# RAPID COMMUNICATIONS

# DOMAINS OF RECEPTOR MOBILITY AND ENDOCYTOSIS

# IN THE MEMBRANES OF NEONATAL HUMAN ERYTHROCYTES

# AND RETICULOCYTES ARE DEFICIENT IN SPECTRIN

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## ABSTRACT

It has previously been shown (Schekman, R., and S. J. Singer, *Proc. Natl. Acad. Sci. U. S. A.* **73:**4075-4079) that receptors in the membranes of neonatal human erythrocytes show a restricted degree of lateral mobility, whereas in adult human erythrocytes the receptors are essentially immobile. This restricted mobility is exhibited, for example, when concanavalin A (Con A) induces a limited clustering of its receptors in the neonatal erythrocyte membrane, resulting in the formation of invaginations and endocytotic vesicles. This does not happen with adult cells. By the use of indirect immunoferritin labeling of ultrathin frozen sections of Con A-treated neonatal blood cells, we now show that the invaginations and endocytotic vesicles do not stain for spectrin, whereas the adjacent unperturbed membrane is heavily stained. The reticulocytes in the neonatal cell population undergo substantially more Con A-induced invagination and endocytosis than do the erythrocytes. These results lend strong support to the hypothesis that specialized discrete domains exist, or are induced, in the membranes of these neonatal cells, in which receptors are laterally mobile, whereas in the remaining (and predominant) part of the membrane the receptors are immobile. Such mobile domains are characterized by an absence of spectrin. During the maturation of the neonatal reticulocyte to erythrocyte, it is proposed that these domains are in large part, but not completely, eliminated.

KEY WORDS    fluid membrane domains ·
immunoferritin · electron microscopy ·
ultracryotomy · concanavalin A

The integral proteins in the membranes of adult human erythrocytes are restricted in their lateral mobility (7, 3, 11), but this appears to be only partially true in the membranes of neonatal human erythrocytes. Following up on early observations of Blanton et al. (2), electron microscope studies from this laboratory (11, and footnote 1)

have shown that (to a limited extent) membrane receptors for the lectin concanavalin A (Con A) can be clustered together by ferritin-labeled Con A into membrane invaginations and then endocytosed, when neonatal erythrocytes are employed but not with adult cells. Similar results were obtained with the blood group A antigen and its antibodies. These observations led to the proposal that "there exist, or are induced upon ligand

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[1] R. Schekman and S. J. Singer. Lectin and antibody-

induced endocytosis in neonatal human erythroid cells. Manuscript in preparation.

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 (11)." It is generally thought (cf. references 1, 3, 14, and 15) that the spectrin complex forms a "scaffolding" under the erythrocyte membrane that is somehow responsible for the lateral immobilization of the membrane proteins. We therefore undertook electron microscope immunoferritin experiments to determine the distribution of spectrin with respect to the Con A-induced membrane invaginations and endocytotic vesicles on neonatal erythrocytes. Intracellular immunoferritin staining of ultrastructurally preserved specimens was made feasible by the development of techniques for ultrathin frozen-sectioning of lightly fixed cells (17, 10, 18). In this communication, we show that membrane invaginations and endocytotic vesicles of Con A-treated neonatal human erythrocytes and reticulocytes are sharply differentiated from unperturbed regions of the cell membrane by an absence of immunoferritin labeling for spectrin.

## MATERIALS AND METHODS

The procedures used to isolate human erythrocyte spectrin, and to prepare and affinity purify the rabbit anti-spectrin antibodies are described elsewhere (13). The antibodies thus obtained were mainly directed to spectrin component 1 protein (13). Goat antibodies to rabbit IgG were also affinity purified. Ferritin, which was purified from fresh horse spleen by the method of Granick (4), was conjugated to the goat anti-rabbit IgG by the method of Kishida et al. (6). Human cord blood samples were freshly collected into citrate-glucose, stored at 4°C, and used within 3 d. The procedure for the Con A stimulation of endocytosis involved incubating the cells with 10 μg of Con A for 10 min at 37°C as described (11).

### Fixation

A 2% formaldehyde solution in 0.1 M phosphate buffer, pH 7.4, was prepared by heating a suspension of paraformaldehyde (Matheson, Coleman and Bell, Los Angeles, Calif.) to ~60°C and adding a small amount of 1 N sodium hydroxide. A 2% glutaraldehyde solution in the same buffer was prepared by diluting 8% glutaraldehyde (EM grade, Polysciences, Inc., Warrington, Pa.). Con A-treated cells were lightly packed by centrifugation, and resuspended in 2% formaldehyde at 4°C. ½ h later, a small volume of 2% glutaraldehyde was added to bring the final concentration to 0.1%. Another

½ h later, the fixation was terminated by a 10-fold dilution with buffer and then by centrifugation, discarding the supernate and resuspending the cells in fresh buffer.

### Frozen Sectioning

The general procedure of ultracryotomy and its improvements for immunoferritin labeling of ultrathin frozen sections are described in our previous papers (17, 18). The Sorvall MT-2B ultramicrotome (DuPont Instruments-Sorvall DuPont Co., Wilmington, Del.) was used with the LTC-2 cryo-attachment. In the present experiments, the temperature range for sectioning was −70° to −80°C and the concentration range of sucrose for the prefreezing infusion into cells as well as for suspending them for sectioning was 1.1-1.2 M in 0.1 M phosphate buffer, pH 7.4.

### Immunoferritin Labeling

Immunoferritin labeling in this study was done indirectly, first with 0.2 mg/ml of the anti-spectrin antibody and, after thoroughly washing unbound antibodies away, with 0.5 mg protein/ml of the ferritin-goat anti-rabbit IgG conjugate. As controls, normal rabbit IgG or rabbit anti-human uterine myosin (13) was employed in place of the anti-spectrin antibodies. The procedure of labeling is described in our previous paper (18). For incubation of frozen sections on a labeling solution, the grid with the sections was floated on a small droplet of the solution suspended on a 4-mm diameter loop. All other steps were taken on 1.5- to 2.5-cm-wide drops of buffers or solutions arranged on sheets of Parafilm (American Can Co., Dixie/Marathon, Greenwich, Conn.).

### Examination

Sections on grids were finally dried in the presence of a small amount of 0.4% dextran with or without additional 0.05-0.1% phosphotungstic acid, pH 7.4. Observations were made in a Philips EM-300 electron microscope.

## RESULTS

Unperturbed regions of the plasma membranes of neonatal erythrocytes were densely stained for spectrin by the indirect immunoferritin procedure (Figs. 1-4). This staining was specific for spectrin because control experiments using rabbit anti-human uterine myosin (*inset,* Fig. 4) or normal rabbit IgG (not shown) in place of the rabbit anti-spectrin showed no staining. Examination of over 40 individual membrane invaginations (Figs. 1-3) and over 100 intracellular vesicles (Fig. 4) in the sections of the Con A-treated neonatal erythrocytes uniformly showed an absence of immunoferritin staining for spectrin on these membrane

regions. On the other hand, intense staining was observed on regions of the membrane immediately contiguous to the invaginations (Figs. 1-3). In the blood of neonatals there are about 10% as many reticulocytes as erythrocytes, whereas in normal adult blood there are only about 1% as many. As observed in our previous experiments on neonatal blood cells (11), invaginations and endocytosis take place more extensively in Con A-treated neonatal reticulocytes than in erythrocytes (Fig. 5). On such reticulocyte invaginations and endocytotic vesicles there is only background staining for spectrin (Fig. 6), although the membrane removed from the invaginations is densely stained (Fig. 7).

## DISCUSSION

In our previous experiments (11) ferritin-labeled Con A was used to induce and detect the clustering and endocytosis of Con A receptors. In the present study, the use of ferritin for the immunostaining of spectrin precluded the use of the ferritin-labeled Con A to discriminate those invaginations and endocytotic vesicles that were induced by Con A clustering. We have determined, however, that there are at least twice as many membrane invaginations on neonatal erythrocytes treated with Con A than on untreated cells. The uniform absence of immunoferritin staining on all of a large number of invaginations which we have examined on Con A-treated neonatal erythrocytes therefore signifies that the Con A-induced invaginations (as well as any that were induced by other unknown mechanisms) were devoid of spectrin staining. This deficiency exists at what appear to be early as well as late stages in the process of invagination, as judged by the increasing curvature of the invaginations (compare Figs. 1 and 3). A uniform absence of staining for spectrin is also observed on all of more than 100 intracellular vesicles in the Con A-treated neonatal erythrocytes. It is therefore highly probable that those vesicles induced by Con A (at least 10-20% of the total [11]), as well as the other vesicles present, are devoid of spectrin staining. Because the ligand-induced invaginations are the precursors of the Con A-containing vesicles, it is reasonable that both should be similarly lacking in staining.

Our results clearly indicate that, whatever the reason, ligand-induced membrane invaginations which become endocytosed are structurally specialized regions of the neonatal erythrocyte and

reticulocyte membrane. Beyond this, however, we may ask, what is the mechanism for such specialization, and in particular, what is the molecular significance of the absence of spectrin staining from these membrane regions? We must first note that the rabbit antibodies to human spectrin employed in these experiments were directed primarily to spectrin band 1 protein and not to band 2 protein (13). Thus, our immunoferritin experiments do not provide information about the disposition of spectrin band 2 protein on invaginations and vesicles. On the other hand, there is much evidence to indicate that spectrin band 1 and band 2 proteins are bound in a 1:1 molecular complex in the erythrocyte membrane (cf. reference 8), and, if a deficiency of the one component exists, it is likely, although not certain, that the other is missing as well. It is conceivable, however, that the deficiency of staining may reflect an inaccessibility, rather than an absence of band 1 protein in those membrane regions that are unstained. In later stages of invagination (Figs. 2 and 3), however, the invaginated membranes appear substantially less electron-dense than the spectrin-containing regions, which is more consistent with an absence of spectrin than with an additional obstruction to its staining. This apparent deficiency of spectrin staining tells us nothing about whether other peripheral proteins, such as actin (13, 16), are also absent from the invaginations and vesicles.

The deficiency of spectrin is consistent with the proposal (11) that the invaginations are discrete domains in which membrane receptors, in contrast to the rest of the membrane, are laterally mobile. In view of current notions that the lateral immobility of erythrocyte membrane proteins is associated with a scaffolding of the spectrin complex under the membrane (cf. references 1, 3, 14, and 15), it is entirely reasonable if such mobile domains specifically lack spectrin.

In neonatal reticulocytes, invaginations and endocytotic vesicles (Fig. 5) induced by treatment with Con A are 20-50 times more numerous than in neonatal erythrocytes (11). These membrane regions are also deficient in spectrin staining (Fig. 6). To accommodate the idea of mobile domains to these facts, we would infer that in the membranes of the reticulocytes the mobile domains are larger and more numerous, and that in the maturation of reticulocytes to erythrocytes, such mobile domains are in some manner eliminated. Evidence supporting such a scheme of reticulocyte matura-
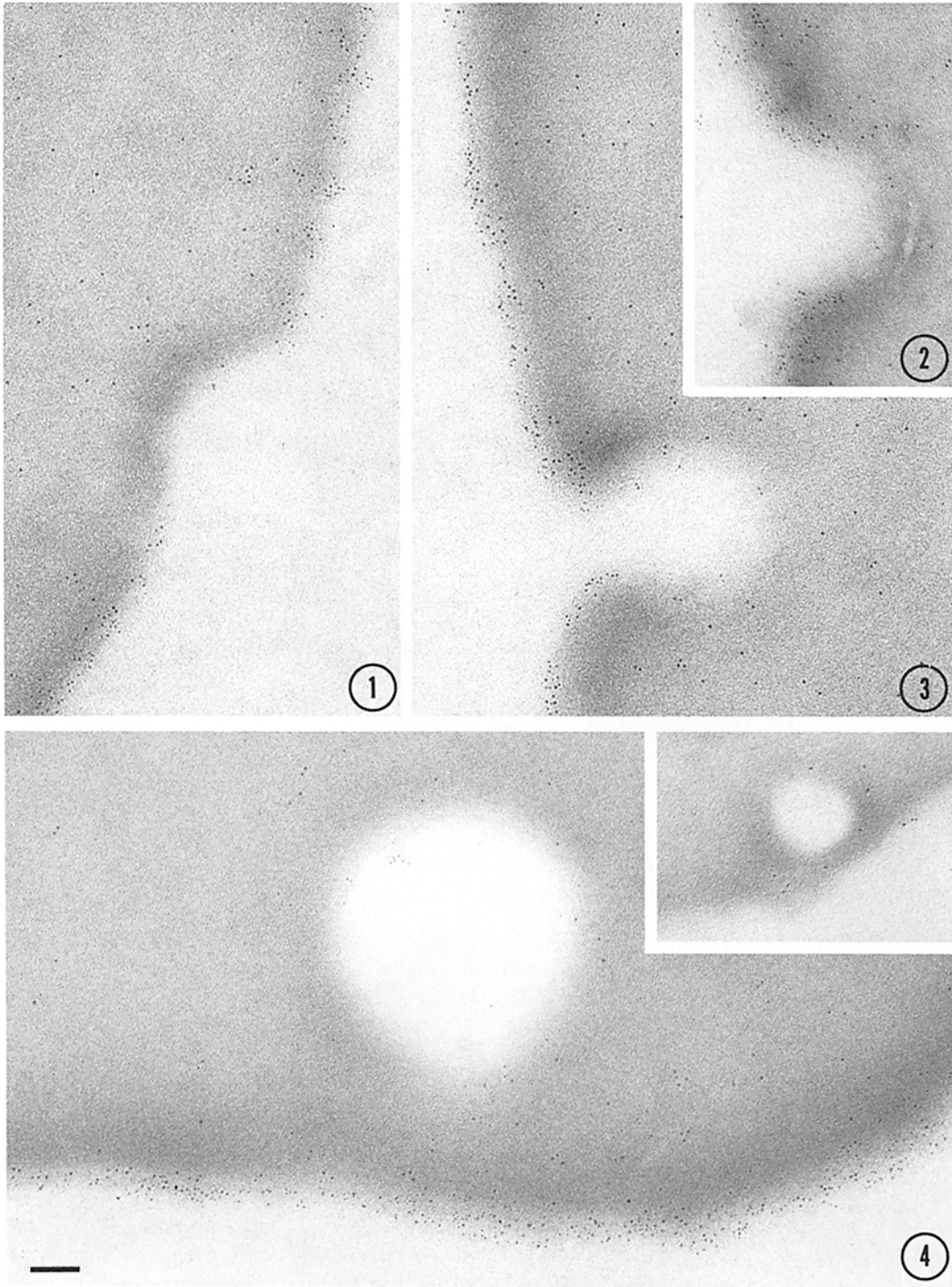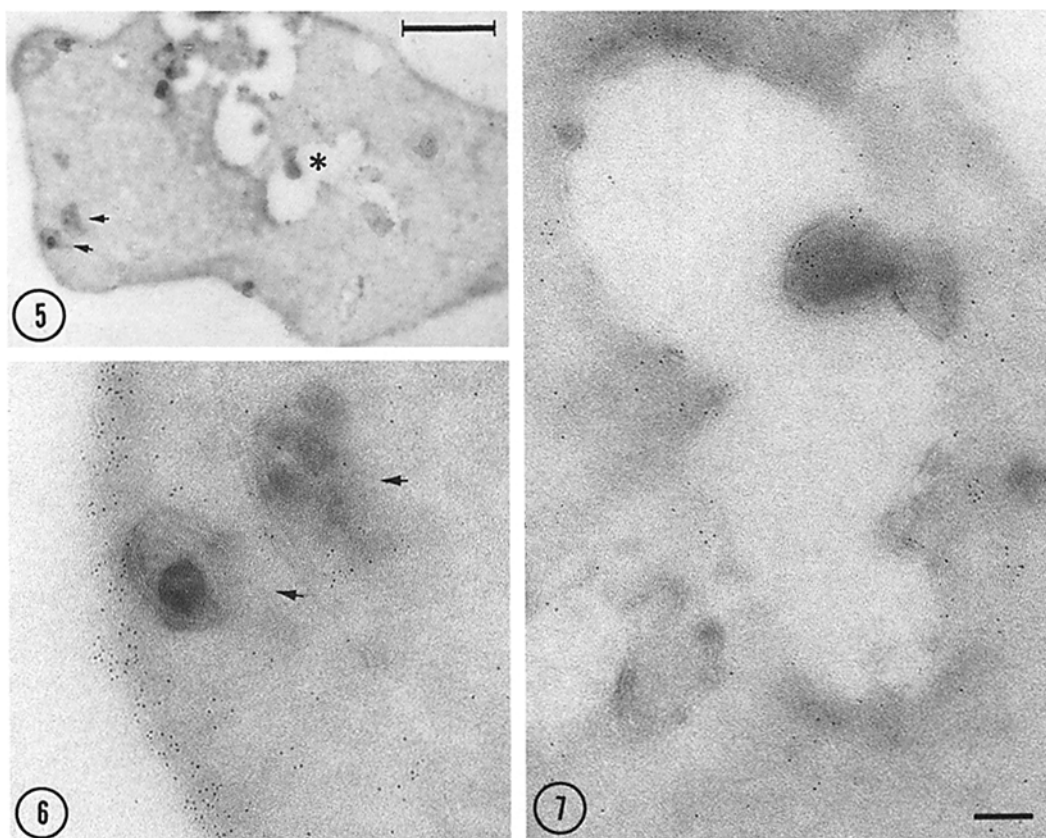
FIGURES 5-7   A Con A-treated reticulocyte in Fig. 5, with portions enlarged in Figs. 6 and 7. The presence of degenerating organelles (e.g. arrows in Figs. 5 and 6) indicates that the cell is a reticulocyte. Dense ferritin staining is seen along the unperturbed periphery of the cell (Fig. 6), but not along the boundary of the vesicles (Fig. 7). The asterisk in Fig. 5 identifies the vesicle in Fig. 7. Fig. 5, × 12,000. Figs. 6 and 7, × 70,000.

tion is presented in the following paper (19).

At least two mechanisms for the formation of these mobile domains are possible: (a) They may exist essentially fully formed and spectrin-free in the neonatal cell membrane just before ligand treatment, or (b) they may exist as nucleation

All figures show ultrathin frozen sections of neonatal human erythrocytes (Figs. 1-4) or reticulocyte (Figs. 5-7), treated with Con A. The sections, except that of the control in the inset of Fig. 4, were indirectly immunoferritin-labeled for spectrin. The control was reacted with rabbit anti-human uterine myosin antibodies, instead of rabbit anti-human spectrin antibodies, in the first step of the labeling process. Scales with and without end marks represent 1 and 0.1 μm, respectively.

FIGURES 1-4   The Con A-induced invagination and endocytosis are presumed to progress in the sequence Figs. 1-4. Ferritin particles, which are present in a continuous and dense array in the peripheral layer of the membrane, abruptly terminate at the sites of invaginations in Figs. 1-3 and are absent around the vesicle in Fig. 4. In the control in the inset of Fig. 4, few ferritin particles are found in the peripheral layer. In all figures, the unperturbed peripheral layer shows an electron density higher than that of the interior, which is still so when the invagination is shallow (Fig. 1). As the invagination becomes deeper (Figs. 2 and 3), however, the high density appears to be left at the surface. The area around the bottom part of the later invaginations or that surrounding the internalized vesicle (Fig. 4) show a density similar to that of the cell interior. All, × 70,000.

sites, perhaps as small imperfections in the spectrin scaffolding, which expand as a result of ligand-induced clustering of receptors. Such expansion would be associated with and accompanied by a clearing of spectrin from these membrane domains. In the former case, one might expect a fairly random assortment of membrane receptors to be present in the preexisting mobile domains. In the latter case, that receptor which was clustered by its ligand would be the major protein component found in the domains. These possibilities should therefore be distinguishable experimentally. It may be noted that the presumed early invagination in Fig. 1 exhibits a density comparable to the extra density on the adjacent unperturbed membrane (although it does not stain for spectrin), whereas the presumed later invaginations in Figs. 2 and 3, and the vesicle membrane in Fig. 4, have a lower density than the adjacent membrane. This might suggest that some components are cleared from the cytoplasmic surface after invagination is initiated. However, the frequency of detection of such early invaginations is low, and insufficient numbers have been studied to know whether this effect is reproducible, or an artifact, for example, of the obliqueness of the section.

How do these results bear on the molecular mechanism(s) of endocytosis on neonatal erythrocytes and reticulocytes? The clustering of Con A receptors in the membrane has been shown to be required for such endocytosis to occur, with the membrane invaginations containing the clustered receptors as the precursors for the formation of the endocytotic vesicles (11). The clustering of a particular receptor, together with the absence or clearing of the spectrin from the clustered region, may alone be responsible for a progressive invagination of that region of the membrane. On the other hand, other mechanochemical factors may also be involved, particularly for the scission of the invagination membrane that generates the vesicle. One interesting possibility is that some factor is localized to those regions of the membrane that are in immediate contact with the spectrin-free domain, forming a "collar" around the domain that exerts a contractile activity responsible for the invagination and endocytosis.

What relationships the ligand-induced clustering and endocytosis with these erythroid cells have with similar processes on other types of cells (cf. reference 12) which do not contain spectrin (9, 5), is another interesting question which remains to be answered.

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