Participation of spectrin in Sendai virus-induced fusion of human erythrocyte ghosts

cell fusion/spectrin meshwork/antispectrin antibody/clustering of intramembrane particles

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ABSTRACT Fusion of washed human erythrocyte ghosts could be induced by the addition of Sendai virus after they were loaded with bovine serum albumin and resealed. Antispectrin antibody purified on a spectrin-Sepharose column and sequestered in the ghosts at 4–5 mg/ml together with the albumin was highly inhibitory for the virus-induced cell fusion, whereas Fab fragments prepared from the same antibody were without effect. The virus-induced aggregation of intramembrane particles of human erythrocytes was also inhibited by the same concentrations of the antispectrin antibody. The virus-induced agglutination of the ghosts and release of bovine serum albumin from the ghosts (which might be caused by fusion of the viral envelope to the erythrocyte membrane) were not inhibited by the sequestered antibody. Therefore, the antibody seems to inhibit fusion at the last step—i.e., fusion between adjacent erythrocyte membranes. Similarities and differences of the mode of participation of spectrin in the virus-induced fusion and in other membrane-linked phenomena of human erythrocytes are discussed.

The importance of membrane fusion in a large number of cellular and subcellular processes is now widely recognized, and efforts have been made to clarify the mechanism of membrane fusion at the molecular level (1). Virus-induced fusion of human erythrocytes (2–6) and erythrocyte ghosts (3, 4) is a suitable system for the detailed study of the mechanism of membrane fusion because erythrocytes are devoid of intracellular organelles (thus minimizing the influence of the cytoplasmic structures) and the molecular architecture of their plasma membrane has been extensively characterized (7–9).

Recently, with the use of freeze-fracture electron microscopy, much evidence has been obtained to indicate that redistribution of intramembrane particles takes place during virus-induced (10) and chemically induced (11–14) cell fusion. Because mobility of membrane glycoprotein(s) (15) or intramembrane particles (16–18) seems to be regulated by spectrin and possibly erythrocyte actin (band 5*) (19) which are peripheral membrane proteins of human erythrocytes, structural perturbation of the spectrin–actin meshwork might be expected to influence the virus-induced fusion reaction.

In this paper, inhibition of the virus-induced fusion by antispectrin antibody loaded into erythrocyte ghosts will be described, as well as the inhibitory effect of the antispectrin antibody on the virus-induced aggregation of the intramembrane particles.

MATERIALS AND METHODS

Reagents. Bovine serum albumin (fraction V) and N-[tris(hydroxymethyl)methyl]glycine (tricine) were purchased from Sigma Chemical Co. Na125I was obtained from Dai-ichi Chemical Co. Iodinated bovine serum albumin (125I-BSA) was prepared by a chloramine-T method (20).

Cells and Virus. Human erythrocytes were obtained from a blood bank (Midori-Juji) and used within 4 weeks after they had been drawn. Sendai virus [hemagglutinating virus of Japan (HVJ)] was grown in the allantoic sac of chick embryos and purified as described (6).

Preparation and Fusion of Erythrocyte Ghosts. Ghosts were prepared according to Dodge et al. (21) with 5 mM sodium phosphate (pH 8.0) as hemolyzing buffer. Packed ghosts were washed once with lysing buffer, incubated with 4 volumes of tricine-buffered saline (T/NaCl; 140 mM NaCl/5.4 mM KCl/40 mM tricine-NaOH, pH 7.6) containing 5% bovine serum albumin and other proteins at 0° for 10 min, and then resealed by incubation at 37° for 60 min. Albumin-loaded ghosts were washed twice with T/NaCl and suspended in T/NaCl to give a concentration of 2% (vol/vol). Fusion experiments were carried out as follows. Ghosts were mixed with virus [5000–10,000 hemagglutination units (HAU)/ml] at 0° for 15 min to allow agglutination; then the aggregates were incubated at 37° for 30 min. The release of intracellular albumin during cell fusion was determined with ghosts loaded with 125I-BSA. After centrifugation, the amount of 125I-BSA in the supernatant was measured in a well-type scintillation spectrometer (Aloka Auto-Well gamma system, JDC-751).

Preparation and Purification of Antispectrin Antibody. Spectrin was extracted from ghosts by dialysis against 1 mM EDTA (pH 10) at 4° for 18–20 hr and further purified by gel filtration on Sepharose 4B. Antibody against spectrin was produced in rabbits. The antisera were first fractionated by ammonium sulfate precipitation and then purified by a spectrin-Sepharose column (2–3 mg of spectrin per ml of packed gel) prepared by the method of Cuatrecasas (22). Antispectrin antibody thus purified was 63% precipitable with purified spectrin and formed a single precipitin band on immunodiffusion against solubilized erythrocyte membrane and purified spectrin. Antispectrin Fab fragments were made and purified by the procedure of Porter (23). Antigen binding activity of the Fab fragments was checked by their ability to prevent precipitation of spectrin–125I-labeled antibody complex near the equivalence point. Control immunoglobulin was prepared from nonimmunized sera by ammonium sulfate precipitation.

Electron Microscopy. The samples were fixed by the addition of an equal volume of 4% glutaraldehyde prepared with the same medium and warmed to the temperature of the re-

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Fig. 1. Restoration of fusion ability of human erythrocyte ghosts by loading with bovine serum albumin. Unsealed ghosts prepared by hypotonic hemolysis were resealed by incubation in isotonic buffer containing 5% bovine serum albumin at 37° for 60 min. These resealed ghosts were agglutinated by HVJ (5000 HAU/ml) at 0° for 15 min and then incubated at 37° for 30 min. (A) Ghosts resealed in the absence of albumin. No fused ghosts can be seen. (B) Ghosts resealed in the presence of albumin. Many fused ghosts, appearing as large spheres, can be observed. (Phase-contrast microscopy; X350.)

action. Fixation was performed for 40 min at 37° or for 2 hr at 4° in the case of nonincubated controls. After three washes with the incubation medium, the fixed samples were suspended in 20% (vol/vol) glycerol in physiological saline at 4° overnight. Then freeze-fracture was performed under standard conditions with a Hitachi freeze-fracture apparatus. Electron micrographs were taken with a Hitachi HU-12 electron microscope.

RESULTS

In contrast to intact human erythrocytes, which can be fused easily by HVJ, ghosts prepared in the hypotonic medium lose their ability to fuse (3, 4). We found that virus-induced fusion of ghosts can be observed when the ghosts are loaded with bovine serum albumin and resealed. When such ghosts were agglutinated by HVJ at 0° and then incubated for 90 min at 37°, large fused ghosts that looked like smooth spheres often could be obtained, whereas no fusion was observed when unloaded ghosts were incubated with the virus (Fig. 1).

To clarify the role of the spectrin meshwork in the fusion process, the fusion ability of ghosts in which purified anti-spectrin antibody was included along with the albumin was examined. As shown in Fig. 2B and C, inclusion of antibody within the ghosts strongly inhibited the fusion, although control immunoglobulin had no effect (Fig. 2A). A relatively high concentration of antibody (1–4 mg/ml) was required for inhibition of the fusion reaction, suggesting that extensive crosslinking of spectrin by antibody was necessary. When ghosts were loaded with Fab fragments of the antibody together with albumin, the fusion of ghosts was not affected (Fig. 2D). Thus, it was the crosslinking of spectrin meshwork by the divalent antibody, rather than binding per se, that was responsible for the antibody effects.

The degree of resealing, which might affect the fusion ability of ghosts, was checked by dextran T-70 discontinuous density gradient centrifugation. After centrifugation, more than 90% of ghosts loaded with albumin and antibody were recovered at the top fraction (i.e., sealed ghost fraction), whereas unsealed ghosts sedimented to the bottom, indicating that most, if not all, of antibody-treated ghosts were resealed.

It is generally accepted that virus-induced fusion involves

Fig. 2. Effect of antispectrin antibody on the virus-induced fusion of erythrocyte ghosts. Unsealed ghosts were resealed in isotonic buffer containing 5% bovine serum albumin and various proteins: (A) Control immunoglobulin (4 mg/ml); (B) antispectrin antibody (1 mg/ml); (C) antispectrin antibody (4 mg/ml); (D) Fab fragments prepared from the antispectrin antibody (4 mg/ml). Resealed ghosts were agglutinated by HVJ (10,000 HAU/ml) at 0° for 15 min and then incubated at 37° for 30 min. (Phase-contrast microscopy; X350.)
at least three different steps: agglutination of cells, fusion between viral envelope and cell membrane (i.e., envelope fusion), and fusion between two adjacent cell membranes. When agglutinability of antibody-treated ghosts was compared to that of untreated ghosts by microscopic observation, no significant difference could be detected (data not shown). Because the virus-induced release of hemoglobin (i.e., hemolysis) could be taken as an indication of envelope fusion in case of intact erythrocytes (6, 24–26), the effect of the antibody on the leakage of intracellular albumin from the ghosts was determined. As shown in Fig. 3, the antibody did not exhibit any significant effect on the leakage of intracellular albumin, suggesting that the interaction between viral envelope and ghost membrane was not affected by the antibody.

It has been shown by Bachi et al. (10) that clustering of intramembrane particles is induced during the fusion of intact human erythrocytes. Therefore, we examined the particles during fusion of intact erythrocytes and their ghosts and the effect of the antispectrin antibody on the distribution of the particles. The particles in the protoplasmic fracture face (PF) were dispersed in antibody-treated ghosts, the same as in intact erythrocytes and untreated ghosts (Fig. 4A). When ghosts loaded with albumin and control immunoglobulin were agglutinated at 0° and then incubated at 37° for 30 min, aggregation of the particles was observed, corresponding to the extensive fusion of the ghosts (Fig. 4B). Similar aggregation of the particles also occurred in virus-induced fusion of intact erythrocytes (Fig. 4D). The particles on the external fracture face (EF) also showed nonrandom distribution. Furthermore, pits that might represent the place where the particles were pulled out were seen on EF and they were found to form clusters corresponding to the distribution of the particles on the PF. On the other hand, the antibody sequestered in the ghosts almost completely inhibited the virus-induced particle aggregation under conditions that inhibited the virus-induced fusion (Fig. 4C).

**DISCUSSION**

In this paper, we report that the antispectrin antibody loaded into erythrocyte ghosts was inhibitory for both HVJ-induced

![Fig. 3](image-url) Effect of antispectrin antibody on the virus-induced leakage of intracellular bovine serum albumin (BSA). Unsealed ghosts were resealed in the isotonic buffer containing 5% 125I-BSA and the following proteins as indicated: C, no addition; □, control immunoglobulin (4 mg/ml); ◆, antispectrin antibody (4 mg/ml). Resealed ghosts were agglutinated by HVJ (10,000 HAU/ml) at 0° for 15 min and then incubated at 37° for different periods.

![Fig. 4](image-url) Freeze-fracture pictures of human erythrocyte ghosts, showing effect of antispectrin antibody and HVJ. (x80,000). (A) PF of ghosts loaded with antibody (5 mg/ml). (B) PF of ghosts loaded with albumin and control immunoglobulin (5 mg/ml) after incubation with HVJ (8000 HAU/ml) at 0° for 15 min and then at 37° for 30 min. Extensive fusion in this sample was observed by phase-contrast microscopy. (C) Same as B except that the control immunoglobulin was replaced by the same concentration of the antibody. Almost no fusion was observed in this sample. (D) PF (center to right) and EF (left) of human erythrocytes after incubation with HVJ (800 HAU/ml) for 15 min at 0° and then for 30 min at 37°.
fusion and clustering of intramembrane particles of the erythrocyte membrane. Based on the evidence described, it can be concluded that the antispectrin antibody inhibited the fusion reaction at the last step—i.e., fusion between adjacent cell membranes. Because Fab fragments of the antibody seques-
tered in the ghosts did not affect the virus-induced fusion and, furthermore, the antibody-to-spectrin ratio required for max-
imal precipitation of spectrin from the solution of purified spectrin was almost the same as that needed for maximal in-
hibition of the virus-induced fusion, extensive crosslinking of spectrin seemed to be required for the inhibition.

As reported by Elgsæter and Branton (16), substantial re-
moval of spectrin is required for the particle aggregation that is induced by a decrease in charge repulsion. Aggregation of surface glycoprotein(s) that is (are) stained by cationic colloidal iron hydoxide also is induced by the addition of antispectrin antibody (0.25–1 mg/ml) at low ionic strength (15). Under almost identical conditions, similar but lesser aggregation of the particles has been observed by freeze-fracture technique (data not shown). Thus, spectrin seems to participate in control of the movement of surface glycoprotein(s) and intramembrane particles. However, the role of this control in biological phe-
omena is not clear from the experiments described in these reports.

Recently, Sheetz and Singer (27) reported that ATP-depen-
dent changes of erythrocyte ghosts to the disk shape and then to the cup shape are markedly accelerated by antispectrin antibody within the ghosts in amounts less than equivalent to the spectrin present. However, when the same antibody was loaded into the ghosts to the spectrin–antibody equivalent point, these changes did not occur. They also found that Fab frag-
ments of the antibody are ineffective. Although the stimulatory effect of the antibody is not clearly observed in the virus-in-
duced fusion, our results agree with theirs as to the inhibitory effect of high concentrations of the antibody and ineffectiveness of the Fab fragments. Thus, spectrin seems to participate in both ATP-dependent shape changes and virus-induced fusion of human erythrocytes. The mode of participation of spectrin is apparently somewhat different in the two phenomena because there is no ATP requirement and Ca<sup>2+</sup> at 1 mM is not appreciably inhibitory (ref. 3; K. Sekiguchi, unpublished data) for the virus-induced cell fusion, whereas the same concentration of Ca<sup>2+</sup> completely inhibits the ATP-induced shape changes. Therefore, changes in membrane structure that are required for shape changes and for membrane fusion may not be iden-
tical.

The redistribution of intramembrane particles that accom-
panies membrane fusion has been reported in the virus-induced (10) and chemically induced (11–14) cell fusion and fusion of the other specialized membranes (28–32). From these and other results, it has been postulated that redistribution of the particles and formation of an area of phospholipid bilayer devoid of the particles are prerequisite for the membrane fusion (10, 11, 28). We confirmed the results of Bächler et al. (10) on the virus-
induced fusion of human erythrocytes and extended them to the fusion of the erythrocyte ghosts. But it remains difficult to de-
cide whether cluster formation of the intramembrane particles is a prerequisite for the fusion reaction or a result of the virus treatment. As reported in this paper, no movement of the parti-
cles was observed in the ghosts loaded with the antibody under conditions that inhibited the virus-induced cell fusion. There-
fore, these results seem to support the hypothesis (10, 11, 28) mentioned above.

Recently, Fowler and Branton (33) reported that mobility of erythrocyte surface glycoproteins labeled with fluorescein

isothiocyanate was greatly increased after the virus treatment. Because mobility of the surface proteins was almost negligible in intact erythrocytes (34, 35), and the spectrin–actin meshwork could play a role in regulation of the mobility of the surface proteins, their results indicate that interaction either between components of the spectrin–actin meshwork or between the meshwork and the surface proteins is modified by the virus action. From our results it is probable that the interaction be-
tween spectrin and the particles is retained to some extent after the virus treatment in the presence of the antispectrin antibody. This is compatible with the fact that spectrin is not released appreciably from the erythrocytes during the fusion reaction (ref. 33; K. Sekiguchi, unpublished data). The relationship be-
tween the virus-induced clustering of intramembrane particles and mobilization of surface proteins reported by them is not well understood at present, but these may be slightly different phenomena. For example, ATP has no effect on the virus-
induced fusion as described above, whereas ATP exhibits some stimulatory effect on the diffusion of the surface proteins (33).

To examine the effect of antispectrin antibody on the fusion of erythrocytes, it was necessary to develop an assay system for the fusion of erythrocyte ghosts. Ghosts prepared by hypotonic hemolysis according to Dodge et al. (21), however, could not be fused by HVJ but could be agglutinated by the virus (data not shown). Although Peretz et al. (3) have shown that ghosts prepared by gradual and partial (90%) hemolysis in the presence of bovine serum albumin still retained their fusion ability whereas ghosts prepared by rapid and complete hemolysis could not undergo virus-induced fusion, they attributed the loss of fusion ability after cell lysis in hypotonic medium to the leakage of some of membrane component(s), which resulted in subsequent structural alteration in the erythrocyte membrane. We found, however, that ghosts prepared by rapid and complete hemolysis regain their fusion ability after loading of bovine serum albumin into the ghosts, although the amount of the virus required to obtain the same degree of the fusion is several times more than that required in case of intact erythrocytes. The role of intracellular albumin in fusion of ghosts is not understood at present, but some other macromolecules, such as ovalbumin and dextran, are also effective to some extent for restoration of fusion ability of ghosts (K. Sekiguchi, unpublished data). With this type of ghost, several treatments that affect the cytoplasmic surface of the ghosts can be easily applied to provide a useful system for the study of the mechanism of cell fusion.

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