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ORIENTATION OF TRANSMEMBRANE POLYPEPTIDES AS REVEALED BY ANTIBODY QUENCHING OF FLUORESCENCE

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We describe here a new method, based on fluorescent techniques, for the determination of the orientation of membrane protein molecules present in vesicles. The method consists of: (a) attachment of a fluorescein derivative to sugar residues of glycoproteins and glycolipids in the cell membrane, and (b) the use of anti-fluorescein antibody, a highly efficient quencher of fluorescein fluorescence, for the quantitative evaluation of sidedness of transmembrane orientation of protein molecules in vesicles. Since antibody molecules do not permeate membranes, quenching is limited exclusively to sites exposed at the external surface of the vesicles. Addition of antibody to a fluorescently-labeled cell suspension results in a full and immediate quenching of the fluorescent signal. The method is highly sensitive (pM protein concentration), rapid and readily applicable to various vesicle preparations. With this method we assessed the orientation of vesicles derived from red blood cell membranes (ghosts) in isotonic medium and followed their inversion from right-side-out to inside-out orientation upon incubation in alkaline, low ionic strength medium.

The study of transmembrane asymmetry of polypeptides in biological membranes has classically resided in the use of impermeant agents of either endogenous or exogenous character. For the case of the red cell membrane, endogenous enzymes such as acetylcholine esterase and glyceraldehyde-3-phosphate dehydrogenase have served, respectively, as intrinsic markers for the external and internal surface of the membrane [1,2]. On the other hand, availability of sialic acid residues to exogenous neuraminidase [3], protease-mediated protein fragmentation [4,5], surface labeling with enzymes or chemical agents [6], and immunochemical detection of defined polypeptide domains [7], have helped to categorize most erythrocyte components in terms of endo- and exofacial membrane domains. Although, in principle, the same approaches are also applicable to sidedness of components in proteoliposomes, their sensitivity and ease of performance are likely to vary, both with the technique and the biological system in question.

An additional promising method which has been used with cells and vesicles relies on tagging of given membrane sites with fluorescent markers and determining their exposure by the use of collision quenchers such as metals and organic substances [8–10]. However, this approach suffers from two major shortcomings, one associated with the partial nature of the quenching effects, and the second with the finite permeability of the membrane to the quenchers. In order to develop a more reliable assay, based on fluorescence quenching as

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Abbreviations; Phosphate-buffered saline, 20 mM sodium phosphate/140 mM NaCl; FTSC, fluorescein-5-thiosemicarbazide.

a means to evaluate sidedness or orientation of membrane components in cells and vesicles, we have utilized fluorescein tagged on galactosyl or sialic acid residues of the red cell membrane, in conjunction with impermeant anti-fluorescein antibodies, as highly efficient quenchers of fluorescein-emitted fluorescence [11,12]. This is schematically depicted in Fig. 1.

Red cells from recently outdated blood were washed with phosphate-buffered saline (pH 7.4) and incubated for 1 h at 37°C with galactose oxidase (Sigma) at 5 units/ml, 70–80% hematocrit [13]. The cells were washed and subsequently reacted at 50% hematocrit for 1 h at 37°C with fluorescein-5-thiosemicarbazide (FTSC) (Molecular Probes) at 0.5 mg/ml phosphate-buffered saline. In some instances the FTSC treatment was replaced by a 2 h reaction with 5-aminofluorescein, 1 mM, in phosphate-buffered saline (pH 8.0) at 37°C, followed by washing of cells and reduction with $NaCNBH_3$, 10 mM, in phosphate-buffered saline (pH 7.4) for 30 min at 5°C. After extensive washes to remove unreacted material, a fraction of the cells was lysed with 30 volumes of ice-cold 5 mM sodium phosphate (pH 8.0), centrifuged at 40000 × *g* for 10 min at 5°C, and washed with the same buffer [14]. The membranes were of a creamy yellow colour, contained 2 mg/ml protein [15] and 3 nmol fluorescein/mg membrane protein, as determined by fluorescence on a Spex Fluorolog II spectrometer (494 nm excitation, 518 nm emission), using fluorescein-albumin or carboxyfluorescein as standards. (The fluorescein content of albumin was determined by absorption at 494 nm using an ϵ_{1M}^{1cm} of 70000.) The hemoglobin-free membranes were kept at 0–5°C in a N_2 atmosphere. Higher fluorescence labeling yields (5 μmol fluorescein/mg membrane protein) were obtained by trypsinizing cells prior to reaction with the fluorescent reagents, using 0.1 mg/ml trypsin (Sigma EC 3.4.21.4, TPCK-treated; non-TPCK-treated preparations of trypsin were often contaminated with chymotrypsin activity) for 30 min at 37°C, followed by washing with phosphate-buffered saline containing 10 mg/ml bovine serum albumin. More than 90% of the fluorescent label was associated with membrane proteins (band 3 and glycophorins, as revealed by polyacrylamide gel electrophoresis), whereas those remaining were associated with glycolipids, as judged by their extractability with organic solvents [16]. Virtually no labeling was obtained by omitting the oxidation step.

Addition of small aliquots of anti-fluorescein-antibodies to a fluorescein-labeled red cell suspension (Fig. 2), resulted in a fast and proportional decrease of fluorescence, with more than 95%

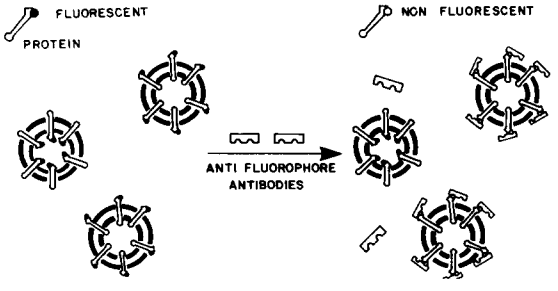

Fig. 1. Schematic representation of protein sidedness in membranes, as evaluated by immunofluorescence quenching. Antibody added to a suspension of vesicles containing opposite transmembrane protein orientation quenches the exofacial label fluorescein tagged on the protein.

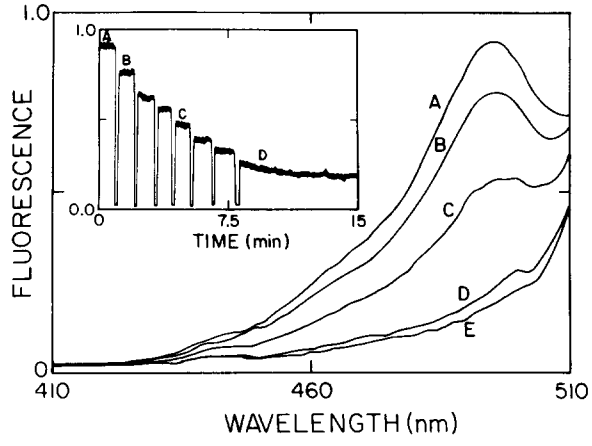

Fig. 2. Immunofluorescence quenching of fluorescein-labeled red blood cells. Anti-fluorescein antibodies were obtained by using fluorescein-albumin [17] as antigen and the protocol described elsewhere for the production of anti-NBD antibodies [18]. The antibodies were diluted back to the original volume of serum, and aliquots of 0 (A), 0.2 (B), 0.8 (C), 2 (D) and 10 (E) μl added to a 2 ml suspension of fluorescein-labeled red cells ($2 \cdot 10^6$ cells/ml phosphate-buffered saline, pH 7.4). Fluorescence excitation spectra were taken at 518 nm emission. The inset represents 494 → 518 nm readings taken after addition of increasing amounts of antibodies, as shown in the main figure, as well as others whose spectra are not shown.

quenching reached at the highest concentrations. Under these conditions, no cell agglutination was observed and, if it occurred at all, it had no demonstrable effect on cell fluorescence. Similar results were obtained using fluorescein-albumin, indicating that virtually all fluorescein fluorophores were exposed at the outer membrane surface and easily accessible to antibodies.

A similar approach was applied to isolated membrane ghost and to membrane vesicles from red cell membranes after alkali treatment (2 mM EDTA (pH 10.5), 1 h at 5°C) and centrifugation. Whereas isolated membranes behaved essentially the same as intact cells, only a minor fraction of the fluorescence was quenched by surplus amounts of antibody to alkali-treated membranes (Fig. 3). In this case, it was only by addition of detergent and disruption of vesicle structures that the remaining fraction of fluorophores could be made accessible to the otherwise impermeant quencher. This result provides support for the idea that, upon alkali-EDTA treatment, a substantial fraction of membrane vesicles undergo inversion [19], thus rendering the fluorophores inaccessible to the impermeant quencher.

Since carbohydrate moieties are present as surface components of the exofacial cell membrane domain, it is clear that tagging of fluorescein groups on these carbohydrates provides entities whose sidedness, with respect to the membrane surfaces, can be easily assessed with the aid of anti-fluorescein antibodies. This approach is applicable for the determination of the orientation of vesicles (right-side-out and inside-out) as well as orientation of polypeptides in isolated or reconstituted membrane systems (Darmon et al., submitted).

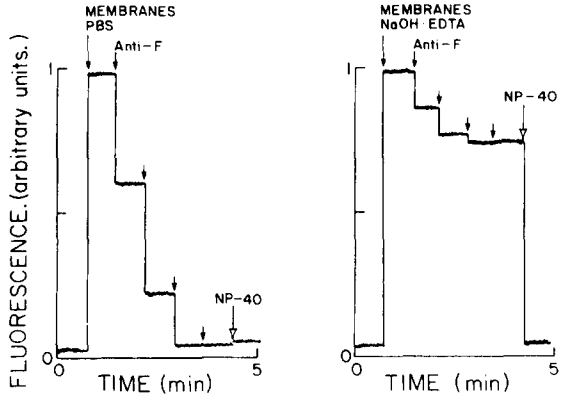

Fig. 3. Sidedness of membrane components in isolated membranes and vesicles. Aliquots of anti-fluorescein (anti-F) antibody were added to phosphate-buffered saline (PBS) suspensions (pH 7.4) of isolated red cell membranes (left) and alkali-treated red cell membranes (right), both containing 3–5 μg/ml membrane protein. After maximal fluorescence quenching was attained, the detergent Nonidet P-40 (NP-40) was added (0.01% final concentration). Fluorescence measurements were followed with time at 25°C at 494 nm excitation and 518 nm emission.

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