SPECTRIN REARRANGEMENT EARLY IN ERYTHROCYTE GHOST ENDOCYTOSIS

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ABSTRACT

The endocytic vacuoles induced in white ghosts were found to be depleted of spectrin and therefore it was proposed that they arose from spectrin-free areas in the erythrocyte membrane. To follow changes in spectrin distribution during endocytosis, affinity-purified rabbit antispectrin antibodies were produced. Quantitative techniques were developed for the use of a highly specific ^{125}I-F(ab')$_2$ antispectrin, and these showed that before the appearance of vacuoles, as assessed by phase microscopy, there was a reproducible decrease in immunoreactive spectrin. To determine whether this spectrin decrease represented a local or diffuse spectrin loss or a spectrin rearrangement, morphologic studies were undertaken using transmission electron microscopy on samples treated with rabbit antispectrin and ferritin-conjugated goat anti-rabbit immunoglobulin. These studies showed that endocytosis was preceded by the creation of extensive spectrin-free areas separated by discrete spectrin-containing zones. Pretreatment of ghosts with alkaline phosphatase blocked all forms of endocytosis and prevented the creation of spectrin-free areas. Therefore, it is proposed that under the impetus of endocytosis inducers, phosphorylated spectrin is redistributed so that spectrin-free zones are created, and that endocytic vacuoles form and fuse in spectrin-free areas.

KEY WORDS endocytosis · spectrin rearrangement · erythrocyte membrane · spectrin phosphorylation · spectrin function

The erythrocyte ghost has been extensively studied, in terms of its composition and function, as a prototype plasma membrane. The proteins of the erythrocyte membrane are classified as being integral or peripheral. The peripheral proteins, spectrin and actin, interact at the membrane cytosol face to form a tough but flexible spectrin-actin cytoskeleton (21, 25). Recent evidence indicates that the integral proteins are attached to the spectrin of the cytoskeleton either directly or indirectly via protein receptors including bands 3a and 4.1 (1, 10) so that the effect of the spectrin-actin cytoskeleton is imposed on the entire membrane (4, 24). Furthermore, there is complicated heterogeneity of membrane spectrin which appears to exist in both polymerized and nonpolymerized forms as well as in phosphorylated and nonphosphorylated forms, and the role of each of these species is not clearly understood (12).

Study of human erythrocytic diseases and genetically determined abnormalities in the erythrocytes of mice has provided considerable information about the function of membrane proteins. For example, irreversibly sickled cells (ISC) have

ISC-shaped spectrin-actin residues (13), and the spectrin-deficient mouse erythrocytes show spontaneous membrane budding and fusion (6). These findings strongly imply that spectrin plays an important role in imparting a degree of structural stability to the membrane. In addition, spectrin plays a key role in modulating erythrocytic shape changes (2, 19). The membrane protein changes which occur during endocytosis in human white erythrocyte ghosts have been studied (7, 16) because endocytosis involves the membrane functions of invagination, constriction, and fusion (7, 16). In a prior study it was demonstrated that alteration of spectrin was an important and perhaps deciding element in the endocytosis process. The fact that the resulting endocytic vacuoles were depleted of spectrin indicated that endocytosis occurred in areas of the membrane that were free of spectrin. When lateral movement of spectrin was blocked by cross-linking with a bivalent antispectrin antibody, endocytosis was completely inhibited (7).

The purpose of this study was to determine whether spectrin rearrangement or depletion was a necessary step in the endocytosis process. This question was pursued with radioiodinated and ferritin-tagged antispectrin antibodies which allowed both the accurate quantification and localization of membrane spectrin during the endocytosis process. The results indicate that spectrin movement is an obligatory and early step in the endocytosis process. Furthermore, phosphorylated spectrin appears to be the form that is capable of undergoing movement or rearrangement under the conditions described.

MATERIALS AND METHODS

Endocytosis

Freshly drawn heparinized human blood was washed in 0.154 M NaCl, and the erythrocytes were lysed by mixing with 40 vol of 5 mM phosphate buffer, pH 8. Ghosts were obtained after two additional washes with the 5 mM phosphate buffer. Ghosts at a protein concentration of 1 mg/ml were incubated with 3 mM Mg-ATP in 50 mM *N*-Tris methyl-2-aminoethane-sulfonic acid (TES) (Grand Island Biological Co., Grand Island, N. Y.) buffer, pH 7.5, to induce endocytosis (8). Alternatively, endocytosis was induced with either 20 ng/ml of trypsin or 0.1 mM EDTA by methods previously described (7). The extent of endocytosis in each experiment was measured qualitatively by phase microscopy and quantitatively by the acetylcholinesterase method (7, 8). The number of ghosts present in each of the samples were counted in the Coulter Counter model ZBI (Coulter Electronics Inc., Hialeah, Fla.). The protein content of ghost membranes was determined by the Lowry method.

Preparation of Antispectrin Antibodies

Antispectrin antibodies were induced in rabbits immunized with purified spectrin by methods previously described (7). The whole serum containing antispectrin antibodies from the rabbits was purified on Ultragel Ac_{22} (LKB Instruments, Inc., Rockville, Md.) affinity columns to which purified spectrin was linked. The antispectrin antibody was then eluted with 0.2 M HCl, pH 2.2, adjusted with 2 M glycene, and dialyzed against 100 vol of 0.1 M phosphate-buffered saline (PBS), pH 7.4.

Measurement of Immunoreactive Spectrin in Ghosts

The $F(ab')_2$ fragment of the purified antispectrin was prepared by digesting affinity-purified antispectrin antibody with 4 mg/ml of Pepsin (Pharmacia Fine Chemicals, Div. of Pharmacia Inc., Piscataway, N. J.) in 0.1 M Na-acetate buffer, pH 4.3, for 8 h at 37°C. The resulting $F(ab')_2$ fragment was isolated either by passing the mixture through a Sepharose-protein A column (Pharmacia), which retarded the Fc fragment, or by gel filtration on Sephadex G-150. The concentrated $F(ab')_2$ antispectrin was then radioiodinated by the method of Jensenius and Williams (9) and used at a sp act of 30–40 μCi/μg. For use in control studies, immunoglobulin was obtained from normal nonimmunized rabbits and the $F(ab')_2$ fragment was prepared. For one set of control experiments the normal nonimmunized $F(ab')_2$ was radioiodinated as described above.

For the quantitative measurement of immunoreactive spectrin, samples of ghosts were taken during the endocytosis process as measured both by phase microscopy and by the acetylcholinesterase method. Ghosts were rapidly added to 10 vol of chilled 5 mM phosphate buffer, pH 8, mixed for 20 s to establish hypotonic conditions, and then glutaraldehyde was added to a final concentration of 0.5–1%. This procedure, described by Seeman (17) and utilized by Nicolson et al. (14) to study spectrin localization with antispectrin antibodies, was used to fix the ghosts under very hypotonic conditions so that the erythrocyte membrane would be permeable to the assaying antibodies used. We tested this method by applying a fluorescent label to our antispectrin and observed that the membrane cytosol face was brightly fluorescent (data not shown). After fixation and extensive washing in PBS to remove the glutaraldehyde, the ^{125}I-$F(ab')_2$ antispectrin was incubated with the ghosts in a 200-ul vol at pH 7.4 for 2 h at room temperature, or at 4°C overnight. The samples were then washed four times, and the residual radioactivity was taken to be a measure of the amount of immunoreactive spectrin available at the specified time. The amount of immunoreactive spectrin was related to the number of ghosts present in the sample.

Topographic Localization of Spectrin in Ghosts

The topographic localization of spectrin in the plasma membrane of ghosts during the endocytosis process was achieved using a ferritin-conjugated goat anti-rabbit IgG. Ghosts undergoing endocytosis were removed and rapidly fixed in hypotonic conditions as described above. Then 150 μg of purified rabbit antispectrin IgG was added to 1 mg of ghost protein. Assuming that 25% of membrane protein is spectrin and what spectrin exists as a dimer of mol wt of ~450,000, there were then $\sim 3 \times 10^{14}$ molecules of spectrin present and 6×10^{14} molecules of rabbit antibody were added, giving a twofold excess of antibody to spectrin. The antibody was then incubated with the fixed ghosts for 2 h at room temperature. In control experiments, normal nonimmunized rabbit IgG was added. After incubation, the samples were extensively washed to remove either unbound antispectrin or unbound immunoglobulin and then an excess of ferritin-labeled goat anti-rabbit IgG (purchased from Miles-Yeda, Rehovot, Israel) was added and the samples were incubated again for an additional 2 h at room temperature. The unbound ferritin was removed by extensive washing with PBS. Samples were then fixed and processed for transmission electron microscopy (TEM) (3).

Effect of Alkaline Phosphatase on Endocytosis

To determine whether the state of spectrin phosphorylation was important in the endocytosis process, ghosts were preincubated with either the hypotonic buffer, enzymes including hexokinase and RNase (Calbiochem-Behring Corp., American Hoechst Corp., San Diego, Calif.) which served as nonspecific controls, or 1.5, 5, and 15-U/ml alkaline phosphatase (*Escherichia coli* derived, purchased from Worthington Biochemical Corp., Freehold, N. J.) (2, 15). The incubations were carried out at room temperature for 20 min and, following the incubation, the samples were washed. Endocytosis was then induced as described above and the extent of endocytosis was measured by phase microscopy and by the acetylcholinesterase method (7, 8). The distribution of spectrin was followed by using TEM and the ferritin conjugate technique described above.

RESULTS

Spectrin Measurement During Endocytosis

A quantitative assay for spectrin was based upon the use of a purified radioiodinated $F(ab')_2$ antispectrin. ^{125}I-$F(ab')_2$ antispectrin provides a sensitive method for spectrin identification because removal of the Fc portion leaves only the specific antibody binding site for interaction with spectrin antigen. Specificity and sensitivity of the $F(ab')_2$ antispectrin used is shown in Fig. 1. Ghost suspensions containing 80 μg of protein were fixed under hypotonic conditions (Materials and Methods); then in one set of control experiments purified spectrin was added in incremental amounts, and in another set of control experiments nonradioiodinated $F(ab')_2$ from nonimmunized rabbits was added in incremental amounts. Subsequently, ^{125}I-$F(ab')_2$ antispectrin was added to all samples which were then incubated and washed as described in Materials and Methods. The addition of 20 μg of purified spectrin to 80 μg of ghost protein (of which 16–20 μg is spectrin) resulted in a 90% inhibition of ^{125}I-$F(ab')_2$ binding (Fig. 1, closed circles). Further addition of purified spectrin to the test mixture showed that 98% of the ^{125}I-$F(ab')_2$ had high affinity for spectrin. Addition of $F(ab')_2$ fragment prepared from normal nonimmunized rabbits did not interfere with the binding of the ^{125}I-$F(ab')_2$ antispectrin to the ghosts (Fig. 1, open circles). Then the binding of radioiodinated $F(ab')_2$ antispectrin was compared with the binding of a radioiodinated $F(ab')_2$ prepared from normal nonimmunized rabbit globulin. Both ma-

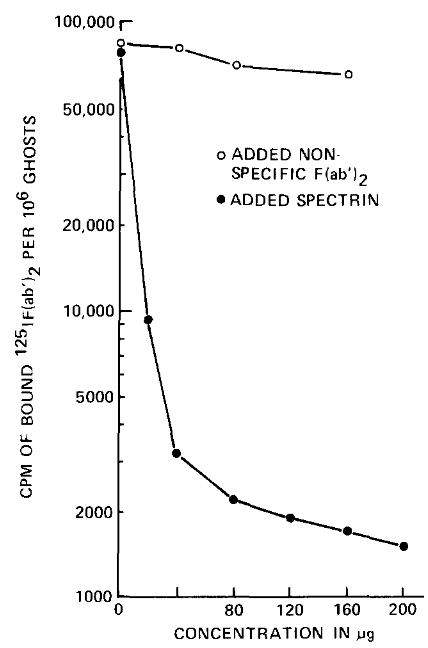

FIGURE 1 Increasing amounts of either purified spectrin (closed circles) or nonradioiodinated $F(ab')_2$ prepared from nonimmunized rabbits (open circles) were added to aliquots of ghosts containing 80 μg of protein, in competitive inhibition studies. The radioisotopic activity of the bound purified $^{125}F(ab')_2$ antispectrin is shown on the ordinate.

terials had the same sp act (see Materials and Methods), and 200,000 cpm of each was added to 20 μl of ghosts containing 80 μg of ghost protein. After incubation and extensive washing, the antispectrin $F(ab')_2$ bound with a value of 105,500 cpm while the comparable value for the nonspecific $F(ab')_2$ was 8,400.

The results of four experiments in which Mg-ATP was used to induce endocytosis are shown in Table I. Blood was obtained from different donors, and duplicate determinations were done for each experimental point. There was a significant decrease in ^{125}I-$F(ab')_2$ antispectrin binding to ghosts during the 8- to 20-min interval, and the trend could frequently be detected by the end of 4 min (Table I). Endocytosis measured either by phase microscopy or by the acetylcholinesterase method (reference 7 and Fig. 4) is usually never detectable before 10 min of incubation and frequently can be accurately detected only after 20 min of incubation. Control samples in which ghosts were incubated only in buffer showed no change in ^{125}I-$F(ab')_2$ antispectrin binding (Table I). After 20–30 min of incubation, there appeared to be a partial restoration of $F(ab')_2$ antispectrin binding which never achieved baseline values (Table I). These findings suggested that early in the endocytic process and generally preceding the appearance of endocytic vacuoles, there was a reproducible decrease in the binding of ^{125}I-$F(ab')_2$ antispectrin to ghosts, and these findings contrasted with the results obtained with control ghosts incubated in buffer where there was no change in ^{125}I-$F(ab')_2$ antispectrin binding during the incubation period (Table I). Endocytosis induced by trypsin may proceed by mechanisms different from those involved in Mg-ATP-induced endocytosis; therefore, three experiments were performed in which trypsin-induced endocytosis was studied (Table II). A decrease in ^{125}I-$F(ab')_2$ antispectrin binding occurred in all experiments and was easily detectable at the 4-min point, becoming most pronounced at

TABLE I

Mg-ATP-Induced Endocytosis, cpm of ^{125}I-$F(ab')_2$ Antispectrin/10^6 Ghosts

Experiment	Time of incubation (min)						P*
	0	4	8	12	20	30	
1	73,657‡	61,502	52,963	59,962	64,850	69,770	<0.0005
	± 327	± 1,603	± 11,305	± 352	± 7,503	± 837	
2	77,787	51,917	20,251	20,639	25,017	27,402	<0.0025
	± 263	± 2,439	± 143	± 4,210	± 448	± 650	
3	74,769	68,770	66,417	58,800	58,560	53,117	<0.001
	± 622	± 425	± 153	± 496	± 549	± 385	
4	74,709	74,967	64,769	59,192	46,797	54,550	<0.025
	± 5,466	± 10,211	± 839	± 241	± 135	± 289	
Control incubation in buffer	77,787	—	78,021	—	79,784	—	

* Comparing 0 time with 12-min point.
‡ Results are expressed as the mean ± SEM.

TABLE II

Trypsin-Induced Endocytosis, cpm of ^{125}I-$F(ab')_2$ Antispectrin/10^6 Ghosts

Experiment	Time of incubation (min)						P*
	0	4	8	12	20	30	
1	88,647‡	65,138	—	60,969	65,312	—	<0.0025
	± 2,068	± 3,855		± 828	± 119		
2	88,121	59,875	43,114	27,708	17,265	29,905	<0.0025
	± 247	± 5,776	± 3,495	± 5,685	± 6,381	± 11,219	
3	78,910	68,675	67,541	53,810	55,208	60,389	<0.01
	± 3,113	± 3,899	± 1,284	± 2,751	± 889	± 771	

* Comparing 0 time with 12-min point.
‡ Results are expressed as the mean ± SEM.

the 12- to 20-min point of the incubation period. After 20 min of incubation, there was again partial restoration of immunoreactive spectrin, and these results are similar to those seen with Mg-ATP-induced endocytosis (Table I).

Spectrin Rearrangement

To determine whether the decrease in spectrin-binding sites was due to a random or a focused depletion of spectrin, experiments were performed in which a purified rabbit IgG antispectrin was allowed to bind to spectrin antigen, after which a ferritin-tagged goat anti-rabbit IgG was added thereby allowing morphologic analysis of spectrin distribution. Omission of antispectrin (Fig. 2*a*) before incubation with ferritin-IgG showed no labeling, indicating that the ferritin conjugate binds uniquely to the rabbit immunoglobulin (see Materials and Methods). When the ghosts were preincubated with normal nonimmunized rabbit IgG, washed, and then incubated with the ferritin-labeled goat anti-rabbit, no labeling of the membrane cytosol face was observed, indicating that a requirement for the assay was the presence of a specific antispectrin antibody. When both antispectrin and ferritin conjugate were used (Fig. 2*b* and *c*), there was a uniform pattern of labeling of spectrin at the cytosol face of ghosts prior to the addition of Mg-ATP.

When ghosts incubated with Mg-ATP for up to 20 min were analyzed, spectrin appeared in discrete areas separated by extensive spectrin-free zones (Fig. 3).

Role of Phosphorylated Spectrin in Endocytosis

Spectrin in the phosphorylated form appears to be the species that interacts with actin (15) and modulates erythrocytic shape changes (2). Therefore, it was reasonable to suppose that dephosphorylation of spectrin might interfere with endocytosis. Ghosts were incubated with various concentrations of alkaline phosphatase. Then, to the control and dephosphorylated ghosts, Mg-ATP, trypsin, or EDTA was added to induce endocytosis. Presumably, neutral enzymes, such as hexokinase and RNase, were also tested in parallel experiments, and in other experiments boiled alkaline phosphatase was tested. Prior exposure of ghosts to alkaline phosphatase blocked all forms of endocytosis (Fig. 4), and the extent of inhibition was dependent on the amount of alkaline phosphatase used. Hexokinase, RNase, and boiled alkaline phosphatase gave the same results as those obtained in the control samples incubated without alkaline phosphatase (latter two not shown) (Fig. 4).

Electron microscope studies were then performed with the ferritin conjugate and antispectrin, and these showed that the addition of Mg-ATP to untreated ghosts resulted in the appearance of spectrin clusters and spectrin-depleted areas (Fig. 5*b*) as before. Prior treatment with 15 μ/ml of alkaline phosphatase completely blocked the expected spectrin rearrangement (Fig. 5*a*). Pretreatment with alkaline phosphatase also blocked the anticipated decrease in immunoreactive spectrin binding (results not shown).

DISCUSSION

In a previous study, membrane internalization in white ghosts was carried to the point where endocytic vacuoles were released and found to be depleted of spectrin. Cross-linking of spectrin with excess bivalent antispectrin blocked the process of endocytosis (7). These observations suggested that spectrin rearrangement might take place before the formation of the endocytic vacuoles. To follow spectrin distribution or disposition in the membrane during early stages of endocytosis, a highly specific purified antispectrin antibody and its $F(ab')_2$ fragment were used.

A decrease in measurable immunoreactive spectrin in the early stages of endocytosis was detected using $^{125}I\text{-}F(ab')_2$ antispectrin (Tables I and II) and could have resulted from actual loss of spectrin, from spectrin rearrangement producing steric hinderance, or both phenomena. It is unlikely that a change in the permeability of the ghost membranes occurred so that the $^{125}I\text{-}F(ab')_2$ antispectrin would have had impaired access to spectrin antigens, because the incubation was performed in hypotonic media and fixation was as described. Spectrin loss cannot account for all of the decrease in immunoreactive spectrin observed (Tables I and II) because later in the process, when endocytic vacuoles could be readily identified, there was a partial restoration of immunoreactive spectrin (Tables I and II). This restoration could reflect a redistribution of spectrin such that more spectrin was available to the $F(ab')_2$ antispectrin probe. Alternatively, loss of a population of spectrin-depleted ghosts could have occurred, leaving relatively spectrin-replete ghosts behind. This possi-

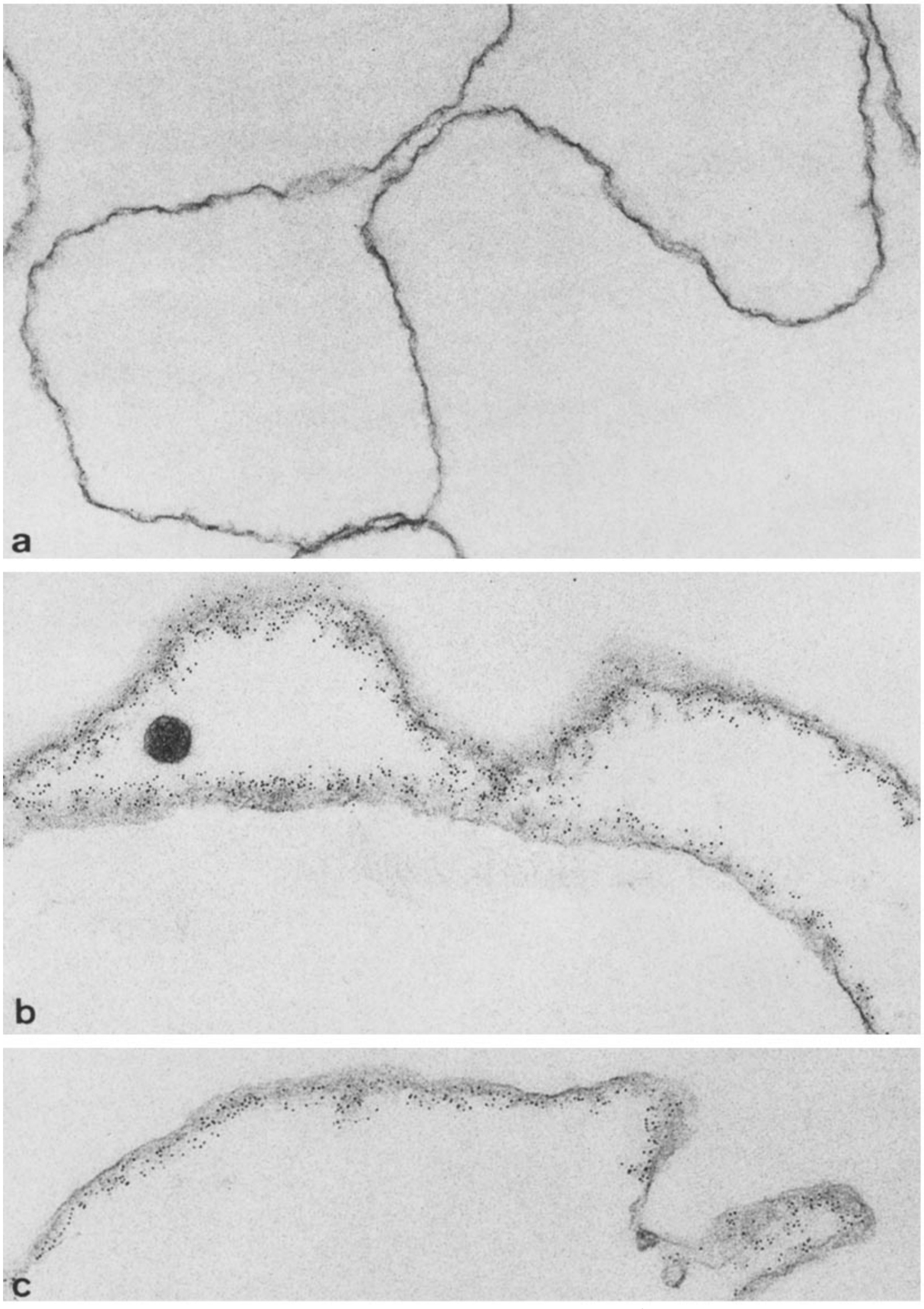

FIGURE 2 (*a*) Untreated ghosts were incubated overnight with goat anti-rabbit IgG conjugated to ferritin. Under these circumstances, there was no ferritin labeling of the membrane. × 50,400. (*b* and *c*) Untreated ghosts were fixed and labeled with an excess of rabbit antispectrin antibodies for 2 h, then washed and incubated with goat anti-rabbit IgG conjugated to ferritin. The uniform distribution of the ferritin particles can be seen along the cytosol face of the membrane. × 60,000.

FIGURE 3 Ghosts were incubated with Mg-ATP for 20 min, fixed, and labeled with rabbit antispectrin antibodies and anti-rabbit IgG-ferritin conjugate. Extensive spectrin-free areas are separated by distinct spectrin-containing zones.

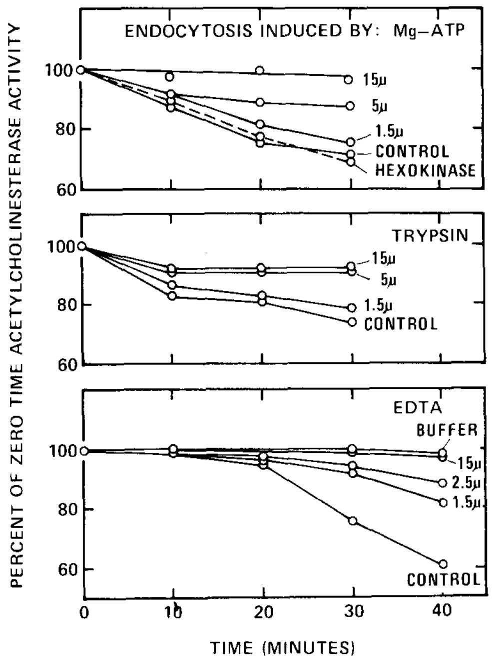

FIGURE 4 Effect of alkaline phosphatase on endocytosis. Endocytosis was induced by Mg-ATP (top panel), trypsin (middle panel), and EDTA (lower panel), and the extent of endocytosis was recorded as the percent decrease in acetylcholinesterase activity on the ordinate. In each case, the control sample contained the inducer of endocytosis and was not pretreated with enzymes. The unitage indicates that the samples had been pretreated with the indicated amounts of alkaline phosphatase to dephosphorylate membrane proteins. In each case, more extensive pretreatment with alkaline phosphatase resulted in incremental graded inhibition of endocytosis. The line-labeled hexokinase indicates that a ghost sample had been pretreated with hexokinase rather than alkaline phosphatase and indicates that nonspecific exposure to enzymes had no inhibitory effect on endocytosis. A "buffer" tube was run with each experiment but is shown only in the lower (EDTA) panel and indicates that in the absence of an endocytic inducer there is no change in acetylcholinesterase activity.

bility appears unlikely because ghost counts were performed regularly, and there was no decrease in cell number. However, the current data cannot definitively resolve this issue.

Morphologic analysis of the patterns of spectrin distribution indicated that before addition of endocytosis inducers, spectrin was homogeneously distributed over the cytosol membrane face as previously described (14) (Fig. 2*b* and *c*). During the early stages of endocytosis and preceding vacuole formation (Figs. 3, and 5*b*), relatively large areas were depleted of spectrin and were separated by discrete spectrin clusters. This pattern of spectrin arrangement observed cannot be reconciled with a random spectrin modification or attack nor with reduced membrane penetrability to the antibody probe, but is consistent with an oriented spectrin attack. Because these spectrin changes were seen with both trypsin and Mg-ATP, they cannot simply represent proteolytic attack on spectrin. Therefore, the creation of spectrin-free areas is thought to be critical in endocytosis.

To test the hypothesis that phosphorylated spectrin was involved in endocytosis (22, 23), ghosts were incubated with increasing amounts of alkaline phosphatase under conditions that are known (2) to dephosphorylate spectrin; however, specific measurements of phosphorylated spectrin were not made. Pretreatment with alkaline phosphatase blocked all forms of endocytosis tested (Fig. 4); moreover the ferritin-labeling method showed that pretreatment of ghosts with alkaline phosphatase blocked creation of spectrin-free areas (Fig. 5*a* and *b*). This observation suggests that the creation of spectrin-free zones is an early and required step in the endocytosis process and raises the interesting possibility that phosphorylated spectrin plays a critical role.

Trypsin and EDTA could modify phosphorylated spectrin interactions to begin the process of endocytosis because of their known ability to attack spectrin. The mechanism by which Mg-ATP induces endocytosis is obscure; however, it could be postulated that the addition of large amounts of Mg-ATP leads to extensive phosphorylation of spectrin (23) which might then interact less readily with itself or more avidly with other proteins.

The experimental results obtained can be used as a basis for formulating a clearer idea of the endocytosis process and for reconsidering some models of erythrocyte membrane function. Agents that cause ghost endocytosis may act initially on phosphorylated spectrin, freeing up loose spectrin interactions and resulting in the formation of spectrin-depleted domains separated by residual spectrin clusters. Spectrin clustering has also been seen in association with the induced clustering of intramembrane particles (20), and pretreatment with antispectrin antibodies blocks the aggregation of intramembrane particles (18). Invagination takes place in the spectrin-free zone. Interestingly, when exocytosis is induced in metabolically depleted erythrocytes, the resulting exocytic vacuoles are

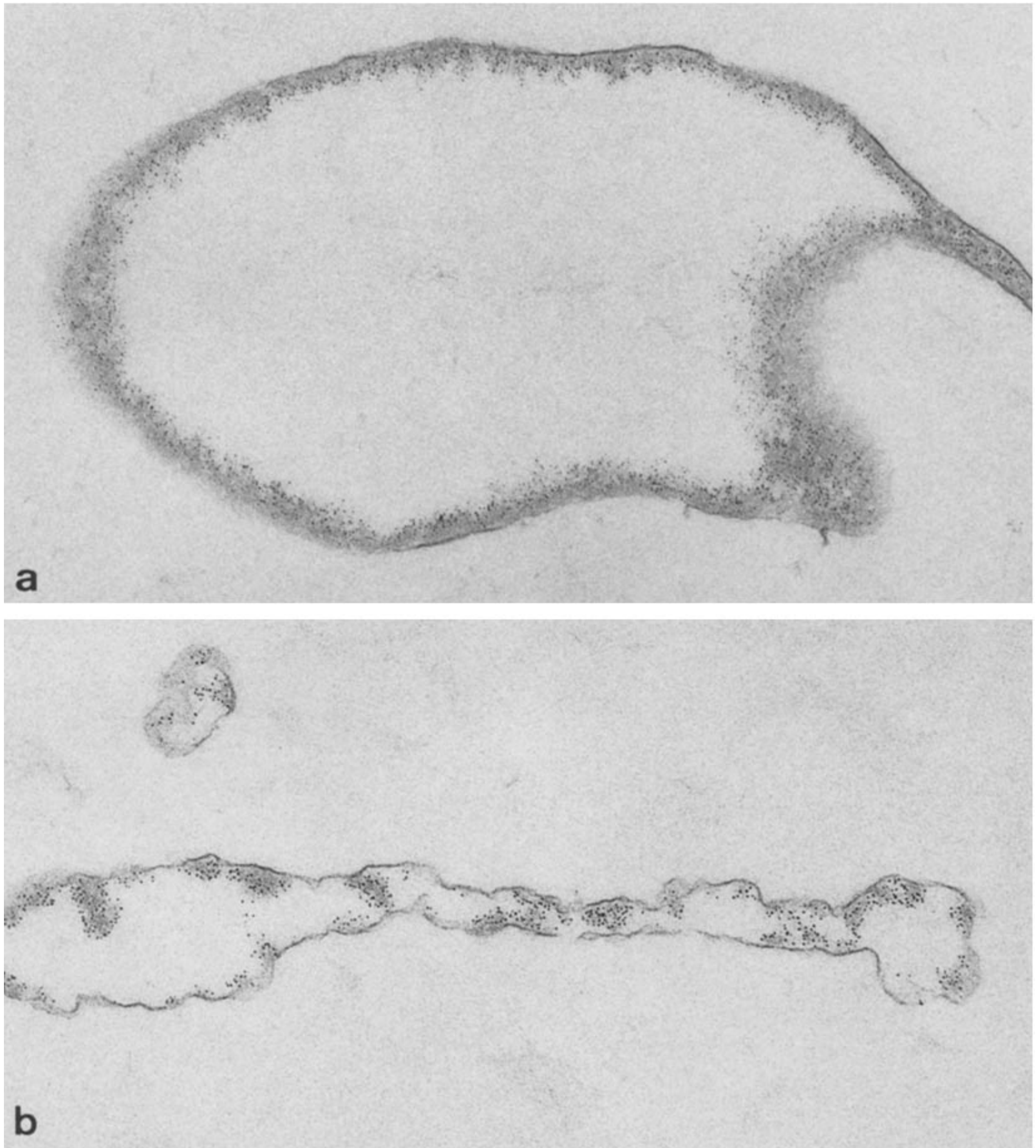

FIGURE 5 (*a*) Ghosts were pretreated with alkaline phosphatase and then exposed for 20 min to Mg-ATP. They were then fixed and stained with the double-labeling method. No spectrin-free zones were observed and a uniform distribution of ferritin was seen along the cytosol face of the membrane. × 48,000. (*b*) Ghosts were exposed for 20 min to Mg-ATP in parallel to the sample shown in Fig. 5*a*. However, no pretreatment with alkaline phosphatase was performed. Note the appearance of spectrin-free areas separated by dense spectrin-containing zones. × 50,000.

free of spectrin and therefore probably also arose from spectrin-depleted membrane segments (11). Subsequently, we propose that membrane fusion occurs in the spectrin-free zone. In fact, spontaneous fusion occurs in the erythrocytes of spectrin-deficient mice (6), and normoblast nuclei are extruded through spectrin-free areas after which fusion takes place (5).

Further study of endocytosis should be directed towards understanding the late events and the patterns of integral membrane protein distribution.

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