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Low-pH association of proteins with the membranes of intact red blood cells. I. Exogenous glycophorin and the CD4 molecule

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Glycophorin and CD4 proteins are tightly associated with intact human erythrocyte membranes after a short-time incubation at low pH (1–2 min, pH lower than 5, 37°C). Flow cytometry and epifluorescence microscope observations showed that after incubation of red cells with fluorescein isothiocyanate (FITC) labeled glycophorin at pH values lower than 5, the erythrocyte membrane and subsequently formed ghost membranes were fluorescent. Unlabeled glycophorin was reacted with mouse erythrocytes using the same low-pH conditions. Flow cytometry and fluorescence microscopy showed that anti-glycophorin monoclonal antibodies were able to recognize the epitopes of glycophorin associated with the mouse erythrocytes. Kinetic experiments showed that the interaction of FITC-glycophorin with red cell membranes can be monitored by a decrease in the fluorescence intensity. Erythrocyte associated glycophorin was not removed from the membranes after 24 h incubation in human plasma (in vitro, 39°C). A glycoprotein extract containing CD4 was isolated from a T4-lymphoma cell line (CEM). This protein extract was incubated with erythrocytes using the same low-pH conditions. Fluorescently labeled monoclonal antibodies against CD4 stained the red cells after association of CD4 with the membranes. Electron microscopy showed 10 nm immunoglobulin G-coated gold beads associated with CD4-bearing erythrocyte membranes after incubation with anti-CD4 antibodies and then with the gold beads. The potential use of the CD4-erythrocyte complex as a therapeutical agent against acquired immune deficiency syndrome (AIDS) is suggested.

Introduction

Proteins which are inserted, covalently bound or tightly associated with the erythrocyte plasma membrane may have important implications in targeting erythrocytes to specific cells as well as for using red cells in the design of new therapeutic agents [1].

Experiments using lipid model membranes indicate that low pH triggers protein penetration into lipid bilayers. Thus, colicin E1 requires acidic pH to form in vitro ion channels in artificial membranes [2], and it was shown that the maximum at pH 4.0 of the channel-forming activity of this protein can be attributed to membrane binding [3]. The requirement of an acidic pH was also shown for the binding and transmembrane channel formation of diphtheria toxin in planar lipid bilayers [4]. Insertion of diphtheria toxin into unilamellar lipid vesicles also became significant at pH values close to 5 [5]. Except for vesicle charge, the process was independent of the nature of the lipid polar groups used.

In this report, the interaction of two intrinsic membrane proteins, glycophorin and CD4, with the erythrocyte membrane at low-pH was investigated. The well-characterized intrinsic membrane glycoprotein, glycophorin, was used as a model for CD4, another intrin-

Abbreviations: AIDS, acquired immune deficiency syndrome; Anti-T4-FITC, FITC-labeled monoclonal antibodies against lymphocyte T4 molecule; Au-IgG-10, 10 nm gold beads coated with immunoglobulin G; BSA, bovine serum albumin; CD4, antigen on helper-inducer T lymphocytes, monocytes; FITC, fluorescein isothiocyanate; Gam-IgG-Pe, phycoerythrin-labeled goat antimouse immunoglobulin G; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; HIV, human immunodeficiency virus; IgG, immunoglobulin G; kDa, kilodalton(s); Leu-3a-Pe, antihuman Leu-3a phycoerythrin conjugate anti-CD4 antibody; OKT-4A-FITC, OKT 4A monoclonal antibody FITC-conjugate anti-human inducer-helper T cell; PBS(7.4), 5 mM sodium-phosphate buffer, 145 mM NaCl (pH 7.4); PMSF, phenylmethylsulfonyl fluoride; RBC, red blood cells; Tris, tris(hydroxymethyl)aminomethane; SDS, sodium dodecyl sulfate.

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sic membrane glycoprotein, since glycophorin is readily available in pure form. The interaction process of glycophorin with the erythrocyte membrane was fast, with half-times between 20 s and 60 s; the rate of the reaction and quantity of protein associated with the erythrocyte membrane increased with decreasing pH between pH 5.0 and pH 3.6. Immunofluorescence, flow cytometry and immunoelectron microscopy observations showed that after the low-pH treatment, CD4 and glycophorin molecules maintain the exposure of functional epitopes, being recognized after association with the erythrocyte membranes by corresponding monoclonal antibodies.

Red blood cells bearing CD4 may have therapeutical applications, since erythrocytes bearing CD4 could be efficient in binding human immunodeficiency virus (HIV) in the blood stream of AIDS patients. They could also form aggregates with HIV-infected cells exposing the viral envelope protein gp120, aggregates which should be eliminated from circulation by the reticuloendothelial system [1].

Materials and Methods

Chemicals. Fluorescein isothiocyanate isomer I (FITC), bovine serum albumin (BSA), glycophorin from human blood type B, MN, MM and NN (predominantly A), phenylmethylsulfonyl fluoride (PMSF), *n*-octyl-4-β-D-glucoside, methyl-α-D-mannopyranoside, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (Hepes) and tris(hydroxymethyl)aminomethane (Tris) were from Sigma (St. Louis, MO). Formvar and phosphotungstic acid were from Electron Microscopy Sciences (Fort Washington, PA). Gold beads coated with immunoglobulin G (Au-IgG-10) were from Boehringer-Mannheim (Indianapolis, IN). The following anti-CD4 monoclonal antibodies were used: monoclonal antibodies anti-T4 molecule lymphocytes FITC conjugated, (Anti-T4-FITC), were from Pel-Freez Clinical Systems (Brown Deer, WI); OKT 4A monoclonal antibodies FITC conjugate anti-human inducer-helper T cell (OKT-4A-FITC) were from Ortho Diagnostic Systems Inc., (Raritan, NJ); anti-human Leu-3a phycoerythrin conjugate (Leu-3a-Pe) antibodies were from Becton Dickinson Monoclonal Center Inc. (Mountain View, CA). The anti-glycophorin A monoclonal antibody 10F7 was kindly provided by Dr. R.H. Jensen from the Biomedical Sciences Division, Livermore National Laboratory, University of California and has been described [6]. Goat antimouse immunoglobulin G labeled with phycoerythrin (Gam-IgG-Pe) was obtained from Molecular Probes Inc. (Junction City, OR). Sodium dodecyl sulfate (SDS), acrylamide, bisacrylamide, nitrocellulose and goat-antimouse-IgG alkaline phosphatase were from Bio-Rad (Richmond, CA).

Glycophorin MN type A (heated at 80 °C for 5 min) showed a single band of its dimer at 66 kDa on 7.5% SDS polyacrylamide gel electrophoresis [7] (results not shown). For immunoblotting the glycophorin was transferred from 7.5% polyacrylamide gels to nitrocellulose and the blots were blocked with 3% gelatin, 20 mM Tris, 500 mM NaCl (pH 7.5) (TBS) for 1 h at 22 °C and incubated with the 10F7 monoclonal antibody to glycophorin A for 3 h at 22 °C. The blots were washed twice with 20 mM Tris, 500 mM NaCl, 0.05% Tween 20 (pH 7.4) (TTBS) and then incubated with goat antimouse IgG alkaline phosphatase for 1 h at 22 °C. The blots were washed again twice with TTBS and once with TBS. The gel was then incubated in the color development reagent BCIP/NBT from Bio-Rad [8] for 15 min until color developed. Using this procedure glycophorin MN was detected as monomer (≈ 32 kDa), dimer (≈ 66 kDa), trimer (≈ 99 kDa) and tetramer (≈ 130 kDa) (results not shown).

Extraction and partial purification of CD4. A T4-lymphoma cell line (CEM) was used as a source of CD4. The membrane bound glycoprotein was detergent solubilized using approx. 1.75×10^8 cells/ml of extraction medium which consisted of 200 mM *n*-octyl-β-D-glucoside, 150 mM NaCl, 10 mM Tris and 0.2 mM PMSF (pH 7.4). Extraction was effected by gently pipetting in the medium for 2–3 min at room temperature. Nuclei were pelleted by centrifugation (12000 × *g*, Sorvall RC-5B centrifuge, for 10 min) and the resulting supernatant was loaded onto a pre-equilibrated lentil-lectin column [9]. The bound glycoproteins were eluted with 0.3 M methyl-α-D-mannopyranoside in an elution medium of 0.05 M Hepes buffer, 0.15 M NaCl and 1% *n*-octyl-β-D-glucoside. After concentration by ultrafiltration, the recovery of CD4-immunoreactivity was assessed using an immunodot blotting method [10]. Aliquots of solutions containing CD4 in the elution medium were used in the experiments.

Red blood cell (RBC) isolation. Erythrocytes were prepared from fresh whole blood obtained from Gulf Coast Regional Blood Center, Houston, TX. A volume of fresh whole blood was diluted with the same volume of 5 mM phosphate buffer (pH 7.4), 145 mM NaCl (PBS(7.4)) and concentrated by centrifugation (approx. 640 × *g*) for 30 min at 4 °C. The supernatant and the buffy coat were removed, the RBC pellet was resuspended in PBS(7.4) and washed three times with PBS(7.4). The RBC concentration in stock solutions was determined in a hemocytometer.

Mouse RBC were isolated from 0.4–0.5 ml of fresh blood taken from the orbital sinus vein of the mouse. The isolation procedure was the same as for human RBC.

Protein labeling with FITC. Covalent binding of fluorescein isothiocyanate to proteins was performed according to Goldman [11]. 0.9 ml of protein suspension

in 0.145 M NaCl (i.e., 0.5 mg protein) was mixed with 100 μl of sodium carbonate buffer (5 mM of 145 mM NaCl, pH 9.5) containing 0.4 mg FITC. This mixture was incubated in the dark at 24°C for 1.5 h. The unbound FITC was then separated from the proteins by filtration through Sephadex G25 columns. The elution buffer was PBS(7.4). The fractions containing the fluorescein-labeled proteins in PBS(7.4) were easily recognized under a fluorescence lamp. Protein concentrations of the samples were calculated from absorbance measurements at 280 nm after correcting the absorbance of FITC at that wavelength [11]. The amount of FITC bound to glycophorin was calculated from the absorption spectrum of labeled proteins in the presence of 0.1% SDS using an absorption coefficient at 496 nm of 80000 for fluorescein bound to protein [12,13]. An average labeling of one FITC molecule per one monomer of glycophorin was obtained. For these calculations we have considered glycophorin to be a monomer although it is known to exist as a dimer in SDS [14].

Flow cytometry. Flow cytometry was performed on a Coulter EPICS Profile instrument. Fluorescein and phycoerythrin fluorescence were measured with a 525 nm and a 575 nm band pass emission filter, respectively. The following histograms were collected for analysis: (i) 90° sidescatter vs. forward angle scatter; (ii) log of green fluorescence vs. number of cells; (iii) log of red fluorescence vs. number of cells.

Measurements of FITC – Glycophorin emission intensity in the absence and presence of Triton X-100. 20 μl FITC-labeled glycophorin solution in PBS(7.4) (1.66 μg/ml final glycophorin concentration) was added to 2 ml of buffer, and the emission spectrum between 490 nm and 600 nm was recorded, excitation at 470 nm. The following buffers were used: 0.02 N sodium acetate buffer, 145 mM NaCl in the pH range from 3.6 to 5.4; 4 mM sodium phosphate buffer, 145 mM NaCl (PBS), for the pH values between 5.8 and 8.0; 5 mM sodium carbonate buffer, 145 mM NaCl for pH 8.5. After recording the fluorescence spectra at different pH values, the same samples were treated with 20 μl of a solution of Triton X-100 with gentle shaking (the final cuvette concentration of Triton X-100 was 0.2%, v/v). After incubation with the detergent for 5 min at room temperature, the emission spectra of the protein/detergent suspensions were recorded.

Measurements of fluorescence polarization were made with a Perkin-Elmer LS-5 spectrofluorimeter equipped with filter polarizers in the excitation and emission beams ($T = 22°C$). Fluorescence was excited at 450 nm, and the emission intensities were measured at 518 nm. Fluorescence polarization degrees were calculated from the equation:

$$p = (I_{0,0} - G \cdot I_{0,90})/(I_{0,0} + G \cdot I_{0,90}) \quad (1)$$

where G is a correction factor which according to Ref. 15 can be obtained as $G = I_{90,0}/I_{90,90}$. $I_{m,n}$ are the fluorescence intensities. The subscripts refer to the positions of polarizers in the excitation and emission beams relative to the vertical (z-axis). A correction for light scatter was made for each fluorescence intensity by subtracting the value obtained for FITC-unlabeled sample, under identical conditions.

Low-pH incubation of proteins with erythrocytes. In a typical assay, 1.5 ml of sodium acetate buffer (acid-base pair 0.02 N $CH_3CO_2H/NaOH$ (pH 4.7), 145 mM NaCl), 60 μl of protein solution (approx. 0.5 μM final protein concentration) and 30 μl of stock RBC suspension (approx. $1.4 \cdot 10^7$ RBC) were mixed and incubated at 37°C for 1.5 min (all the solutions prior to the mixing were equilibrated at 37°C). After incubation, the RBC were concentrated by centrifugation of the reaction mixture at 3000 rpm, for 4 min, in an Eppendorf centrifuge. The RBC pellet was resuspended in PBS(7.4) and washed twice with PBS(7.4).

Kinetic measurements of protein–erythrocyte interaction. The time changes in fluorescein emission intensity, after adding RBC suspensions to FITC-labeled protein solutions, were monitored at different pH values. A typical assay mixture in the cuvette contained 2 ml of buffer (pH indicated in the text), 7 μl of protein extract (about 30 nM protein final concentration) to which 10 μl of the stock RBC suspension was added (about $5 \cdot 10^6$ cells) at time zero. The pH values indicated in the text were obtained in separate experiments from mixtures of the three components. Fluorescence measurements were performed on an SLM-8000 spectrofluorometer in a thermostated sample holder equipped with a magnetic stirrer. Fluorescein was excited at 460 nm and emission was monitored at 518 nm. After addition of RBC to the cuvette, the changes in protein FITC fluorescence were recorded. Triton X-100 was added (0.2% (v/v) final concentration in the cuvette) after steady-state was reached (spectra were recorded after 4 min of equilibration). The changes in FITC fluorescence (after subtracting the filter effects due to the addition of RBC) were normalized to the FITC fluorescence intensities of the samples after the Triton X-100 treatment. Triton X-100 solubilizes all proteins and lipids into micelles, offering to all FITC-labeled proteins in the mixture a similar hydrophobic environment. Thus, the percentage of proteins associated with the RBC from the initial amount of proteins in the cuvette, can be defined as:

$$\% \text{ association} = [(F_{eq} - F_T)/(F_i - F_T)] \times 100 \quad (2)$$

where F_i is the initial FITC fluorescence of the mixture immediately after the sharp drop in the fluorescence due to filter effects of erythrocytes; F_{eq} is the fluorescence intensity at equilibrium, and F_T is the fluorescence of the Triton X-100-treated sample. Stained

cells were viewed using an epifluorescence Zeiss Universal microscope and photographs were taken on Kodak Ektachrome 800 film.

Incubation of erythrocytes with antibodies. The staining of cells with antibodies was performed at room temperature. In the case of glycophorin experiments, $3 \cdot 10^7$ mouse RBC were incubated with 6 μg of primary antibody 10F7 against glycophorin, in a total volume of 10 μl PBS(7.4) for 30 min, washed twice and incubated with 10 μg of secondary antibody Gam-IgG-Pe in 100 μl PBS(7.4). After two washings with PBS(7.4), the erythrocytes were resuspended in 100 μl PBS(7.4) and kept at 4°C prior to analysis by flow cytometry. As a control, in the studies of glycophorin recognition by anti-glycophorin, the mouse RBC were incubated at low pH in the presence of BSA (same concentrations as for glycophorin), since the presence of BSA reduced the hemolysis. Incubation with anti-CD4 antibodies is described below.

Electron microscopy. After low-pH incubation with CD4-extract, human erythrocytes ($1.4 \cdot 10^7$ cells in 20 μl PBS(7.4)) were incubated with a mixture of anti-T4-FITC (≈ 2 μg), OKT-4A-FITC (≈ 0.5 μg), and Leu-3a-Pe (≈ 0.5 μg) for 15 min at 24°C. Erythrocytes were concentrated by centrifugation and resuspended in 0.5 ml of PBS(7.4). 30 μl of an undiluted suspension of 10 nm gold beads coated with goat antimouse immunoglobulin G (Au-IgG-10) were added. Erythrocytes were incubated with the beads for 30 min at 24°C. After washing twice with PBS(7.4) aliquots were loaded into gold cups (Balzers Union BB 113 142-1) and frozen by immersion in liquid propane. Samples were transferred to and remained in liquid nitrogen until fractured. In control experiments, erythrocytes which had been incubated at low pH in the presence of BSA were treated with anti-CD4 antibodies and Au-IgG-10 as described. All samples were fractured at −110°C in a Balzers BAF 400 D freeze-fracture apparatus. Etching time was 90 s at −110°C. Pt-C replicas were recovered on Formvar covered 300 mesh Cu grids and cleaned with bleach. Replicas were examined using a Philips 420 electron microscope operated at 100 kV.

For negative staining, erythrocytes with CD4 were incubated with the same mixture of monoclonal anti-CD4 antibodies followed by Au-IgG-10 beads. After washing with PBS(7.4), 10^7 cells in 10 μl PBS(7.4) were lysed by the addition of 0.5 ml of phosphate buffer, (5 mM, pH 8.0). The resulting ghosts were stained with a 2% aqueous solution of phosphotungstic acid (pH 7.2). In control experiments, erythrocytes which had been incubated at low pH in the presence of BSA were treated with anti-CD4 antibodies and Au-IgG-10, lysed and stained as described for the CD4-containing erythrocytes.

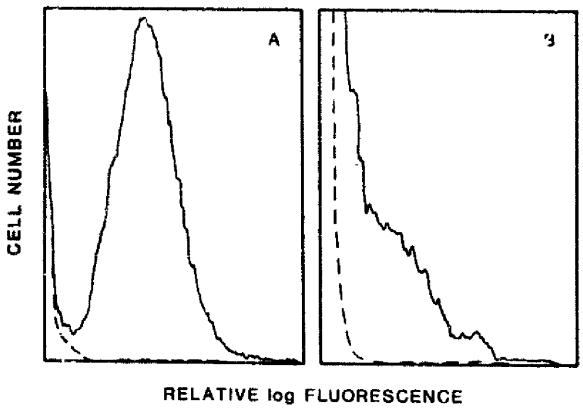

Fig. 1. (A) Fluorescence histogram of human erythrocytes after interaction with FITC-labeled glycophorin. FITC-glycophorin was incubated at pH 4.7 with RBC as described in Materials and Methods. The fluorescence intensity of a RBC population of 10000 cells was analyzed by flow cytometry (———). The graph displays the relative log of FITC fluorescence versus cell number. Control (- - - - -) represents the fluorescence background of erythrocytes that had been incubated at low pH in the absence of FITC-glycophorin and presence of BSA. (B) Fluorescence histogram of mouse erythrocytes after glycophorin–RBC interaction and incubation with fluorescently labeled anti-glycophorin antibodies (———) represents the fluorescence profile of 10000 mouse erythrocytes containing glycophorin in the membranes after being incubated with anti-glycophorin 10F7 and Gam-IgG-Pe antibodies. Control (- - - - -), represents mouse RBC incubated at low pH with BSA and then carried through the incubation with antibodies as in the case of glycophorin.

Results

Fluorescence emission of RBC after incubation with FITC-labeled glycophorin

Short incubation (1.5 min) of RBC with FITC-glycophorin at pH 4.7 resulted in a uniform staining of the erythrocytes (photographs not shown). Erythrocyte ghosts formed by hypotonic lysis after incubation of RBC with FITC-glycophorin also showed fluorescence associated with the ghost membranes (data not shown). To examine the fraction of the human RBC population which was stained with FITC-glycophorin, erythrocytes were analyzed by flow cytometry after the low-pH incubation in the presence of glycophorin and subsequent washings (Fig. 1). These studies indicated that 100% of the red blood cells were fluorescent, with a sharp cell population profile. To determine whether the glycophorin molecules were in a state which could be recognized by glycophorin antibodies after the low-pH incubation with RBC, we used mouse RBC. Using the same low-pH procedure, glycophorin molecules (not FITC-labeled) were incubated with mouse erythrocytes and then incubated with antiglycophorin 10F7 antibodies. The presence of these antibodies on the surface of the mouse RBC was shown using the second fluorescently labeled antibody, Gam-IgG-Pe (Fig. 1B). Since single-cell fluorescence was measured by the flow cytometer, Fig. 1B shows that the glycophorin epitopes

are present on the mouse erythrocyte surface. Incubation of glycophorin-bearing mouse RBC with antiglycophorin 10F7 and Gam-IgG-Pe antibodies at higher antibody concentrations, induced the formation of large cell aggregates (photographs not shown).

Effect of Triton X-100 on the fluorescence spectra of FITC-labeled glycophorin

Non-ionic detergents such as Triton X-100 have been used as a replacement for the lipid bilayers in determining the conformation of integral-membrane proteins [16–20]. Thus, the differences that occur in the fluorescence spectrum of a protein solution after addition of mild non-ionic detergent show the direction of fluorescence changes associated with protein-membrane association events.

To address the question of whether glycophorin interacts with Triton X-100 micelles, we measured the FITC-glycophorin polarization degree before and after addition of Triton X-100. Thus, after addition of Triton X-100 to a FITC-glycophorin solution, the polarization degree of FITC-glycophorin increased from $p = 0.07 \pm 0.01$ to $p = 0.16 \pm 0.02$ at pH 7.4 (PBS(7.4)) and from $p = 0.24 \pm 0.02$ to $p = 0.37 \pm 0.02$ at pH 4.7. These increases in polarization degree after detergent addition indicate the formation of Triton-glycophorin complexes at physiological and low pH values.

The addition of Triton X-100 to FITC-glycophorin solutions at different pH values caused strong changes in the fluorescein emission intensity compared to that measured in the absence of detergent at the same pH (Fig. 2). As can be seen in Fig. 2B, depending on pH, Triton X-100 had two opposite effects on FITC fluorescence of glycophorin in aqueous suspensions: it caused a decrease in the fluorescence below pH 5.4 and an increase in the fluorescence intensity above pH 5.4. These results indicate that an interaction of FITC-glycophorin molecules with the hydrophobic region of the erythrocyte membrane will be shown by a decrease in fluorescence intensity, if the pH of the reaction is below 5.4, but that an interaction at pH values greater than pH 5.4 will cause an increase in FITC fluorescence. Experiments similar to those presented in Fig. 2 showed that Triton X-100 reduces the fluorescence intensity of free FITC in the entire pH range studied.

Time course of the low-pH interaction of glycophorin with erythrocytes

When a human RBC suspension was added to a FITC-glycophorin solution in sodium acetate buffer (final pH of the mixture being 4.7), an initial fast drop in FITC fluorescence occurred, due to filter effects of the red blood cells. The fluorescein emission continued to decrease, reaching steady-state conditions in about 5 min (half lifetime of 40 s) (Fig. 3). The addition of Triton X-100 to the mixture (Fig. 3A, curve 3) resulted

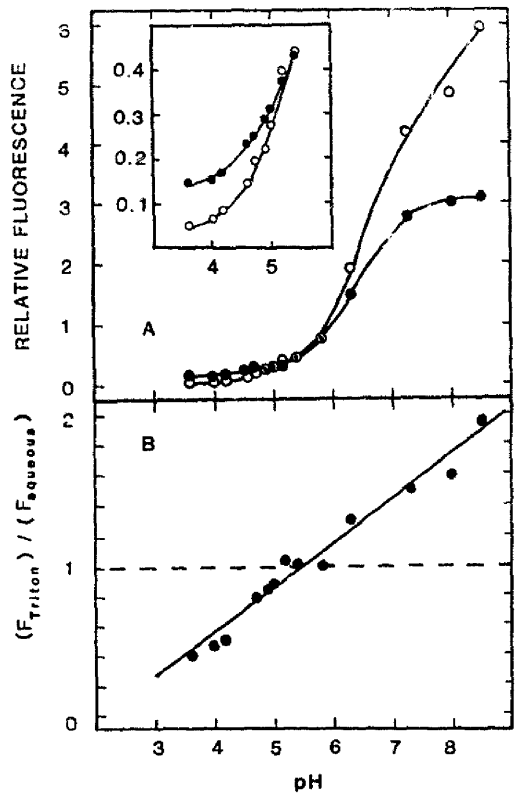

Fig. 2. (A) pH dependence of FITC-glycophorin emission intensity in the absence and presence of Triton X-100. ●, pH dependence of fluorescein emission intensity at 518 nm of FITC-glycophorin in the absence of Triton X-100. ○, pH dependence after addition of Triton X-100. The inset in Fig. 2A is another scale representation of the fluorescence intensities in the pH interval between 3.5 and 5.4. (B). From the data in Fig. 2A, the ratios of $(F_{Triton}/F_{aqueous})$ are represented as a function of pH. $F_{aqueous}$ and F_{Triton} represent the fluorescence intensity of FITC-glycophorin at 518 nm before and after addition of Triton X-100, respectively. Lines were drawn for clarity only and have no theoretical significance.

in a further decrease of the fluorescence intensity at 518 nm and a stronger appearance of a fluorescence intensity maximum at 560 nm. The fluorescence intensity at 518 nm of the Triton X-100 treated sample was considered to be a reference value for the determination of the percentage of association (see Eqn. 2). The analysis of the Triton X-100 effect on FITC-glycophorin fluorescence (Fig. 2) indicated that the reduction in FITC fluorescence intensity during incubation with RBC (Fig. 3) was related to conformational changes of glycophorin, changes which occurred due to increased hydrophobicity of the protein surrounding. Thus, the results in Fig. 3 suggest that FITC-glycophorin interacts with the lipid component of the erythrocyte membrane.

pH dependence of glycophorin interaction with erythrocytes

Experiments similar to those presented in Fig. 3 were performed at different pH values. From the recorded

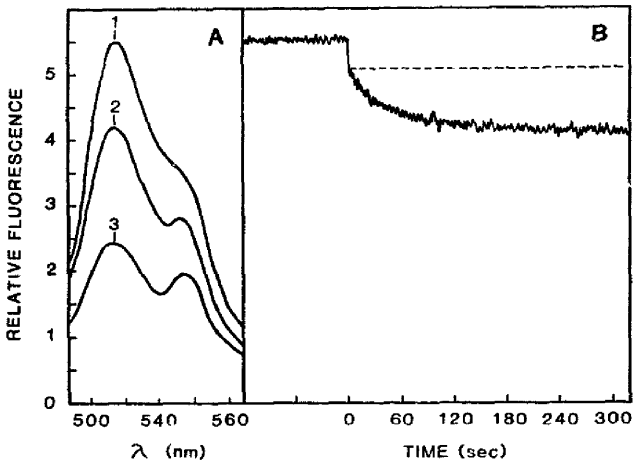

Fig. 3. (A) Emission spectra of FITC-glycophorin before (curve 1) and after (curve 2) mixing of a FITC-glycophorin solution with human erythrocytes at pH 4.7. Curve 2 represents the emission spectrum of the mixture at equilibrium. Curve 3, emission spectrum after reacting the mixture with 1% (v/v) Triton X-100. (B) Time dependence of the changes at 518 nm of FITC-glycophorin intensity after adding human erythrocytes to the glycophorin suspension. The continuous line corresponds to the changes in fluorescence intensity occurring at pH 4.7 during the transition from curve 1 to curve 2 in Fig. 3A. The dashed line (pH 7.4) is an example of no changes in fluorescence intensity which was the case when the incubation was performed at pH values between 5.8 and 8.5.

curves of the time-evolution of FITC-glycophorin emission intensity, we determined interaction half-times and the relative amount of membrane-associated protein (Fig. 4). These results show that the glycophorin-association process with RBC is substantial below pH 5 and that no interaction between glycophorin and erythrocytes occurred in the pH range from 5.4 to 8.5. Epifluorescence microscope observations of erythrocytes incubated with FITC-glycophorin at pH values higher than 5.4 (30 min, 37°C) showed no fluorescent cells.

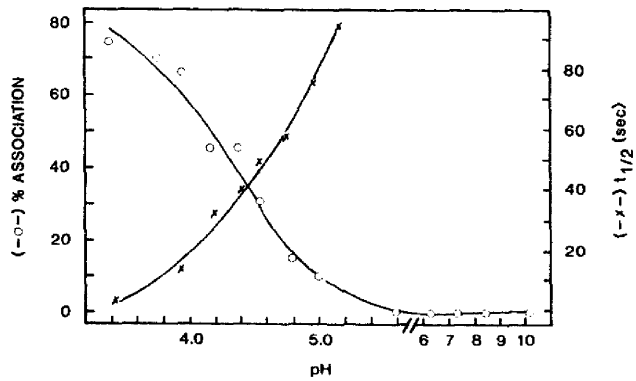

Fig. 4. Kinetic parameters of FITC-glycophorin interaction with human erythrocytes as a function of pH. Human RBC were reacted with FITC-glycophorin suspensions at different pH values at 37°C. The percentage of associated glycophorin (○) was calculated using Eqn. 2. The half-time values, $t_{1/2}$, (×) were measured from the decrease in FITC fluorescence intensity in time.

Stability of glycophorin-membrane association

After incubation of erythrocytes with FITC-glycophorin suspension and subsequent washings in PBS(7.4), the red blood cell pellet was resuspended in human plasma. After 0.1, 1, 2, 3, 4, 18, 20 and 24 h of incubation in blood plasma (first 4 h at room temperature and the next 20 h at 39°C), the red blood cells were concentrated by centrifugation and the supernatant was assayed for the presence of FITC fluorescence. After recording the fluorescence spectra, the supernatant and erythrocyte pellet were mixed and the incubation was continued. The next measurements were performed in the same way. These measurements showed no difference in the fluorescence intensity of the supernatants assayed at the indicated time points of plasma incubation, i.e., no release of FITC-glycophorin from the RBC. Further, no change in the fluorescence of the FITC-glycophorin-labeled RBC was observed under the light microscope (after 24 h).

CD4 molecules associate with RBC membranes

Human RBC were incubated with a CD4-containing glycoprotein extract at low pH using the same conditions as those described for glycophorin. Cells incubated at low pH with the CD4-containing glycoprotein extract were fluorescently labeled with anti-CD4 monoclonal antibodies. The fluorescence was weak but uniformly distributed (photographs not shown). These experiments showed that CD4 molecules, after interaction with erythrocyte membranes, have exposed epitopes that can be recognized by the monoclonal antibodies.

Freeze-etching of erythrocytes which had been incubated at low pH with the CD4 extract, then with a mixture of mouse monoclonal antibodies to CD4 and finally with 10 nm gold beads coated with goat anti-mouse IgG, showed the 10 nm gold beads at the erythrocyte surface (Fig. 5a,b,c, arrows point to the beads). Because they could be confused with membrane proteins in the etched protoplasmic face of a laterally fractured erythrocyte, the beads were unequivocally identified only in the case of cross-fractures. In control cells, i.e., erythrocytes incubated at low pH in the presence of BSA, no gold beads were observed at the cell surface after incubation with anti-CD4 antibodies then Au-IgG-10 (Fig. 5d).

If the erythrocyte is fractured tangentially, exposing an area containing gold beads, the total number of beads attached at the cell surface can be calculated, thus providing an estimate of the number of CD4 epitopes on an erythrocyte surface (Fig. 5c). With an average of three gold beads per projected area of 12600 nm^2 (in Fig. 5e, the bar represents 112 nm and a depth

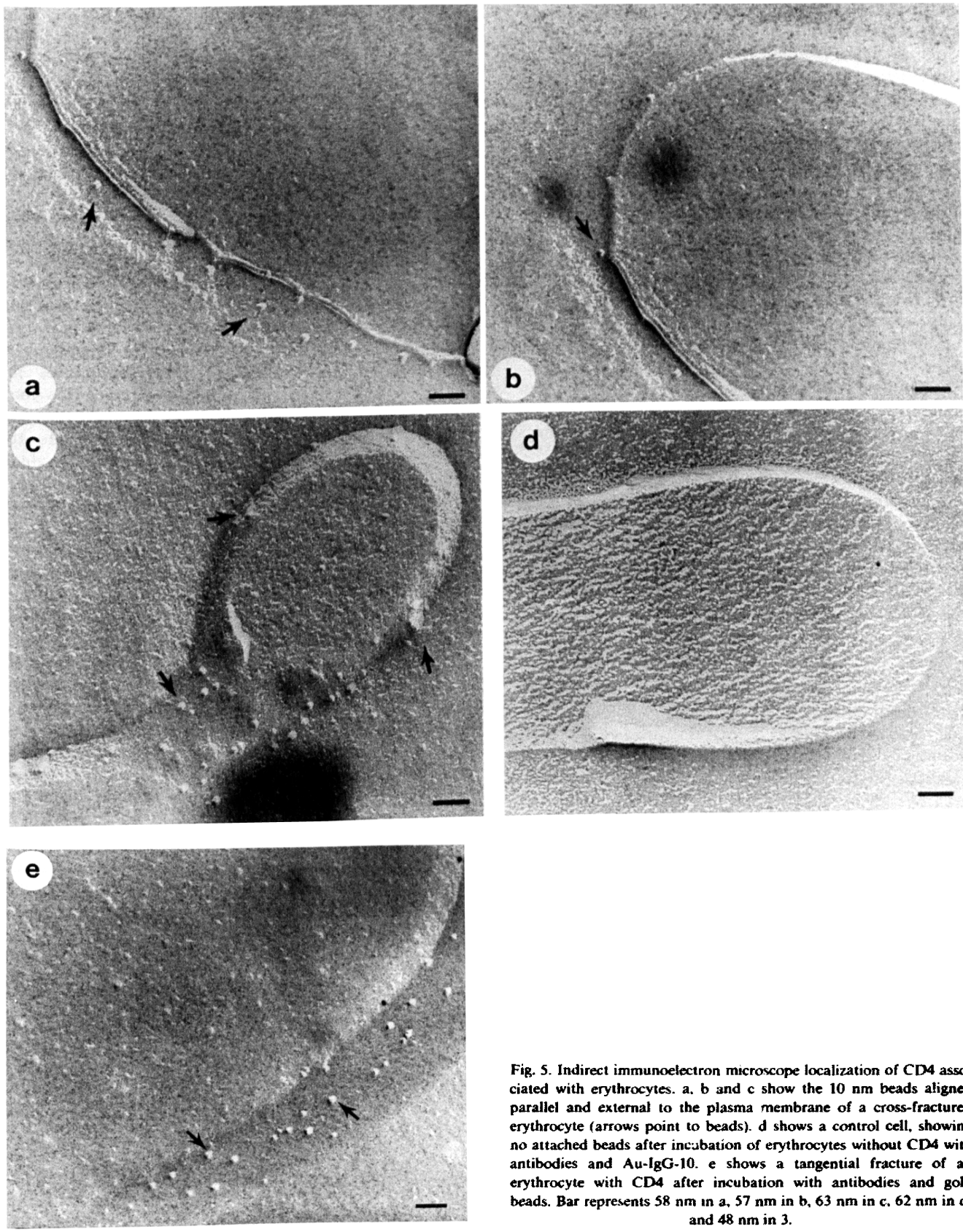

Fig. 5. Indirect immunoelectron microscope localization of CD4 associated with erythrocytes. a, b and c show the 10 nm beads aligned parallel and external to the plasma membrane of a cross-fractured erythrocyte (arrows point to beads). d shows a control cell, showing no attached beads after incubation of erythrocytes without CD4 with antibodies and Au-IgG-10. e shows a tangential fracture of an erythrocyte with CD4 after incubation with antibodies and gold beads. Bar represents 58 nm in a, 57 nm in b, 63 nm in c, 62 nm in d, and 48 nm in 3.

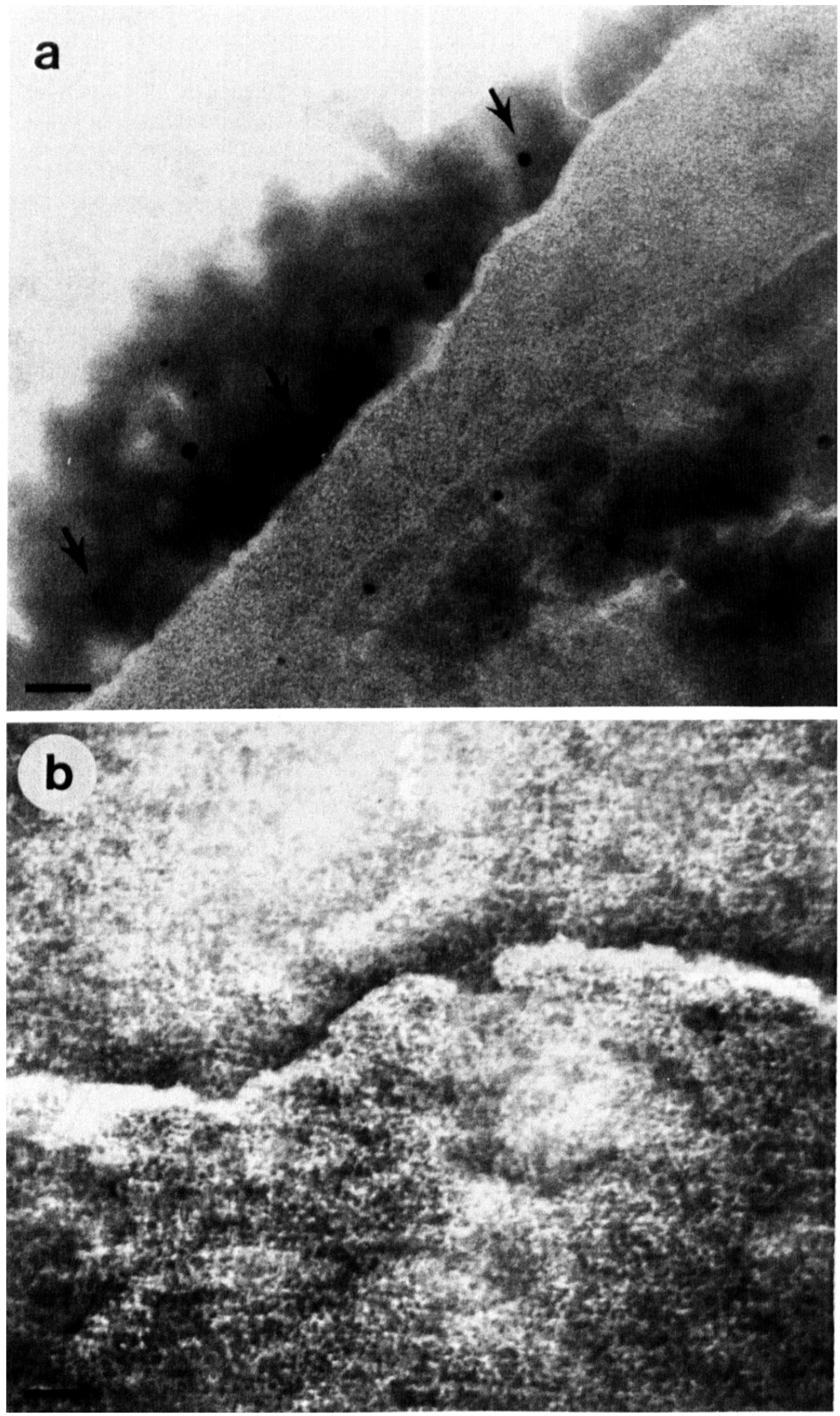

Fig. 6. (a) Negatively stained erythrocyte ghost carrying 10 nm gold beads which identified functional CD4 epitopes in the intact erythrocyte (arrow points to beads). (b) No gold beads are attached to the surface of control erythrocyte ghosts. Bar represents 36 nm in a and 52 nm in b.

of 10 nm at the edge is assumed), and taking a value of 145 μm^2 as the surface area of the erythrocyte, [21], a value of 34500 gold beads per erythrocyte is obtained.

Fig. 6a shows that Au-IgG-10 beads remained attached at the erythrocyte ghost membrane after lysis of the erythrocytes (arrows point to beads). In control experiments (Fig. 6b), no gold beads were found associated with ghost membranes.

Discussion

The use of low-pH provides a method to achieve an association of membrane proteins with the red blood cell membrane. The short time of incubation (1.5 min) seems to have no damaging effects on the protein structure and on the erythrocyte membrane. For example, scanning electron microscopy indicated no significant change in erythrocyte shape after proteins were associated with the RBC membrane as a result of the low-pH incubation (data not shown). Freeze-fracture analysis showed no significant aggregation of membrane proteins after the low-pH incubation in the presence of proteins (data not shown). The fact that glycophorin and CD4 proteins were recognized by monoclonal antibodies indicates that the integrity of the epitopes was not lost during low-pH treatment and that the epitopes are accessible at the surface of the RBC (see Figs. 1, 5 and 6).

It was proposed that the event of insertion of intrinsic membrane proteins into the membranes is facilitated by membrane fusogenic conditions [23,24]. We point out that the conditions reported here for protein interaction with membranes are exactly the same as those reported earlier as being membrane fusogenic [25–28].

The fluorescence assay used to study the interaction of proteins with membranes is based on the changes of FITC fluorescence which occur when FITC-labeled integral membrane proteins are transferred from an aqueous medium to a more hydrophobic surrounding. Thus, if an integral membrane protein inserts into a membrane, conformational changes are expected. These conformational effects, we believe, are responsible for the changes in FITC fluorescence and polarization degrees when Triton X-100 was added over suspensions of FITC-glycophorin molecules at different pH values (Fig. 2). Non-ionic detergents, such as Triton X-100, are widely used in the study of membrane protein conformation in hydrophobic environments using ultraviolet circular dichroism spectra, i.e. as in Ref. 18, or by the changes in the protein tryptophan intrinsic fluorescence i.e. as in Refs. 20 and 29. In our system we cannot use such techniques due to the presence of erythrocyte membrane proteins. For glycophorin in aqueous solution, such intrinsic protein fluorescence studies cannot be performed since glycophorin contains no tryptophan [30].

Results which are presented in the following article [22] suggested that low-pH induces a perturbance of the erythrocyte membrane which facilitates the protein–membrane interaction: these perturbances are thought to be the protein–membrane interaction sites and these perturbances, in the absence of proteins in the surrounding aqueous media, are thought to develop into the observed 'openings' in the erythrocyte membrane [22].

Functional CD4 epitopes were identified by immunoelectron microscopy using freeze-etching analysis for erythrocytes and using negative staining analysis for ghosts. These procedures involved multiple washings and centrifugations, and exposure to low salt concentrations in the preparation of ghosts, thus indicating that the identified epitopes arise from a stable membrane-CD4 association.

Should the red blood cells bearing CD4 be long-lived in the circulation, they may be envisioned as potential scavengers of HIV-infected cells expressing the viral envelope protein, gp120, as well as of free virus or circulating gp120 in the blood stream [1]. Measurements of the life span of such erythrocytes are currently underway. Whatever the efficiency of CD4-bearing erythrocytes as a therapy in AIDS disease, the low-pH association process of membrane proteins with the red blood cell membranes may contribute to the understanding of in vivo protein–membrane interaction mechanisms.

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