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Clustering and endocytosis of membrane receptors can be induced in mature erythrocytes of neonatal but not adult humans

(membrane protein mobility/ferritin-lectin conjugates/succinyl concanavalin A)

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ABSTRACT Concanavalin A (Con A) is taken up by endocytosis in mature erythrocytes of newborn humans but not in adult red cells. Thin sections of neonatal cells incubated with ferritin-conjugated Con A at 37° show ferritin clusters on invaginations at the surface and in intracellular vesicles, but such invaginations and vesicles are absent with adult cells. The endocytosis induced by ferritin-conjugated Con A is inhibited at 0°, and by methyl-α-D-mannopyranoside at 37°. Succinylation of Con A, which is known to convert it from the tetrameric to dimeric form, renders Con A inactive in cell agglutination and endocytotic vesicle formation, presumably by reducing the number of oligosaccharide chains simultaneously bound by a single Con A molecule. Ferritin-conjugated succinyl Con A binds to neonatal erythrocytes but does not induce endocytosis; if, however, antibodies to ferritin are now added, endocytosis occurs. These results are consistent with a greater lateral mobility of at least a fraction of Con A receptors in the membrane of the intact neonatal erythrocyte compared to the adult. The results also support the hypothesis that the clustering of receptors is obligatory for endocytosis to occur. No discernible difference was found in the sodium dodecyl sulfate/polyacrylamide gel patterns of the membrane proteins of the neonatal and adult cells.

The current generally accepted view of the structure of biological membranes is of a two-dimensional fluid solution of integral proteins embedded in a lipid bilayer (the fluid mosaic model, ref. 1). Rapid and extensive lateral mobility of the protein constituents of many membranes has been demonstrated (2). In particular, the binding of a multivalent ligand (such as an antibody or a lectin) to specific receptors in a cell membrane often leads to a lateral clustering followed by an endocytosis of the ligand-bound receptors (3, 4). It has been appreciated for some time (4, 5), however, that the membrane of the intact erythrocyte of the adult human is atypical in this respect. Antibodies and lectins bind to receptors on the erythrocyte surface, but do not normally induce any redistributions or endocytosis of the receptors. On the other hand, there is a report (6) that the intact mature erythrocyte of the *newborn* human does show ferritin-lined endocytotic vesicles when ferritin-labeled antibodies directed to the blood group A antigen are bound to the cell surface. This observation, made before the fluidity of membranes was appreciated, suggested that the mobility of receptors in the membrane of the neonatal erythrocyte might be significantly greater than in the corresponding adult cells (7). Aside from its medical interest, this possibility seemed of great potential relevance to at least two general and fundamental problems in cell biology: (*i*) the molecular mechanisms that restrict the lateral mobility of membrane components; and (*ii*) the molecular mechanisms involved in endocytosis. We have therefore investigated the interactions of several different ligands with neonatal and adult erythrocytes, and report here our results using the lectin concanavalin A (Con A) and its derivatives.

MATERIALS AND METHODS

Con A was purified from jack bean meal by the method of Agrawal and Goldstein (8) and stored as a lyophilized powder at −20°. Ferritin was purified from fresh horse spleen by the method of Granick (9) and stored as a sterile solution at 4°. Rabbit antiserum to horse ferritin was a generous gift from W. Davis; the IgG fraction was purified by ammonium sulfate precipitation and DEAE-cellulose chromatography (10). Rabbit anti-Con A IgG was purified from immune serum by the same technique. Succinic anhydride was obtained from Matheson, Coleman, and Bell; [^{14}C]succinic anhydride and Na^{125}I were from New England Nuclear; glutaraldehyde was from Polysciences and was used without further purification; and chloramine T was from Sigma. Other materials were from sources previously described (11).

Preparation of Modified Forms of Con A. Succinyl Con A (sCon A) was made by a modification of the procedure described by Gunther *et al.* (12). In order to preserve primary amino groups required for glutaraldehyde conjugation to ferritin only the second succinic anhydride derivatization was performed. This abbreviated treatment resulted in four succinyl groups per Con A monomer. Nevertheless this procedure abolished Con-A-mediated erythrocyte agglutination without affecting red cell binding capacity (see Table 1). Succinyl Con A made by this modification chromatographed on gel filtration columns at a position relative to unmodified Con A that was consistent with a dimeric subunit structure. Con A and sCon A were labeled with ^{125}I by the chloramine T procedure of Hunter (13). The iodinated protein (100 μg, 0.5 mCi of ^{125}I) was adsorbed to a 0.5 ml (0.6 cm diameter) column of Sephadex G-50 (fine). Protein was eluted with 0.03 M glycine·HCl (pH 2.0) and then neutralized with 1 M Tris·HCl (pH 8.0). Specific activities ranged from 2 to 5 × 10^6 cpm/μg. Con A iodinated and purified by this procedure had hemagglutinating activity similar to unmodified Con A.

Preparation of Ferritin Conjugates. Con A was conjugated to ferritin and purified as previously described (11). An aliquot of ^{125}I-labeled Con A was included as a marker for yield and Con A concentration in the isolated product. The gel filtration fractions containing ferritin-Con A (F-Con A) conjugate were pooled and concentrated by centrifugation at 190,000 × *g* for 90 min at 4°. When the conjugation reaction contained 40 mg/ml of ferritin, 20 mg/ml of Con A, and 0.04% glutaraldehyde the final product contained between 5 and 10% of the starting Con A. Succinyl Con A (40 mg/ml) was conjugated to ferritin (40 mg/ml) with 0.07% glutaraldehyde for 1 hr at 22°. The resulting product was purified as above, except that the

Abbreviations: Con A, concanavalin A; F-Con A, ferritin-conjugated Con A; sCon A, succinylated Con A; F-sCon A, ferritin-conjugated succinyl Con A.

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Table 1. Binding and agglutination properties of normal and modified forms of Con A

Ligand	Antibody	Lectin bound/assay* µg (µg added)		Agglutination midpoint† (µg/ml, 0.5% cells)		Temperature (°C)
		Adult	Neonatal	Adult	Neonatal	
Con A	—	—	—	1.25	5.0	0
		—	—	5.0	12.5	22
		0.25 (10)	0.38 (10)	12.5	12.5	37
sCon A	—	0.23 (10)	0.30 (10)	>500		37
sCon A (250 µg)	Anti-Con A	—	—	2.5	—	22
sCon A	Anti-ferritin	—	—	No agg.	—	22
F-sCon A		—	—	>500	—	22
F-sCon A (250 µg)	Anti-Con A	—	—	No agg.	—	22
F-sCon A	Anti-ferritin	—	—	0.33	—	22
Ferritin (450 µg)	Anti-ferritin	—	—	No agg.	—	22

* Incubations were carried out as described in the *Materials and Methods* section for 10 min at 37°. Each tube contained about 1 µCi of ^{125}I-labeled lectin. Unbound material was removed by repeated centrifugation and washed samples were withdrawn for radioactivity determination. Binding was >90% prevented by incubation in the presence of 0.1 M methyl-α-D-mannopyranoside.

† Incubation and washing were carried out as above and cells were diluted 50-fold to 1% (vol/vol). Aliquots (50 µl) of the diluted cells were distributed into microtiter test plate wells. The amount of ligand which each aliquot had been exposed to is noted in parentheses in column 1. These aliquots were then mixed with 50 µl portions of buffer, lectin, or diluted antibody and agglutination midpoints were noted after 1 hr at the indicated temperature.

concentrated material was resuspended in 20 volumes of buffer and the centrifugation step was repeated. The final yield of ferritin-succinyl-Con A (F-sCon A) varied between 3 and 5%. In each preparation <5% of the total Con A was free.

Erythrocyte Incubations and Electron Microscopy. Human adult and cord blood samples were freshly collected into citrate–glucose, stored at 4°, and used within 3 days. The citrated blood was centrifuged and washed twice in saline and resuspended to 50% cells. Incubations were performed in conical 1.5 ml polyallomer tubes. Reaction mixtures contained: 200 µl 0.05 M Tris·HCl (pH 7.5), 0.10 M NaCl, 1 mg/ml of bovine serum albumin; 50 µl of 50% blood; and $CaCl_2$, glucose, and adenosine each at a final concentration of 1 mM. After designated incubations the cells were sedimented in a Beckman Microfuge and resuspended in isotonic sodium phosphate (pH 7.4) containing 1% glutaraldehyde. After 1 hr at 0° cells were washed and post-fixed for 2 hr at 0° in 2% osmium tetroxide. Fixed cells were then dehydrated through graded ethanol and propylene oxide and embedded in Epon. Thin sections (60–80 nm) were cut with a Dupont diamond knife on a Sorvall MT2-B ultramicrotome. Sections were stained with lead citrate and uranyl acetate and observed by transmission electron microscopy with the Philips EM 300 instrument. Erythrocytes were readily distinguished from reticulocytes by the absence of ribosomes.

RESULTS

Con A Binding and Agglutination of Human Erythrocytes. Neonatal erythrocytes bind 30–50% more Con A (measured with ^{125}I-labeled and unlabeled Con A) than do adult cells (Table 1). This difference is not reflected in increased agglutinability. Adult and neonatal cells have identical hemagglutination midpoints at 37° (Table 1); adult cells are somewhat more sensitive than neonatal cells to lectin-mediated agglutination at lower temperatures.

Con A Induces Receptor Clustering and Endocytosis in Neonatal Erythrocytes. Visualization and quantitation of Con A-induced clustering and endocytosis can be achieved using a ferritin conjugate of Con A as an electron microscope marker. Clustering is measured by counting invaginations on the cell surface to which ferritin molecules are attached, and endocytosis by counting ferritin-bound vesicles in thin sections of treated cells (Fig. 1). Neonatal erythrocytes contain vesicles in addition to those that are induced by Con A (Table 2, ref. 6). These vesicles are found in thin sections of untreated cells and this accounts in part for the high ratio of total vesicles to ferritin-containing vesicles in F-Con-A-treated cells. It is also evident in Fig. 1a and b that in general only a fraction of the surface of an invagination or an endocytotic vesicle is occupied by ferritin particles, and it is therefore likely that some of the vesicles that do not contain ferritin in sections of F-Con-A-treated cells may have contained ferritin but have been sectioned through regions that happened not to exhibit any ferritin. Therefore, the number of F-Con-A-induced vesicles is probably underestimated in the following data.

Table 2 shows several characteristics of the F-Con A reaction, as measured by the average number of ferritin-containing invaginations and vesicles per cell section. Con-A-induced invagination and endocytosis are specific for the neonatal cell, with adult red cells showing no invaginations and one-tenth the number of ferritin-containing vesicles. A competitive inhibitor of Con A binding, methyl-α-D-mannopyranoside, clearly reduces invagination and endocytosis, as does incubation at 0°. The cells do not respond to ferritin alone. Since only about 1% of the cell volume is represented in each cell thin section and the vesicles are around 100 nm in diameter, 0.2 vesicle per cell section is equivalent to the presence of 10 to 20 ferritin-containing vesicles per cell. In a separate report[‡] we will provide evidence that the structures we have scored as vesicles are indeed enclosed within the cell and not just cross sections through invaginations.

In contrast to erythrocytes, reticulocytes (recognized by the presence of ribosomes in stained sections) when reacted with F-Con A showed 20 to 50 times the number of ferritin-containing vesicles per cell section.[‡] Thus, though only about one in five erythrocyte sections showed a ferritin-containing vesicle, reticulocytes often showed 10 to 20 ferritin-containing vesicles per cell section.[§]

‡ R. Schekman and S. J. Singer, in preparation.

§ For this reason we suspect that Fig. 6 in ref. 6 is a reticulocyte and not an erythrocyte thin section.

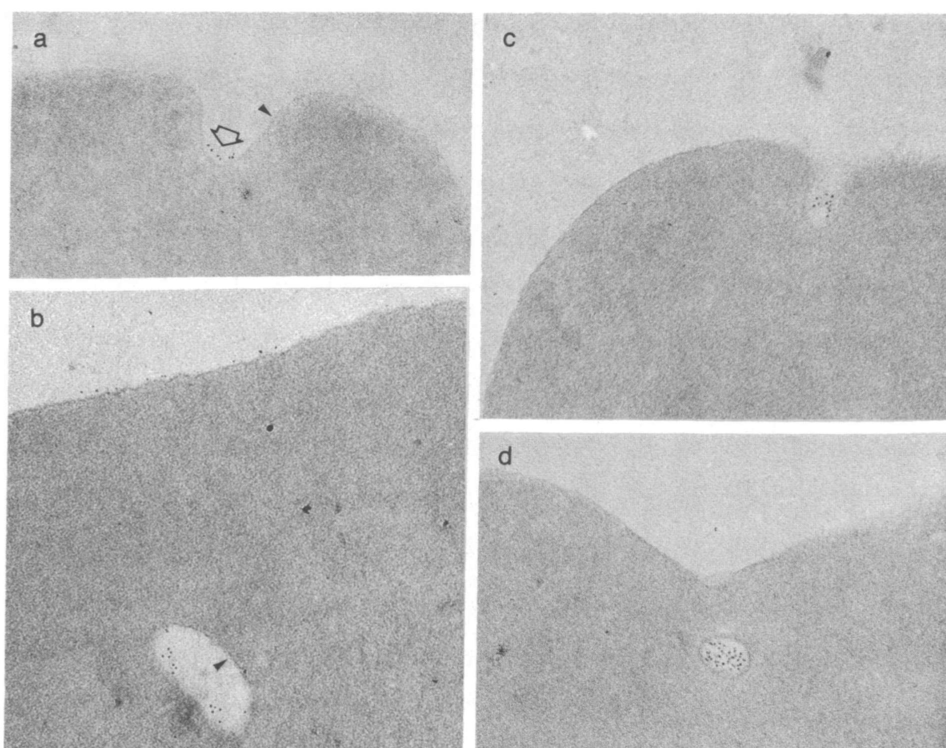

FIG. 1. Ferritin-containing invaginations and vesicles in neonatal erythrocyte thin sections. F-Con-A-induced (a) invagination and (b) vesicle. F-sCon-A-treated cells with (c) invagination and (d) vesicle induced by anti-ferritin antibody. Large arrow, ferritin cluster in invagination; small arrows, ferritin-free regions of invagination and vesicle membrane. Magnification, ×49,000.

Endocytosis Requires Ligand-Mediated Receptor Clustering. The density of F-Con A molecules inside vesicles and on nascent invaginations is greater than on the rest of the neonatal red cell periphery (Fig. 1). This behavior is consistent with multivalent lectin-induced redistribution of mobile receptors. In order to test the prediction that receptor mobility and clustering are necessary for endocytosis we prepared a modified form of Con A. Treatment with succinic anhydride converts tetrameric Con A to a dimer (12). This form retains an affinity for mannose-terminated oligosaccharides but will no longer agglutinate cells or redistribute surface receptors. Ferritin-conjugated succinyl Con A (F-sCon A) will also not agglutinate red cells (Table 1). Although anti-Con A antibody will cause sCon-A-treated cells to agglutinate, it will not agglutinate F-sCon-A-coated cells. In this case ferritin probably interferes with antibody binding to Con A. Anti-ferritin antibody will, however, agglutinate F-sCon-A-treated cells, whereas it has no effect on sCon-A-bound cells (Table 1).

Under conditions where the neonatal cell does, and the adult cell does not, respond to F-Con A by the induction of invaginations and endocytosis, neither cell responds to F-sCon A (Table 3). Endocytosis is restored to F-sCon-A-treated neonatal cells upon the addition of anti-ferritin antibody (Fig. 1c and d), with no effect produced with adult cells. Both ferritin-con-

Table 2. Characteristics of F-Con-A-induced invaginations and endocytosis

Cells	Incubation	Total vesicles/ cell section*	Ferritin invaginations/ cell section	Ferritin vesicles/ cell section
Neonatal	Untreated	0.62	0	0.01
Neonatal	F-Con A† (10 μg, 37°)	1.79	0.060	0.17
		1.84	0.055	0.19
		0.90	0.067	0.16
Neonatal	F-Con A (10 μg, 0°)	1.16	0.005	0.04
Neonatal	Methyl-α-D-mannopyranoside (0.1 M) + F-Con A (10 μg, 37°)	0.82	0.005	0.04
Neonatal	Ferritin (50 μg, 37°)	1.08	0	0.04
Adult	F-Con A (10 μg, 37°)	0.25	0	0.02

Incubations (1 hr) were performed as described in the *Materials and Methods* section. Stained thin sections were viewed at a magnification of ×16,000. Only erythrocytes were scored. Only vesicles which spanned the section were counted and ferritin-containing vesicles were scored only if the ferritin was clearly focused and contained within a vesicle boundary.

* Two-hundred-fifteen cell sections counted in each.

† Three different cord samples.

Table 3. Endocytosis requires clustering of Con A receptors

Cell	F-Con A (10 μg)	F-sCon A (60 μg)	Antibody to ferritin (μg)	Ferritin invaginations	Ferritin vesicles
Neonatal	+	—	0	13	37
Neonatal	—	+	0	1	6
Neonatal	—	+	4	7	31
Neonatal	—	+	22	4	19
Neonatal	—	+	118	2	16
Adult	—	+	0	0	2
Adult	—	+	4	0	4
Adult	—	+	22	0	0
Adult	—	+	118	0	0

Incubations were carried out as described in the *Materials and Methods* section at 37° for 10 min. Unbound conjugate was removed by centrifugation and cells were resuspended in fresh reaction buffer with the specified additions. After 1 hr further incubation at 37°, the samples were fixed and processed as before. Two-hundred-twenty cell sections were counted in each.

taining vesicles and invaginations appear at a low anti-ferritin antibody concentration, but decrease in frequency as the antibody concentration is raised. This decrease may be attributed to the formation of smaller antigen–antibody aggregates in antibody excess (3).

Similar Membrane Proteins in Neonatal and Adult Cells. Detergent-solubilized membranes prepared from neonatal and adult cells show qualitative and quantitative similarity in the major membrane polypeptides as determined by sodium dodecyl sulfate/polyacrylamide gel electrophoresis (Fig. 2).

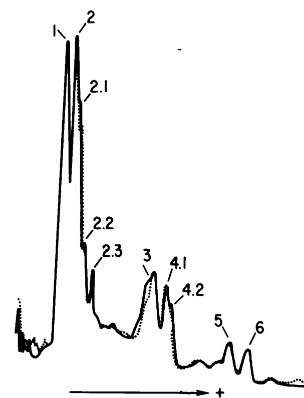

FIG. 2. Detergent-solubilized neonatal and adult erythrocyte membrane proteins resolved by sodium dodecyl sulfate/polyacrylamide gel electrophoresis. Membranes were prepared, solubilized, and electrophoresed by the methods of Fairbanks *et al.* (14), except that gels containing a gradient of acrylamide concentration (3.5–8%) were used. Gels were stained with Coomassie blue and destained, and protein positions were scanned at 550 nm. Adult membrane proteins, solid line; neonatal membrane proteins, dotted line. The band numbers are in accordance with Steck (15). The diminished staining of lower molecular weight bands may be due to higher acrylamide concentration.

DISCUSSION

We have demonstrated that membrane receptors for Con A are more mobile in the plane of the membrane of intact neonatal erythrocytes than in the membrane of adult cells. This increased mobility is indicated by the F-Con-A-induced clustering of the receptors into areas that form invaginations on the neonatal cell surface. The clustering occurs at 37° but is greatly reduced at 0°, as would be expected from the marked increase in effective lipid viscosity at the lower temperature (16). The same specimens that show ferritin particles lining the external surface of invaginations also show intracellular vesicles with ferritin particles on their internal surfaces, which presumably are formed from such invaginations (see below). Neither invaginations nor endocytotic vesicles are observed under similar circumstances with normal intact adult erythrocytes.

One possible explanation for this mobility difference is that Con A receptors in the membrane are of several kinds, and that a specific mobile receptor is present on neonatal erythrocytes that is absent on adult cells. However, we have shown‡ that the blood group A antigen (6) and the $Rh_0(D)$ antigen on neonatal erythrocytes are also induced to cluster and undergo endocytosis by appropriate antibody treatments, whereas no such effects are observed with adult cells. It appears, therefore, that the different receptor mobilities reflect generalized structural differences in the membranes of the neonatal and adult cells.

On the other hand, this does not require that there necessarily be bulk differences between the two membranes, such as an overall difference in lipid fluidity. Indeed, studies with the fluorescent probe perylene have shown (M. Keahry, J. Yguerabide, and S. J. Singer, in preparation) that the apparent lipid fluidities in the two cell ghosts are indistinguishable. The clustering of Con A receptors on neonatal erythrocytes is very unlike the "capping" phenomenon seen in comparable experiments with lymphocytes (3, 4) and proerythroblasts (17). Small and discretely separated F-Con A clusters are formed on the neonatal erythrocyte surface, in contrast to the large collection of aggregated receptors that constitutes a cap. The endocytotic vesicles that form are relatively small, whereas such vesicles formed within lymphocytes are much larger. Furthermore, the maximum extent of endocytosis of F-Con A and ferritin-conjugated antibodies to group A antigen is reached when only a small amount (<1% in the latter case‡) of the bound ligand is internalized, and this limit is not enhanced by the addition of secondary antibody‡. Taken together, these considerations suggest that only a small fraction of the Con A receptors on the neonatal cell are mobile.

These observations are consistent with the proposal that there exist, or are induced upon ligand binding, ***discrete domains*** in the intact neonatal cell membrane within which receptor proteins can exhibit lateral mobility. These mobile domains would be interspersed within a matrix of immobilized receptors, and might be so few and far between as to remain essentially independent of one another. Receptor clustering and endocytosis would be confined within those domains. It is further proposed that such mobile domains are absent, or cannot be induced by ligand binding, in the membrane of the normal intact adult erythrocyte. [On the other hand, under nonphysiological conditions of stress, adult erythrocytes can exhibit clustering of their membrane receptors (18, 19), and endocytosis (20), and may therefore be constrained to form mobile domains under such circumstances.]

How might the mobility of membrane receptors be restricted and how might this inhibition be partially relaxed in the neonatal cell? The restriction may be attributable to the proteins of the spectrin complex that are attached peripherally to the cytoplasmic surface of the erythrocyte membrane (5, 21, 22). There is some evidence that this attachment is to the intramembranous particles which span the thickness of the membrane, and that these particles project protein-bound oligosaccharide chains at the exterior surface of the membrane

to which lectins can bind (21). Recent work in this laboratory (M. Sheetz and S. J. Singer; W. Birchmeier and S. J. Singer; in preparation) has led to the proposal that the spectrin complex, consisting of components 1 and 2 (spectrin), component 5 (actin, refs. 23 and 24), and perhaps other proteins, undergoes metabolically regulated aggregation–disaggregation reactions while remaining bound to the membrane. When highly aggregated, the complex would then closely tie together many attached intramembranous particles, not only thereby immobilizing them, but causing them to serve as intramembranous barriers to the lateral motion of other integral proteins in the membrane.

Upon examining the membrane proteins of the two cells, we found no detectable significant difference (Fig. 2), indicating that very similar, if not identical, amounts of the spectrin complex were bound to both membranes. It is possible, however, that the spectrin complex may be somewhat less highly aggregated on the membrane of the neonatal than of the adult cell because of the different metabolic states of the two cells. This less aggregated network might then allow the formation of mobile domains within the neonatal membrane.

In this connection, it is of great interest that reticulocytes in blood of newborns, when reacted with ferritin conjugates of Con A or anti-A antibodies, exhibit a greatly increased number of ferritin-containing vesicles per cell section compared to mature neonatal erythrocytes[‡]. As this difference probably reflects a greater mobility of receptors in reticulocyte than in erythrocyte membranes, it may be that the mobile domains formed in the former membrane are more numerous and larger than in the latter. It is known, however, that the proteins of the spectrin complex are no longer synthesized in the reticulocyte (25), and if the spectrin complex regulates receptor mobility, the decrease in mobility that occurs during the maturation of the reticulocyte to the erythrocyte may reflect a change in the state of aggregation of the spectrin complex rather than in its amount. This work therefore points to two interesting membrane comparisons: adult with neonatal erythrocytes, and erythrocyte with reticulocyte.

Finally, these studies bear directly on the mechanisms of endocytosis. The neonatal erythrocyte, lacking microtubules, smooth muscle myosin-like proteins (26), and many other structural and enzymatic components present in other eukaryotic cells, is a relatively simple system for such study. We have shown that endocytosis of Con A receptors does not occur without a multivalent Con-A-induced aggregation of its receptors into discrete clusters in the fluid membrane. F-sCon A, because of its reduced valence (12), is presumably incapable of forming multiple bonds with several Con A receptors, and hence cannot induce receptor clustering, and therefore no endocytosis can occur. However, if bivalent anti-ferritin antibodies are then bound to the F-sCon-A-treated cells, clusters of Con A receptors are now formed by the bridging of multivalent ferritin molecules, and this results in endocytosis. Similar treatments of adult erythrocytes have no effect because there are no domains of Con A receptor mobility in the membrane. It seems likely therefore, that in this and other systems, cluster formation is obligatory for endocytosis to occur (7, 27). The suggestion is that the formation of such clusters transmits a signal across the membrane that *locally* activates an intracellular endocytotic mechanism, so that only the membrane in the vicinity of the cluster is involved in endocytosis; if this hypothesis is correct, the nature of the signal and the mechanism that is activated remain to be discovered.

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