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Cytochrome b_5 and a recombinant protein containing the cytochrome b_5 hydrophobic domain spontaneously associate with the plasma membranes of cells

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Both cytochrome b_5, isolated from rabbit liver microsomes, and LacZ:HP, a recombinant protein consisting of enzymatically active *Escherichia coli* β-galactosidase coupled to the C-terminal membrane-anchoring hydrophobic domain of cytochrome b_5, were shown to spontaneously associate with the plasma membranes of erythrocytes and 3T3 cells. Association was promoted by low pH values, but proceeded satisfactorily over several hours at physiological pH and temperature. About 150 000 cytochrome b_5 molecules or 100 000 LacZ:HP molecules could be associated per erythrocyte. These proteins were not removed from the membrane by extensive washing, even at high ionic strength. After incubation with fluorescently labeled cytochrome b_5 or LacZ:HP, cells displayed fluorescent membranes. The lateral mobility of fluorescently labeled cytochrome b_5 and LacZ:HP was measured by photobleaching techniques. In the plasma membrane of erythrocytes and 3T3 cells, the apparent lateral diffusion coefficient D ranged from $1.0 \cdot 10^{-9}$ to $8 \cdot 10^{-9}$ cm^2 s^{-1} with a mobile fraction M between 0.4 and 0.6. The lateral mobility of these proteins closely resembled that reported for lipid-anchored proteins and was much higher than that reported for Band 3, an erythrocyte membrane-spanning protein with a large cytoplasmic domain. These results suggest that the hydrophobic domain of cytochrome b_5 could be employed as a universal, laterally mobile membrane anchor to associate a variety of diagnostically and therapeutically useful recombinant proteins with cells.

Introduction

It would be of considerable interest for a variety of scientific, diagnostic and therapeutic applications to develop a procedure permitting arbitrary proteins to be associated with the external face of the plasma membranes of intact cells. Although isolated membrane proteins do not in general reinsert spontaneously into membranes, a few examples of spontaneous insertion are known. These include colicin E1, a protein coded for by the ColE1 plasmid, which induces ion leakage in target bacteria as reviewed in Luria and Suit [1], and C9 which, during complement fixation, integrates into cell membranes and polymerizes to form a torus, resulting in an aqueous channel and cell lysis [2].

Membrane proteins can readily be incorporated into the lipid bilayers of liposomes, for example when detergent is dialyzed from a mixture of lipid and protein. In this procedure, reconstitution occurs simultaneously with the formation of membrane bilayers. However, most membrane proteins do not spontaneously associate with or insert into lipid bilayers of preformed liposomes, although some exceptions to this are known (for a review, see Jain and Zakim [3]). Also, most membrane proteins are not directly inserted into membranes in living cells. In recent years, extensive investigations have begun to elucidate the pathways by which proteins are inserted into cellular membranes *in vivo* as reviewed in Lee and Beckwith [4] and Walter and Lingappa [5]. These pathways do not in general involve direct spontaneous insertion and turn out to be very complex. Possibly such pathways are needed because

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the energetics of transporting hydrophilic segments of polypeptides into or through lipid bilayers are unfavorable. Such considerations may have inhibited research into possible mechanisms which could be employed for direct association of polypeptides into biological membranes.

Cytochrome b_5, an integral membrane protein, has two distinct domains linked together by a connecting peptide. The amino-proximal domain contains a heme ring which is involved in electron transfer; the carboxyl-proximal domain is hydrophobic and serves to associate the protein with membranes [6]. The hydrophobic domain contains no charged amino acids except two acidic residues very near the carboxyl terminus (including the C-terminal carboxyl group itself). *In vivo*, cytochrome b_5 may associate spontaneously with membranes, since it is synthesized on free polysomes and since signal recognition protein is not required for association [7].

Cytochrome b_5 has the interesting property of spontaneously associating with preformed phosphatidylcholine vesicles, artificial membrane bilayers [8,9] and liver microsomal membranes [10]. Plasma membranes obtained from hemolyzed rabbit or sheep erythrocytes and incubated with cytochrome b_5 were reported not to show detectable cytochrome b_5 bound [11], but inside-out vesicles prepared from erythrocyte ghosts did [12].

In the light of this evidence for spontaneous association with some membranes at least, we decided to investigate whether the hydrophobic domain (HP) of cytochrome b_5 could be used as a universal membrane association sequence. We first demonstrated that spontaneous association of fluorescently labeled cytochrome b_5 with the plasma membranes of erythrocytes and other cells did in fact occur. Subsequently, a recombinant gene was constructed coding for LacZ:HP, *Escherichia coli* β-galactosidase carrying the hydrophobic domain of cytochrome b_5 at the carboxyl terminus. For both cytochrome b_5 and LacZ:HP, lateral mobility was measured by photobleaching techniques. These results support the idea that the hydrophobic domain acts as a membrane anchor and confers on both proteins spontaneous and stable association with the cell surface and high mobility in the plane of the membrane.

Materials and Methods

Purification of cytochrome b_5

Rabbit liver cytochrome b_5 was isolated and purified using the method of Spatz and Strittmatter. To minimize proteolytic degradation all the procedures were carried out at 4°C in buffers containing 1 mM PMSF (Sigma Chemical Co.). After DEAE-anion exchange and G-75 gel filtration chromatography, a major product consistent in size (16.7 kDa) and electrophoretic mobility to that of cytochrome b_5 was obtained (Fig. 1A), which was divided into aliquots and stored at −70°C. Cytochrome b_5 stored at 4°C for twelve months had a molecular mass of 12 kDa, consistent with proteolytic loss of the HP domain (Fig. 1A).

Purification of the LacZ:HP fusion protein

The construction of the LacZ:HP gene fusion has been described previously [13]. *E. coli* containing the plasmid pUR278 were grown in LB medium containing ampicillin and kanamycin at a concentration of 50 μg/ml. At an optical density at 600 nm of 0.3, the cultures were induced by adding iso-propyl thiogalactoside to 1 mM and growth was allowed to continue to an optical density at 600 nm of 1.0 [13]. The cells were harvested by centrifugation and disrupted in a French pressure cell at 16000 psi in Tris acetate (TA) buffer (10 mM Tris, 1 mM EDTA) containing 1 mM $MgCl_2$ and 10 mM β-mercaptoethanol. Unlysed cells were removed by centrifugation (25000 × *g* for 10 min in the SS34 rotor) and the pressate centrifuged at 180000 × *g* for 1 h at 4°C in the 70Ti rotor to collect the membranes. The membranes were extracted by sonication in TA buffer containing 1 mM $MgCl_2$, 10 mM β-mercaptoethanol and 1% sodium cholate (pH 8.1). This crude membrane extract could be stored at −70°C for three months without loss of β-galactosidase activity.

LacZ:HP was purified by gel filtration on Sepharose 4B coupled with DEAE-anion exchange chromatography and also by substrate (*p*-aminophenyl-D-thiogalactoside) affinity chromatography [14] with equivalent results. Protein purified by the latter procedure was used for the experiments reported here (Fig. 1B). The crude membrane extract (4 mg protein/ml) was mixed with 10 volumes of TA buffer containing 1 mM $MgCl_2$, 10 mM β-mercaptoethanol, 0.5% sodium cholate and 1.5 M NaCl, pH 7.5 (buffer N). Following sonication, insoluble material was removed by ultracentrifugation (100000 × *g* for 30 min in the 70Ti rotor) and the clarified supernatant immediately applied to the affinity column which had been pre-equilibrated with buffer N. After thoroughly washing the column with buffer N, the bound fusion protein was eluted with 0.1 M sodium borate (pH 9.2), 0.5% sodium cholate, 1 mM $MgCl_2$ and 10 mM β-mercaptoethanol. Most contaminating protein, but less than 5% of the loaded β-galactosidase activity, was found in the flow through fractions. The eluted fractions containing β-galactosidase activity were pooled and dialyzed extensively against 10 mM potassium phosphate buffer (pH 7.2) containing 1 mM $MgCl_2$ and 10 mM β-mercaptoethanol. The column was regenerated by thoroughly washing with buffer N.

Fluorescent labeling of cytochrome b_5

Cytochrome b_5 was fluorescently labeled with either Texas red (the sulfonyl chloride derivative of sulforho-

damine 101 [15] or FITC [16]. Briefly, 6 ml of a stock solution of cytochrome b_5 (0.15 mg/ml) was dialyzed against distilled water for 12 h at 4°C, lyophilized and resuspended in 2 ml of 0.2 M Tris buffer (pH 9.1), 0.15 M NaCl. Following addition of either 1 mg of Texas red (Molecular Probes Inc.), or FITC, the mixture was stirred overnight at 4°C. Conjugated protein was separated from the unreacted fluorophore by gel filtration on a Sephadex G-25 column (0.5 × 60 cm) eluted with 0.01 M Tris acetate buffer (pH 8.1), 0.1 M NaCl, 0.1 mM EDTA and 0.02% NaN_3. Labeled protein elutes in the void volume with good separation from free label. UV illumination of SDS-polyacrylamide gels established that the single Coomassie-stained band corresponded to the single fluorescent band.

Fluorescent labeling of LacZ:HP

A 1.0 mg/ml solution (2 ml) of LacZ:HP in 50 mM sodium carbonate buffer containing 1% sodium cholate (pH 9.0), was reacted with a 500-fold molar excess of FITC for 4 h at room temperature in the dark. Following incubation, the reaction mixture was chromatographed on a PD-10 Sephadex G25 column (Pharmacia) and dialyzed against 10 mM potassium phosphate buffer in the dark at 4°C. Labeling was monitored by visualizing the labeled protein with UV light after electrophoresis on SDS-polyacrylamide gels. The extent of labeling was measured spectrophotometrically [17] and normally corresponded to 2–3 fluorescent groups per protein molecule.

Radioiodination of cytochrome b_5

Radioiodinations (using an early preparation of cytochrome b_5 which was less purified than the later preparation shown in Fig. 1a) were carried out using the chloramine T method [18] and the procedure of Bolton and Hunter [19]. In the first procedure, to 1 ml of a stock solution of cytochrome b_5 (0.15 mg/ml) in 0.01 M Tris acetate buffer (pH 8.1), 0.1 M NaCl, 0.1 mM EDTA and 0.02% NaN_3 were added two iodobeads (Pierce Chemical Co.), followed immediately by 200 μCi of $Na^{125}I$ (ICN) in 2 μl. After incubation for 1 min at room temperature, the unreacted iodide was separated from the iodinated cytochrome b_5 by gel filtration on a Sephadex G-25 column (1.5 × 30 cm) eluted with reaction buffer. In the Bolton and Hunter procedure, to a 1 mCi vial of Bolton and Hunter reagent (ICN) was added 0.5 ml of a solution of cytochrome b_5 (1.5 mg/ml) in 0.05 M sodium borate buffer (pH 8.5). The reaction was allowed to proceed for 12 h at 4°C and then terminated by the addition of 100 μl of a 1 mg/ml solution of glycine. Unreacted Bolton and Hunter reagent was separated from the radio-labeled protein by gel filtration on a Sephadex G-10 column (1.5 × 15 cm) eluted with reaction buffer containing 0.1% (w/v) gelatin.

Erythrocytes

Freshly drawn erythrocytes (type O, Rh^+), collected in 10 ml vacutainer tubes containing sodium heparin were centrifuged and washed three times with PNK (8 mM Na_2HPO_4, 1 mM KH_2PO_4, 136 mM NaCl, 3 mM KCl (pH 7.4), containing 10 mM glucose and 5 mM inosine (PNKGI). The cells were resuspended at a hematocrit of 50% and stored at 4°C in PNKGI for no more than 2 weeks.

Resealed erythrocytes were prepared by a modification of the preswell-lysis technique [20]. Briefly, 4 volumes of hypotonic PNKGI (200–205 mosm) were added to washed packed erythrocytes. After incubation at room temperature for 5 min, the cells were centrifuged at 2000 × g for 3 min. This step was repeated once and the cells lysed by addition of 5-times the packed cell volume of 10 mM phosphate buffer (pH 7.4), and incubated at room temperature for 5 min. They were resealed by the addition of 1.54 M KCl to 0.15 M, incubated at 37°C for 30 min, centrifuged for 3 min and resuspended three times at 3000 × g. The resealed cells generally retained 10–20% of the original hemoglobin.

Incubation of erythrocytes with radiolabeled cytochrome b_5

To 10 μl packed erythrocytes (1×10^8 cells) or resealed erythrocyte ghosts was added approximately 3000 cps of either chloramine T radio-iodinated or Bolton and Hunter radio-iodinated cytochrome b_5. The incubations were carried out in Tris acetate buffer (pH 8.1 at 37°C) for 2 h in a total volume of 0.2 ml. The cells were then washed three times in Tris acetate buffer, and the extent of association of cytochrome b_5 with the red blood cells was estimated by gamma counting. Following this, the erythrocyte membranes were isolated, detergent extracted with Triton X-100 [21], and analyzed by SDS-polyacrylamide gel electrophoresis and autoradiography.

Incubation of erythrocytes with fluorescently labeled cytochrome b_5

To 10 μl packed erythrocytes or resealed erythrocyte ghosts was added 190 μl of a fluorescently labeled stock of Texas red cytochrome b_5 in the appropriate buffer. Following incubation using defined conditions of temperature, pH and time (generally 37°C, pH 5 or 7, and 2 h of incubation), the cells were thoroughly washed three times and aliquots removed and visualized by fluorescence microscopy or used for quantitative fluorescence spectrophotometry. To establish whether ionic interactions may contribute to the observed results, labeled cells were incubated for 5 min in the presence of 1 M NaCl at room temperature, washed thoroughly and again visualized by fluorescence microscopy.

Quantitative fluorescence spectroscopy was done on an SLM 8000 Photon Counting Spectrafluorometer. Resealed erythrocytes were lysed in 2 ml of distilled water and placed in an acryl cuvette. Excitation of the Texas red fluorophore was carried out at 550 nm and emission spectra collected over the 560–700 nm range. Peak heights at the emission maximum (610 nm) were used to quantitate erythrocyte associated fluorescence. To estimate the number of molecules of cytochrome b_5 associated with the erythrocyte membranes, a calibration curve relating fluorescence intensity to the concentration of Texas red-labeled cytochrome b_5 was also constructed (Fig. 4A). To quantitatively measure the fluorescence associated with individual labeled red cells, an ACAS 570 interactive laser cytometer (Meridian Instrument, Inc.) was used.

Lateral mobility measurements

The lateral mobility of cytochrome b_5 and LacZ:HP was measured by the fluorescence microphotolysis (photobleaching) technique (for a review, see Peters and Scholz [22]). Cytochrome b_5 or LacZ:HP was associated with the membranes of human erythrocytes or resealed erythrocyte ghosts by incubating the cells with either FITC-labeled cytochrome b_5 (5 μM) or LacZ:HP (5 μM) in PNK buffer for 2 h at 37 °C (pH 7.0). The cells were then thoroughly washed and resuspended in PNK buffer (pH 7.4). In some experiments incubations were carried out in PNK titrated to pH 5.0 (see Table I). To assess whether detachment of the membrane skeleton from the lipid bilayer would affect the mobility of cytochrome b_5 within the membrane, some incubations were carried out with erythrocyte

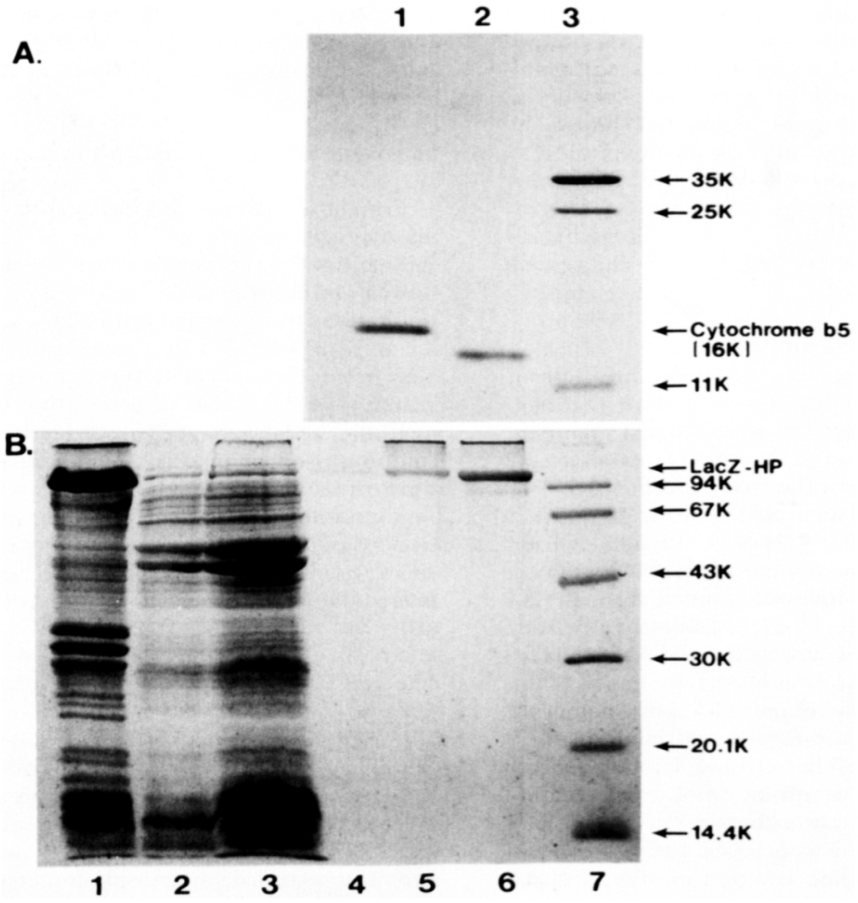

Fig. 1. Electrophoretic analysis of purified proteins. (A) Coomassie blue-stained SDS-polyacrylamide gel (10%) of samples from a cytochrome b_5 purification. The left lane corresponds to rabbit liver cytochrome b_5 (−80 °C stock) and the center lane to cytochrome b_5 stored at 4 °C for more than 1 year. Lambda phage proteins as molecular weight markers are run in the right lane. (B) Coomassie blue stained SDS-polyacrylamide gel (10%) of samples from a LacZ:HP affinity purification. The lanes correspond to: 1, crude membrane extract; 2 and 3, column flow through fractions; 4–6, 3, 10 and 30 μl of the affinity purified LacZ:HP fusion protein; 7, protein molecular mass standards.

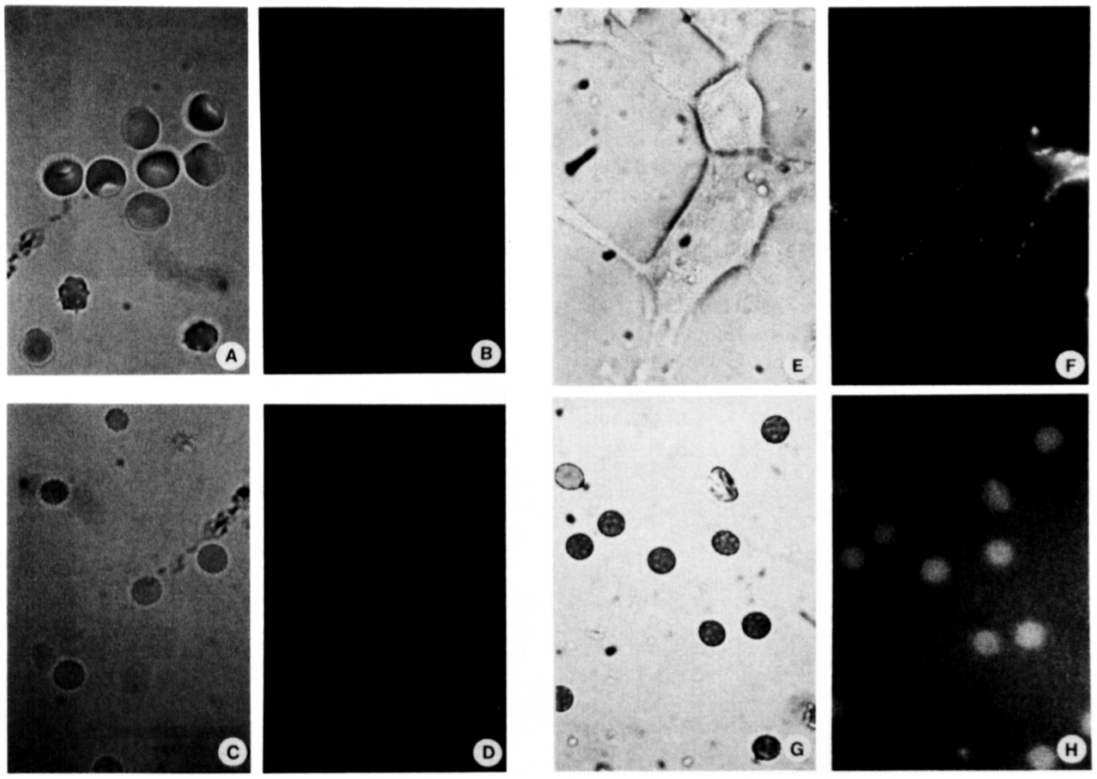

Fig. 2. Cells labeled with fluorescently labeled cytochrome b_5 and LacZ:HP. The photographs show the same cells viewed by phase contrast (A, C, E, G) and fluorescence microscopy (B, D, F, G). The images correspond to: (A) and (B), normal red cells labeled with fluorescently labeled cytochrome b_5. (C) and (D), resealed (1:5) red cell ghosts labeled with cytochrome b_5. (E) and (F), 3T3 fibroblasts labeled at 0°C with fluorescently labeled LacZ:HP. (G) and (H), normal red cells labeled with fluorescently labeled LacZ:HP.

ghosts internally loaded with trypsin (100 mg/ml), 3T3 fibroblasts in monolayer culture were incubated with cytochrome b_5 (5 μM) or LacZ:HP (5 μM) for 2–3 h at 4°C (pH 7.0). The cells were then thoroughly washed and resuspended in PNK (pH 7.4).

For mobility measurements labeled erythrocytes or ghosts were introduced into a flat glass capillary (microslide, pathlength 50 μm, Camlab, Cambridge, U.K.). This was sealed and mounted on a slide which was positioned on the stage of a photobleaching apparatus [23]. A 100-fold magnifying oil-immersion objective was used to focus the laser beam on the surface of an erythrocyte or ghost adhering to the glass/water interface. A circular membrane area of 2 μm diameter was uniformly illuminated by the 488-nm line of an argon ion laser. Fluorescence of the illuminated area was measured at a low excitation energy (approx. 10 nW). Then, the fluorophores in the illuminated area were partially bleached by increasing the beam power to about 1 mW for 1/30–1/15 s. Fluorescence recovery was monitored at the initial small beam power. The recovery curve was analyzed for the apparent lateral diffusion coefficient D and the mobile fraction M according to Axelrod, et al. [24] using the approach of Soumpasis [25] and a non-linear least-squares fitting routine. Mobility measurements on 3T3 cells were performed on monolayers, as grown in the culture dish, employing a 40-fold water immersion objective. All measurements were carried out at 22 ± 2°C.

Results

Purity of cytochrome b_5 and LacZ:HP

Both cytochrome b_5 and LacZ:HP used in these studies were highly purified, as demonstrated by electrophoresis on SDS-polyacrylamide gels (Figs. 1A and 1B). Prolonged storage at 4°C resulted in a reduction in molecular mass consistent with cleavage and loss of the HP segment. LacZ:HP was enzymatically active displaying approximately 200000 u/mg of β-galactosidase activity. The purified proteins were found to spontaneously associate with preformed phosphatidylcholine liposomes [26].

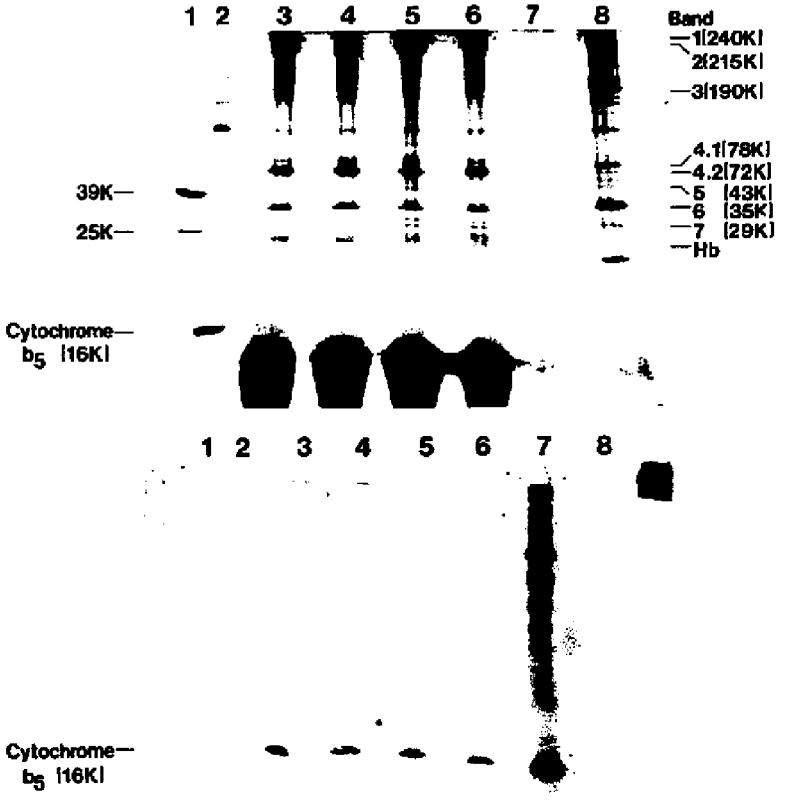

Fig. 3. SDS-polyacrylamide gel (top) and autoradiograph (bottom) of radio-iodinated cytochrome b_5 recovered from red cell membranes. The lanes correspond to: 1, lambda phage proteins as molecular weight markers; 2, rabbit liver cytochrome b_5 stock; 3–6, cytochrome b_5 extracted from red cell membranes with detergent; 7, Bolton and Hunter radio-iodinated cytochrome b_5; 8, normal red cell membranes extracted with detergent.

Association of cytochrome b_5 and LacZ:HP with the plasma membrane of erythrocytes and 3T3 fibroblasts

Cytochrome b_5 and LacZ:HP spontaneously associate with both normal and resealed erythrocytes and 3T3 cells under physiologically relevant conditions (pH 7 and 37 °C). Fluorescence microscopic studies with erythrocytes preincubated with fluorescently labeled cytochrome b_5 or LacZ:HP (Fig. 2) showed that the membranes of all the cells were fluorescent and had approximately equal intensities, indicating that there is relatively little variation between cells. Association is therefore a property of all erythrocytes in the population. Consistent with the idea that the proteins were associated with the membranes, the fluorescent protein was distributed completely around the erythrocyte membrane in a ring of fluorescence and remained associated with the membranes after the erythrocytes were hypotonically lysed, as demonstrated either by centrifugation or by floatation of the membranes in density gradients.

Most of the erythrocytes had small areas or dots of more intense fluorescence in their membranes (Fig. 2D) and most of the 3T3 cells had larger dots of fluorescence. Two possible explanations for this effect are that the individual protein molecules may localize preferentially at particular sites on the membranes or that aggregates of the protein may also associate with cells. Both cytochrome b_5 and LacZ:HP aggregate, apparently by hydrophobic interactions involving the HP portion of the molecule. Aggregation is favored by high salt concentrations and by pH values less than 6. Aggregates of lipophilic chemicals are known to associate with erythrocyte membranes [27]. To remove possible protein aggregates which might be present, aliquots of the fluorescently labeled protein solutions were centrifuged for 30 min at $100000 \times g$. However, when the supernatant was incubated with erythrocytes, small areas of localized fluorescence were still observed.

Membrane-associated cytochrome b_5 or LacZ:HP could not be removed from erythrocytes by repeated centrifugation in PNK or by washing with 1–2 M NaCl. This was assessed directly by fluorescence microscopy

of the erythrocytes before and after the various treatments and by enzymatic assay of LacZ:HP. Fluorescent cytochrome b_5 or LacZ:HP was not detected in the supernatants. Erythrocytes washed with 1–2 M NaCl were crenated but appear as fluorescent as unwashed cells. BSA (1–10 mg/ml) did not prevent association.

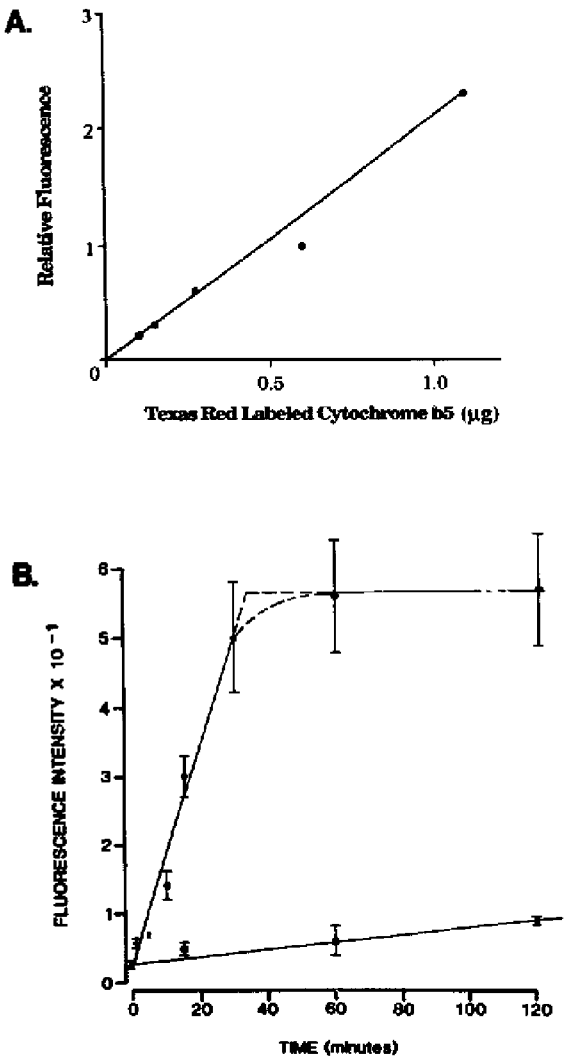

Fig. 4. Association of fluorescent cytochrome b_5. (A) Calibration curve. The fluorescence associated with varying concentrations of fluorescently labeled cytochrome b_5 was determined by fluorescence spectrophotometry. (B) Kinetics of association of cytochrome b_5 with erythrocyte membranes at pH 5 (●) and pH 7 (■). Incubations were carried out at 37 °C with Texas red-labeled cytochrome b_5 (5 μM) in either PNK buffer (pH 7) or sodium acetate buffer (pH 5). Buffers were adjusted to an osmolarity of 310–315 mosm with 5 M sodium chloride. Values plotted are means ± S.E. The pH 7 time points are the average of four determinations, as are the 0,2, and 5 min time points at pH 5. The remainder of the pH 5 time points are the average of six determinations.

Although red cells became very fluorescent after incubation with FITC or Texas red-labeled cytochrome b_5 or LacZ:HP at concentration of 25 μg/μl or less, various other proteins at the same concentration do not label the red cells. After incubation with high concentrations (0.2–2 mg/ml) of fluorescent proteins, including commercial β-galactosidase at concentrations greater than 1 mg/ml, red cells can be made minimally fluorescent. This binding can be greatly reduced or eliminated by addition of HSA during incubation, but HSA does not prevent association of LacZ:HP or cytochrome b_5. High salt washes (1–2.5 M NaCl) remove these other proteins, but not cytochrome b_5 or LacZ:HP.

About 62% of the added enzymatic activity of LacZ:HP was associated with the red cells, whereas only 9% of normal β-galactosidase synthesized from the parent plasmid was associated. Little or no association was observed for commercial β-galactosidase. Association of LacZ:HP was also demonstrated by Western blotting of the membrane proteins after hypotonic lysis of the red cells. Normal β-galactosidase did not associate with red cells as measured by Western blots.

Cytochrome b_5 radiolabeled with chloramine T did not associate with erythrocyte membranes. Since this labeling procedure specifically modifies tyrosine residues, 40% of which are located in the hydrophobic domain of cytochrome b_5, the properties of the hydrophobic domain probably had been altered. Membrane association was observed when cytochrome b_5 labeled with the Bolton and Hunter reagent was used. In this case, radiolabeling is accomplished utilizing free amino groups, all of which are located exclusively in the amino terminal portion of cytochrome b_5. Following incubation, the membranes were isolated, detergent extracted and analyzed by autoradiography of SDS-PAGE gels. Although other radiolabeled components are present in the radio-iodinated cytochrome b_5 stock solution, the membrane bound radioactivity was found to correspond exclusively to radiolabeled cytochrome b_5 (Fig. 3). This result, together with the finding that detergent is required to solubilize erythrocyte membrane associated cytochrome b_5, supports the conclusion that cytochrome b_5 is closely associated with the erythrocyte membrane. It also excludes the possibility that fluorescent labeling of the membranes could result from some contaminant of the cytochrome b_5 preparation or by transfer of the fluorescent probe to the membrane.

Association kinetics of cytochrome b_5

The kinetics of association was estimated by fluorescence microscopy, including the ACAS system, and quantitated by fluorometric analysis of solubilized membranes (see Materials and Methods). The kinetics of association of Texas red-labeled cytochrome b_5 with

erythrocyte membranes was measured both at neutral and acidic pH values (Fig. 4B). At pH 5.0 the association reaction had a half-time of about 20 min and was completed after about 60 min of incubation. At pH 7.0 the rate of association was much slower. About 150000 cytochrome b_5 molecules were bound per erythrocyte ghost at pH 5.0. The pH dependence of association measured fluorometrically after a 2-h incubation with Texas red-labeled cytochrome b_5 (5 μm) at 37 °C, is shown in Fig. 5B. Association increased about 5-fold between pH 9 and 5 with a particularly steep increase between pH 5 and 6. pH values below 5 were not employed, since the erythrocytes became fragile. The temperature dependence of cytochrome b_5 association is shown in Fig. 5A (pH 5.0). At 0 °C essentially no association was observed with intact erythrocytes or resealed ghosts. Association increased steeply with increasing temperature and did not reach saturation at the highest temperature studied (42 °C). These findings are similar to results with liposomes reported by Vaz et al. [28] who found that the association of cytochrome b_5 with dimyristoylphosphatidylcholine vesicles had a half-time of about 25 min at 35–40 °C and that the rate of association increased about 8-fold between 20 and 35 °C.

Exchange of cytochrome b_5 between erythrocyte membranes and liposomes

When phosphatidylserine liposomes (125 μg) were incubated with $1 \cdot 10^8$ erythrocytes (approx. 45 μg lipid) fluorescently labeled with Texas red cytochrome b_5, the cells became less fluorescent indicating that some of the cytochrome b_5 had been extracted by the liposomes. The majority of the protein, however, remained associated with the erythrocytes. This observation suggests that cytochrome b_5 associated with erythrocytes is partially transferable, as is known to be the case with cytochrome b_5 associated with liposomes [29].

Association kinetics of LacZ:HP

The association of LacZ:HP with erythrocyte membranes was studied by microfluorometry employing the ACAS system. Fig. 6A shows fluorescence images obtained of erythrocyte ghosts after incubation with FITC-labeled LacZ:HP for 0, 17 and 120 min, respectively. The distribution of fluorescence intensities corresponding to the images of Fig. 6A is given in the histogram of Fig. 6C. Association kinetics (Fig. 6B), derived from this and other histograms, are similar to those of cytochrome b_5, although a saturation was not apparent after 120 min of incubation. The concentra-

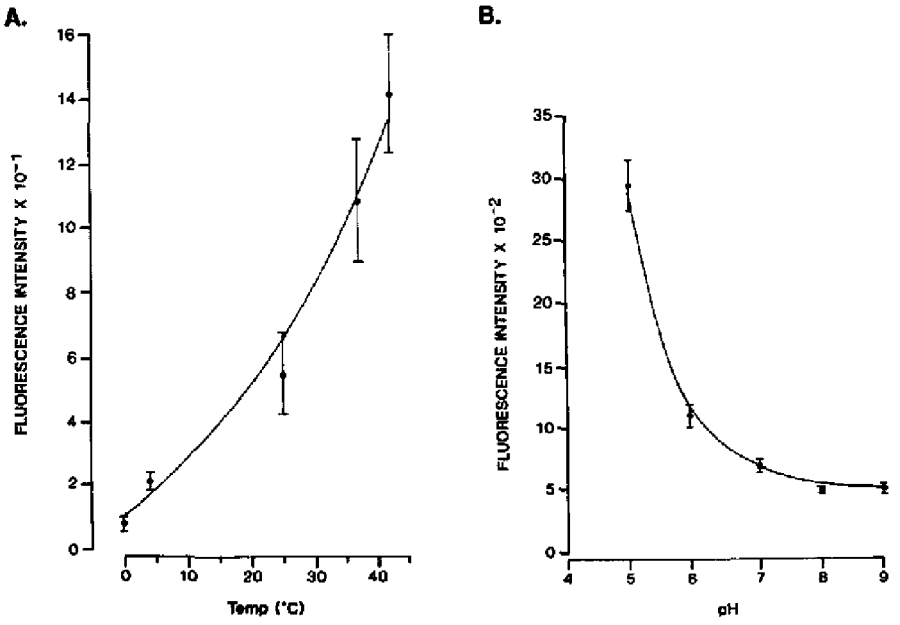

Fig. 5 (A) Temperature dependent binding of cytochrome b_5 to erythrocyte membranes. Incubations were carried out under conditions described in (Fig. 5B) for 2 h in 0.2 M sodium acetate buffer (pH 5). Values plotted are means ± S.E. ($n = 4$). (B) pH dependent association of cytochrome b_5 with erythrocyte membranes. Incubations with Texas red-labeled cytochrome b_5 (5 μM) were carried out for 2 h at 37 °C in the respective buffer, 0.2 M sodium acetate (pH 5) 0.2 M phosphate (pH 6 and 7), or 0.2 M Tris-HCl buffer (pH 8 and 9). Buffers were adjusted to an osmolarity of 310–315 mosm with 5 M sodium chloride. Values plotted are mean ± S.E. ($n = 8$ for pH 5, 7 and 9, and $n = 6$ for pH 6 and 8).

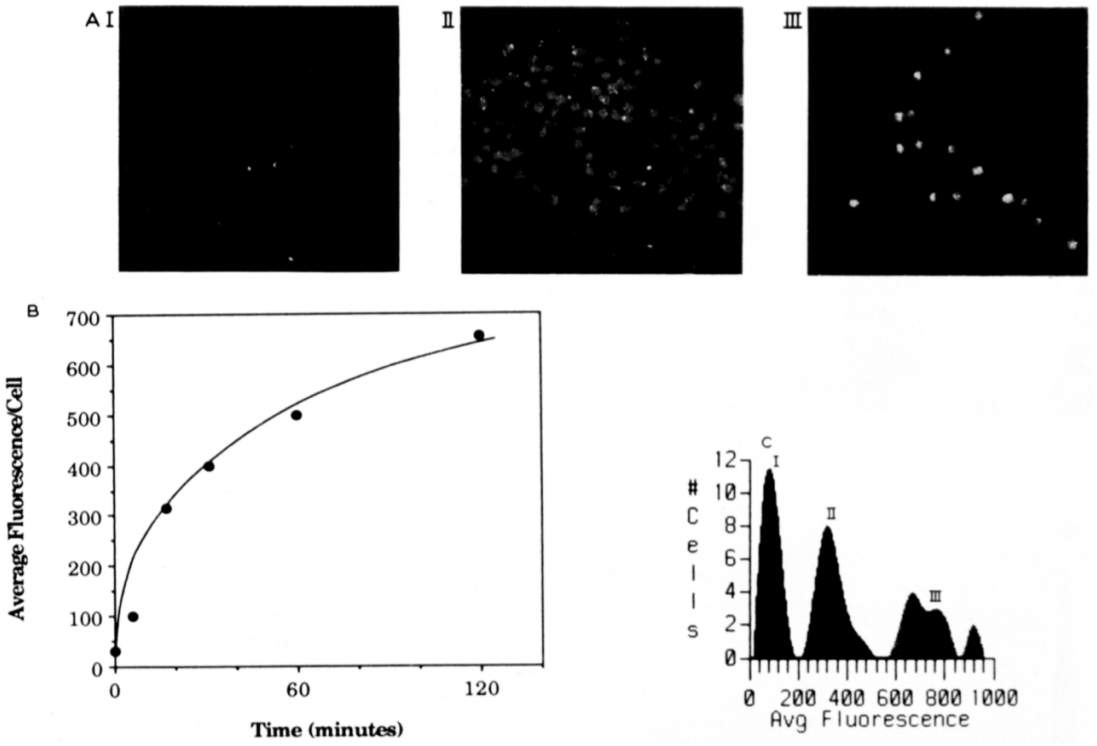

Fig. 6. Microfluorometric analysis of parameters related to the binding of LacZ:HP to erythrocytes using the ACAS system. (A) Fluorescent image of erythrocytes labeled with FITC LacZ:HP after (I) 0 min, (II) 17 min and (III) 120 min. (B) Time course for the association of LacZ:HP with erythrocytes derived from the average fluorescence/cell determined per time point as in Fig. 6C. (C) Histogram showing the distribution of fluorescence intensities for erythrocytes labeled with FITC LacZ:HP. Peaks I, II and III correspond to the level of fluorescence associated with the cells after incubation for 0, 17 and 120 min, respectively.

tion dependence of LacZ:HP association is shown in Fig. 7. The fluorescence associated with the erythrocytes after a 1-h incubation with LacZ:HP increased with increasing LacZ:HP concentration, reaching saturation at about 1.5 μM. Transfer of LacZ:HP between erythrocyte ghosts was studied by incubating LacZ:HP labeled ghosts for one hour with a 10-fold excess of unlabeled ghosts. Fig. 8 shows that about half of the fluorescence was transferred.

Lateral mobility of cytochrome b_5 and LacZ:HP in the plasma membrane of erythrocytes and 3T3 fibroblasts

The lateral mobility of cytochrome b_5 and LacZ:HP was studied by photobleaching techniques. For this purpose intact erythrocytes, resealed ghosts or 3T3 fibroblasts were incubated with FITC-labeled cytochrome b_5 or LacZ:HP and photobleaching measurements were carried out at 23°C. Typical fluorescence recovery curves together with the fitted theoretical curves are shown in Fig. 9. The results of measurements under various experimental conditions are listed in Table I.

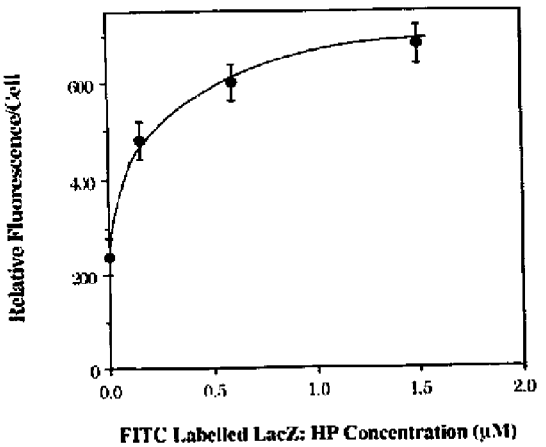

Fig. 7. Concentration dependence of the association of LacZ:HP with erythrocytes. FITC LacZ:HP was incubated with erythrocytes (5×10^7 in a total volume of 100 μl) for 2 h at 37°C. in PNK buffer (pH 7) under conditions of varying LacZ:HP concentration. Fluorescent labeling of the cells was quantitated by microfluorometry using the ACAS system.

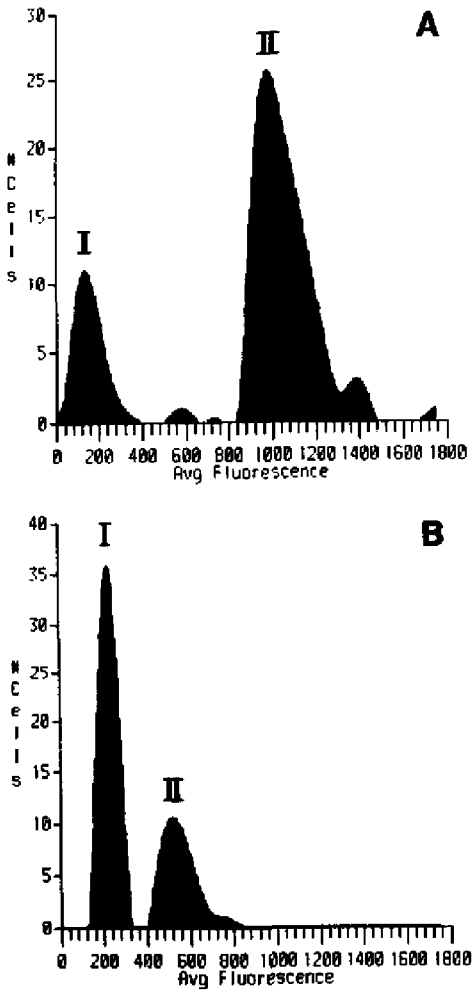

Fig. 8. Microfluorometric study of the exchange of LacZ:HP between erythrocytes using the ACAS system. (A) Histogram of the distribution of fluorescence intensities in a population of FITC LacZ:HP-labeled erythrocytes. Peak I results from the autofluorescence of the cells. Peak II corresponds to the fluorescence resulting from erythrocytes labeled with FITC LacZ:HP. (B) Histogram of the distribution of fluorescence intensities in the population of FITC lacZ:HP-labeled erythrocytes from (A), after incubation with a large excess of unlabeled erythrocytes for 1 h at 37 °C. As a result the average fluorescence/cell in Peak II is significantly diminished.

After incubation at neutral pH the apparent lateral diffusion coefficient D of cytochrome b_5 in the erythrocyte membrane was $(1.0 \pm 0.3) \cdot 10^{-9}$ cm^2 s^{-1} and the mobile fraction was 0.52 ± 0.02. This may be compared with the mobility of erythrocyte membrane proteins and lipids (for a review, see Peters [30]). Band 3 protein, an integral protein of the erythrocyte membrane with a large cytoplasmic portion, has a D value of $(1–10) \cdot 10^{-12}$ cm^2 s^{-1} and a M value of 0.1–0.4 at 23 °C. Lipid probes have D values around $1 \cdot 10^{-8}$ cm^2 s^{-1} with M values approaching 1.0.

LacZ:HP in its enzymatically active tetrameric form has a molecular mass of about 500 kDa as compared to 16 kDa for cytochrome b_5. Despite this, LacZ:HP was observed to have a substantially larger D-value in erythrocytes, $(6.2 \pm 2.1) \cdot 10^{-9}$ cm^2 s^{-1}. The M value observed for LacZ:HP was slightly smaller (0.41 ± 0.06) than that of cytochrome b_5 (0.50). However, a difference in mobility between cytochrome b_5 and LacZ:HP was not observed in the plasma membrane of 3T3 fibroblasts where both proteins had D values of $(1–2) \cdot 10^{-9}$ cm^2 s^{-1} and M values between 0.40 and 0.50.

An attempt to determine the origin of the mobile and immobile fractions was made by incubating cytochrome b_5 or LacZ:HP-labeled erythrocytes with an excess of unlabeled erythrocytes. Although part of the membrane-associated cytochrome b_5 and LacZ:HP was transferred to the unlabeled erythrocytes, no significant changes in the D or M values for the residual protein was observed. Furthermore, the transferred fluorescent cytochrome b_5 also had similar values for D and M. (The latter measurements could be not be performed with LacZ:HP as the level of fluorescence was too weak).

When erythrocytes or resealed ghosts were labeled with cytochrome b_5 at pH 5.0, D and M values (always measured at pH 7.0,23 °C) were $(2.3 \pm 0.4) \cdot 10^{-9}$ cm^2 s^{-1} and 0.52 ± 0.10, respectively. Degradation of the membrane skeleton by entrapment of high concentrations of trypsin in resealed ghosts [31] had no significant effect on D or M, indicating that the immobile fraction of cytochrome b_5 is not the result of interaction with the cytoskeleton.

Discussion

This study shows that both cytochrome b_5 and the recombinant protein LacZ:HP, which contains the hydrophobic membrane-anchoring domain of cytochrome b_5, spontaneously associate with the plasma membranes of erythrocytes and other cells. Fluorescent erythrocytes which are easily visible in the fluorescence microscope can be readily prepared by incubation of cells with either fluorescently labeled cytochrome b_5 or LacZ:HP. The association of cytochrome b_5 with the membrane was confirmed by the recovery of ^{125}I-labeled protein with the molecular weight of cytochrome b_5 from erythrocyte membranes. Importantly, the amounts of membrane-associated cytochrome b_5 and LacZ:HP can be quite large, corresponding to approx. 150000 molecules of cytochrome b_5 or 100000 of LacZ:HP bound per erythrocyte.

Once associated with the plasma membrane, cytochrome b_5 and LacZ:HP remained associated with the membrane even after repeated washing with

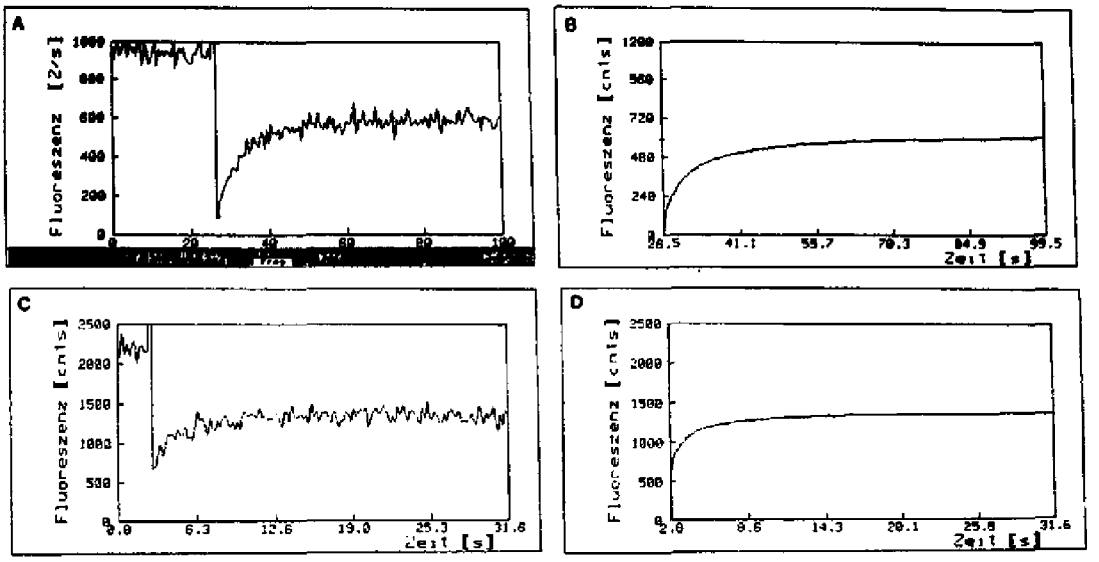

Fig. 9. Measurement of the lateral mobility of cytochrome b_5 and LacZ:HP by fluorescence photobleaching recovery. Curves A and C correspond to the original recovery curves for cytochrome b_5 and LacZ:HP, respectively. Curves B and D correspond to the computer fitted curves.

physiological saline or 2 M NaCl, conditions which remove many proteins loosely associated with membranes [10]. This result, together with the finding that detergent is required to solubilize the erythrocyte-associated cytochrome b_5, suggests that the association of cytochrome b_5 and LacZ:HP with the membrane is based on the interaction of the hydrophobic domain of cytochrome b_5 with the lipid membrane phase. Also supporting this conclusion was the observation that modification of the tyrosine residues, some of which are located in the hydrophobic segment, severely inhibited association, whereas radioiodination of free amino

TABLE I

Diffusion constants (D) and mobile fractions (M) obtained from microphotolysis studies

The values obtained for the respective diffusion constant (D) in 10^{-9} cm^2 s^{-1} and their corresponding mobile fractions (M) calculated as a percentage are listed for the microphotolysis studies carried out with erythrocytes and 3T3 fibroblast cells. A minimum of six measurements ($n \geq 6$) were made for each experimental set, and all values quoted are means ± S.D. Values listed under the columns cytochrome b_5 (a) and cytochrome b_5 (b) represent those originating from two separate studies.

	Cytochrome b_5 (a)		Cytochrome b_5 (b)		LacZ:HP	
	D	M	D	M	D	M
Erythrocytes	2.3 ± 0.4	51.7 ± 10.0	1.0 ± 0.3	52.4 ± 1.9	6.2 ± 2.1	40.5 ± 5.8
Erythrocyte ghosts	2.8 ± 0.6	62.5 ± 9.3	–	–	–	–
Erythrocyte ghosts internally loaded with 100 mg ml^{-1} trypsin	2.5 ± 0.7	44.9 ± 14.0	–	–	–	–
Labeled erythrocytes after incubation with an excess of unlabeled erythrocytes	–	–	1.0 ± 0.2	54.7 ± 4.5	5.8 ± 2.4	39.8 ± 10.4
Erythrocytes labeled by protein exchange	–	–	1.7 ± 0.4	62.3 ± 4.4	–	–
3T3 fibroblasts	8.2 ± 1.8	55.8 ± 10.0	1.1 ± 0.6	46.3 ± 10.7	1.6 ± 0.7	41.6 ± 9.1

groups, which are located exclusively within the amino terminal domain, did not.

The association between cytochrome b_5 and LacZ:HP and the plasma membrane was further characterized by photobleaching measurements. Membrane-spanning proteins usually have diffusion coefficients between 10^{-9} and 10^{-10} cm^2 s^{-1} [32]. However, integral membrane proteins of erythrocytes have very low mobility, as reviewed by Peters [30], as a consequence of interaction of the cytoplasmic portion of the membrane protein with the spectrin-actin membrane skeleton (for review, see Branton et al. [33]). For example, Band 3 protein, which is the predominant integral protein of the erythrocyte membrane, has a very low mobility with an apparent diffusion coefficient of $(1–10)\cdot 10^{-12}$ cm^2 s^{-1} and a mobile fraction of 0.1–0.4 at 23°C. Detachment of the cytoskeleton by trypsin treatment increases the mobility of Band 3 to $4\cdot 10^{-10}$ cm^2 s^{-1} [31,34]. In spherocytic mouse erythrocytes with a defective cytoskeleton Band 3 mobility was reported to be $2.5\cdot 10^{-9}$ cm^2 s^{-1} as compared with $4.5\cdot 10^{-11}$ cm^2 s^{-1} in normal mouse erythrocytes [35].

Non-erythrocyte transmembrane proteins in erythrocyte membranes such as the Sendai fusion protein and the Sendai HN protein are also relatively non-mobile ($D = (3.1–3.3)\cdot 10^{-10}$ cm^2 s^{-1}) [36]. This suggests that proteins which span the erythrocyte membrane might be substantially immobilized by entrapment within the membrane skeleton, even if they do not directly interact with the cytoskeletal proteins or with proteins such as Band 3. Cytochrome b_5, with a lateral diffusion coefficient of $(1.0–2.8)\cdot 10^{-9}$ cm^2 s^{-1}, and LacZ:HP with a lateral diffusion coefficient of $(5.8–6.2)\cdot 10^{-9}$ cm^2 s^{-1} are very mobile. These values indicate that the proteins in the mobile fraction do not interact with the membrane cytoskeleton and consequently do not expose a substantial domain on the cytoplasmic membrane face. The membrane insertion model for cytochrome b_5 proposed by Strittmatter and Dailey [37], lends support to this hypothesis, predicting that only the C-terminal 4 amino acid residues are likely to remain exposed at the external rather than the cytoplasmic surface of the membrane.

Lipid-anchored membrane proteins are also characterized by high mobilities. The Thy-1 antigen is anchored in membranes by means of a phosphatidylinositol tail and has a diffusion coefficient of $(2–4)\cdot 10^{-9}$ cm^2 s^{-1} in fibroblasts and lymphoid cells [38–40]. The rapid mobilities observed for both cytochrome b_5 and LacZ:HP suggest that they, like Thy-1, may interact with only the outer leaflet of the membrane and that the hydrophobic domain functions in a manner similar to the lipid anchor of Thy-1. Their high mobilities are consistent with the view that the amino acid residues flanking the hydrophobic region on both sides are exterior to the cell and that the hydrophobic region does not pass completely through the membrane. Possibly supporting this suggestion is the observation that part, but apparently not all, of the cytochrome b_5 associated with erythrocytes can be removed by incubation with liposomes or other unlabeled erythrocytes. However, incubation of liposomes with erythrocytes is known to remove not only loosely inserted proteins such as acetylcholinesterase [41] but also some integral membrane proteins including Band 3 and glycophorin [42] and, conversely, incubation of liposomes bearing Band 3 can be used to insert functional Band 3 into erythrocytes [43].

In the plasma membrane of erythrocytes and 3T3 cells both cytochrome b_5 and LacZ:HP have relatively small mobile fractions (0.40–0.50). This is somewhat surprising if their membrane association were based on the interaction of the hydrophobic domain of cytochrome b_5 with the outer leaflet of the lipid bilayer. Membrane lipids usually have mobile fractions approaching 1.0 and the hydrophobic domain of cytochrome might be expected to be similarly mobile. Proteins constituting the non-mobile fraction might be transmembrane with the short C-terminal segment in the cytoplasm. However there is no necessity to assume a conformational difference between the proteins of the mobile and non-mobile fractions since mobility parameters very similar to those reported in this study have been described for lipid-anchored proteins. Thy-1 [40] has a mobile fraction of 0.43–0.55 in the plasma membrane of lymphoid cells ($D = (2.1–3.9)\cdot 10^{-9}$ cm^2 s^{-1}) and VSG [44], a variant surface glycoprotein of trypanosome Brucei, has a mobile fraction of 0.56 in BHK cells ($D = 0.66\cdot 10^{-10}$ cm^2 s^{-1}). The immobile fractions of cytochrome b_5 and LacZ:HP as well as those of lipid-anchored proteins may result from an interaction of these proteins with the constituents of the external face. Supporting this idea, VSG has a large mobile fraction (0.80) in the plasma membrane of trypanosoma Brucei, while the diffusion coefficient remains virtually unchanged ($0.7\cdot 10^{-10}$ cm^2 s^{-1}). In contrast to erythrocytes and many other mammalian cells trypanosoma Brucei has no glycocalix.

The experiments reported here demonstrate that both cytochrome b_5 and LacZ:HP can associate with the outer aspect of the plasma membranes of erythrocytes and other cell types, where the proteins behave as mobile membrane proteins. We anticipate that a variety of hybrid proteins bearing the cytochrome b_5 hydrophobic domain, prepared either biochemically or genetically, could be associated with cellular membranes. Among the practical uses of this technique might be development of antibody-bearing erythrocytes capable of circulation for a substantial time, which might be useful in various diseases including AIDS. We are currently studying the fate of cytochrome b_5 and LacZ:HP in 3T3 cells after endocytosis.

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