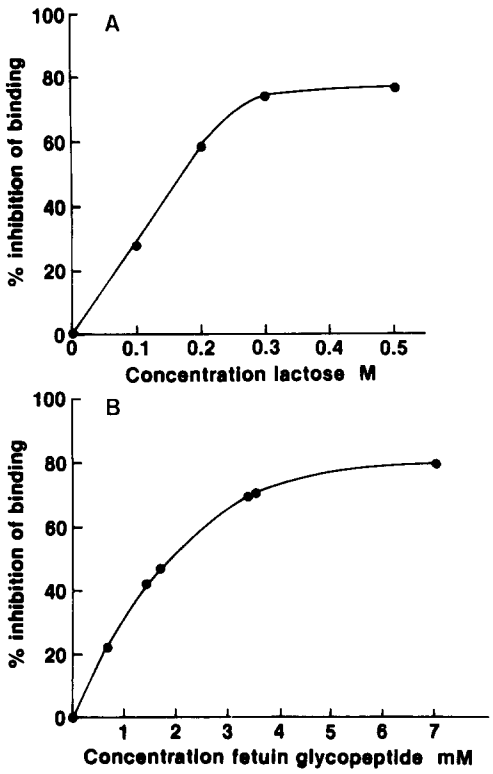

Fig. 3. Inhibition of ^{125}I-labelled RCA 120 binding to reconstituted Band 3 by increasing concentrations of lactose (A) and desialylated fetuin glycopeptides (B). Glycopeptide concentration is normalized for galactose content. Lectin concentration was 160 μg/ml and binding was measured at 22°C.

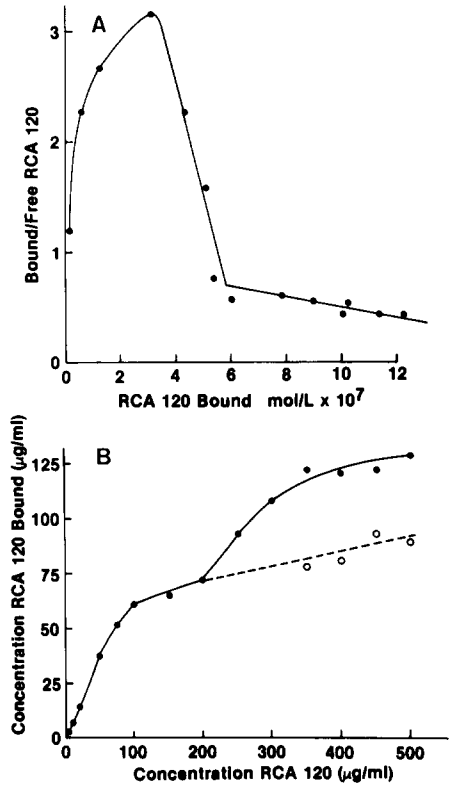

Fig. 4. Binding of ^{125}I-labelled RCA 120 to reconstituted Band 3. Vesicles containing 15 μg of Band 3 (4:1, w/w lipid/protein ratio) were incubated with varying concentrations of ^{125}I-labelled RCA 120 at 22°C, in the absence or presence of 40% (v/v) ethyleneglycol. (A) Scatchard plot of the binding data in the absence of ethyleneglycol. (B) Binding curves determined in the absence (●——●) and presence (○——○) of ethyleneglycol.

with 50% inhibition being achieved at a concentration of approx. 2 mM, normalized for galactose content (Fig. 3B). The sugar sequences of the major and minor glycopeptides of desialylated fetuin (*N*-linked via asparagine, Ref. 19 and *O*-linked via serine or threonine, Ref. 34) greatly resemble the terminal oligosaccharides of Band 3 [14,15]. The inhibition data thus suggests that RCA 120 might recognize an extended carbohydrate sequence on glycoprotein oligosaccharide chains rather than a single sugar residue, a suggestion also put forward by others [4,35].

Fig. 4B shows the binding curve determined for interaction of ^{125}I-labelled RCA 120 with reconstituted Band 3, and clearly demonstrates that two distinct types of binding site are present. A high-affinity binding mode exists, which is saturable at around 100–200 μg/ml lectin, and a second much-lower-affinity nonsaturable mode becomes evident only at high lectin concentrations. The high-affinity contribution to binding shows strong positive cooperativity at low lectin concentrations, as shown by the downwardly concave nature of the Scatchard plot in Fig. 4A. The association constant for binding determined from the Scatchard plot is very high ($K_a = 1.1 \cdot 10^7$ M^{-1}) and the maximum number of high-affinity sites determined from the binding data equals the number of outward-facing Band 3 molecules, implying that each glycoprotein is capable of binding one molecule of lectin. Thus, reconstituted Band 3 provides very-high-affinity binding sites for RCA 120 in lipid bilayers, and the characteristics of binding are very similar to those seen for both

succinyl-concanavalin A binding to this same receptor in lipid bilayers and concanavalin A binding to Band 3 in human erythrocytes. It thus appears that positive cooperativity is related to the molecular nature and surface arrangement of the glycoprotein in the bilayer, rather than the properties of the particular lectin under study. These observations support our previous suggestion that clustering of this particular glycoprotein in the bilayer may be responsible for positively cooperative lectin binding [36].

The much lower affinity binding ($K_a = 4.5 \cdot 10^5$ M^{-1}) can be almost completely abolished by addition of 40%, v/v ethyleneglycol, suggesting that hydrophobic forces might be involved in this type of interaction. This low-affinity binding does not represent binding of RCA 120 to the lipid bilayer itself. Binding experiments carried out with vesicles composed of phospholipid alone showed that only about 4 μg RCA 120/ml incubation mixture is bound to lipid at a lectin concentration of 400 μg/ml, whereas the low-affinity contribution to binding results in the binding of about 40 μg lectin/ml incubation mixture at this concentration (See Fig. 4B). This observed low affinity of RCA 120 for lipid bilayers is in agreement with previous reports in the literature [37,38]. The low-affinity binding observed with our reconstituted system probably represents hydrophobic binding of RCA 120 to another site on the Band 3 molecule, perhaps a hydrophobic peptide domain. Thus, our observations on this particular reconstituted system suggest that lectins may interact with cell surfaces in a variety of different ways. Possible membrane associations include high-affinity 'specific' receptor-mediated binding and lower-affinity hydrophobic binding to both nonglycosylated domains of membrane proteins and phospholipids.

The nature of the receptor for RCA 120 in human erythrocytes

Binding studies on reconstituted Band 3 described above indicate that the glycoprotein binds RCA 120 with very high affinity, suggesting that it may form the major receptor for this lectin in the intact human erythrocyte. To investigate this possibility, we have quantitated binding of ^{125}I-labelled RCA 120 to intact human red cells and the resulting data are shown in Fig. 5. The binding was of very high affinity ($K_a = 1.9 \cdot 10^7$ M^{-1}) and in contrast to the situation seen in reconstituted vesicles, was more than 98% inhibitable by the addition of 0.1 M lactose. The value of the association constant that we have measured agrees with previous reports [33,39] and is very similar to that found for binding of the related lectin RCA 60 to human erythrocytes [35]. A Scatchard plot of the binding data is concave downwards at low lectin concentrations (Fig. 6), indicating positive cooperativity, and the number of lectin-binding sites per cell can be estimated to be $7 \cdot 10^5$. Adair and Kornfeld [33] reported $7 \cdot 10^5$ high-affinity sites for RCA 120 binding to erythrocytes and $5 \cdot 10^5$ additional much-lower-affinity sites and another group reported $1 \cdot 10^6$ total sites, again with evidence of two classes of receptor [35]. The reason for these discrepancies is not clear, although loss of terminal sialic acid from glycophorin exposes galactose residues which can bind RCA 120 (see below), and this phenomenon could account for the second lower-affinity class of receptors observed by others. It is also possible that the lectins used in these previous studies, which were labelled using chloramine T, had a modified sugar specificity or biological activity due to protein damage. The labelling method we have used for lectin radioiodination is known to produce minimal oxidation damage relative to other techniques [40].

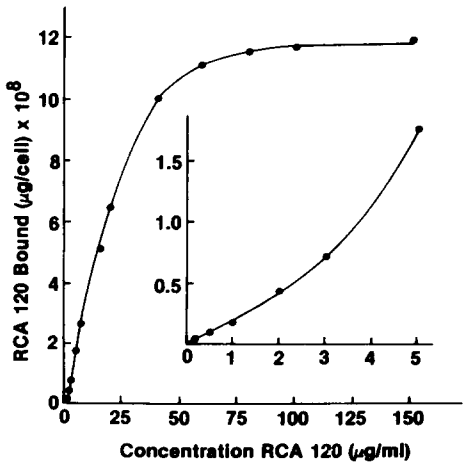

Fig. 5. Binding of ^{125}I-labelled RCA 120 to intact human erythrocytes at 22°C. Inset shows binding measured at very low lectin concentrations.

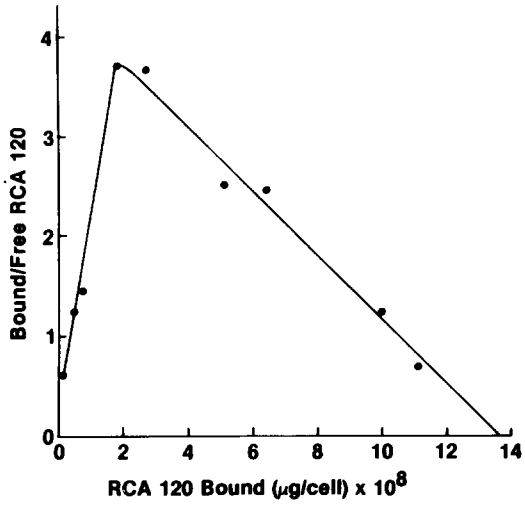

Fig. 6. Scatchard plot of the ^{125}I-labelled RCA 120 binding data shown in Fig. 5.

The molecular identity of the RCA 120 receptor on the human erythrocyte membrane is still unclear. Band 3 possesses several terminal galactose residues which may be available for lectin binding, and RCA 120 affinity columns have been used to isolate both Band-3-derived oligosaccharides [14,41] and a glycoprotein fraction from erythrocytes thought to be Band 3 itself [33]. The number of Band 3 molecules per cell has been estimated to be approx. $1.2 \cdot 10^6$ [42], so there would seem to be an adequate number of copies of this glycoprotein to act as receptors for RCA 120. Another possibility for the erythrocyte RCA 120 receptor is the major sialoglycoprotein glycophorin. There is some evidence that RCA 120 can interact with glycophorin-derived glycopeptides [43] and liposomes containing glycophorin have been reported to be agglutinated by the lectin [44]. However, other workers have provided convincing evidence that native glycophorin binds RCA 120 weakly or not at all, although removal of its terminal sialic acid exposes large numbers of galactose residues which can interact with this lectin [33,45]. In addition, the number of glycophorin copies per erythrocyte ($5 \cdot 10^5$, see Ref. 46) is substantially lower than the number of lectin-binding sites, thus even if glycophorin is able to bind some RCA 120 in the intact cell (perhaps through desialylation of some oligosaccharide chains), it cannot be the major receptor. Consideration of all the available evidence suggests that glycophorin in its native state may carry a small number of low-affinity sites for RCA 120, but the similarity in binding characteristics observed for interaction of the lectin with reconstituted Band 3 and intact erythrocytes (very high K_a, positively cooperative) supports the view that this glycoprotein forms the major receptor in the human erythrocyte. The number of receptors per erythrocyte that we have determined, relative to the known estimates of Band 3 numbers, suggests that each Band 3 dimer can bind one molecule of lectin in the intact cell. Since reconstituted Band 3 can bind RCA 120 in a stoichiometric fashion, steric or other constraints at the erythrocyte membrane surface may be responsible for the difference in lectin/receptor ratio between the intact cell and our model systems.

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