ISOLATION OF HUMAN ERYTHROCYTE MEMBRANES AND THEIR OUTER SURFACE PROTEINS BY COVALENT FRACTIONATION ON SOLID SUPPORT

Yuval ESHDAT and Aya PRUJANSKY-JAKOBOVITS
Department of Biophysics, The Weizmann Institute of Science, Rehovot, Israel

Received 5 March 1979

1. Introduction

An understanding of the molecular basis of the structure and function of biological membranes requires their isolation and complete characterization. Current methodology for purification of these membranes relies almost exclusively on centrifugation techniques [1]. We describe here a novel method for the isolation of human erythrocyte membranes based on the covalent binding of the cells to a solid support via cleavable disulfide bonds. After attachment, the cells are lysed, washed and the resultant membranes detached from the support by mild reduction with dithiothreitol. The application of this method to the isolation and identification of outer cell surface proteins is described.

2. Materials and methods

AH-Sepharose 4B (Agarose beads containing aminohexyl side chains, ~10 μmol/ml) was obtained form Pharmacia, dimethyl 3,3′-dithiobispropionimidate HCl and methyl 4-mercaptobutyrimidate HCl were purchased from Pierce, 2,2′-dithiopyridine was a product of Aldrich, and phenylmethanesulfonyl fluoride was obtained from Sigma. Other chemicals, purchased from commercial sources, were of the highest purity available and were used without further purification.

Human erythrocytes, obtained from freshly drawn blood, were centrifuged at 2500 × *g* for 5 min. The pellets were suspended in 5 mM phosphate buffer containing 150 mM NaCl (PBS, pH 8.0) and centrifuged as above. After each centrifugation the buffy coat was removed by aspiration. This operation was repeated 5 times to obtain the washed cells.

For comparative studies, human erythrocyte ghosts were isolated by a conventional method [2]. Sodium dodecyl sulfate (SDS)–polyacrylamide gel electrophoresis and staining were according to [3] as modified [2].

An activated solid support carrying cleavable reactive imidoester groups was prepared by mixing equal volumes of AH-Sepharose 4B beads and 40 mM dimethyl 3,3′-dithiobispropionimidate in PBS (pH 8.6) at 23°C for 15 min. Excess free reagent was removed and the pH adjusted by washing the activated beads with ice-cold PBS (pH 8.0). The activated beads were immediately used for binding the washed cells. Unless otherwise mentioned, all operations were at 4°C.

3. Results and discussion

The preparation of human erythrocyte membranes by covalent fractionation on a solid support is illustrated in fig.1. The attachment of cells to beads was accomplished by gentle agitation of activated beads (5 ml) with an equal volume of a 10% suspension of red blood cells (~10^9 cells/ml) in PBS (pH 8.0) (fig.2). After 10 min at room temperature or 20 min at 4°C, the cell-coated beads were allowed to settle and the supernatant containing free cells was aspirated. The excess of reactive imidoester groups was inactivated by suspending the beads for 30 min in 5 vol. 50 mM ethylamine in isotonic solution (pH 8.0). The

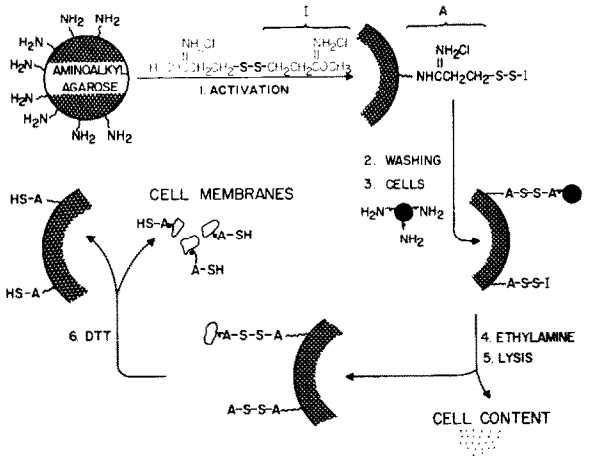

Fig.1. Schematic illustration of cell membrane isolation by covalent fractionation on a solid support. Aminoalkyl agarose beads were activated (step 1) by reaction with imidoester groups (I) of the reversible crosslinking reagent 3,3'-dithiobispropionimidate, to form chemically stable amidine groups (A) which link dithioimidate groups to the beads. Following washing (step 2), the active beads were mixed with cells (step 3), leading to amidination of the beads' imidoesters by amino groups of cell surface components. The excess of imidoesters on the beads were deactivated via amidination by ethylamine (step 4), the covalently bound cells were lysed (step 5), and the cell content removed. Reduction by dithiothreitol (DTT) (step 6) of the disulfide bonds linking the resultant cell membranes and the beads, permitted the isolation of the cell membranes.

remaining free cells were removed by permitting the beads to settle and then removing the supernatant. The beads were washed 3–5 times with 5 vol. PBS (pH 8.0) in the same fashion.

The bound cells were rapidly lysed by suspending the coated beads in 30 vol. 5 mM phosphate buffer (pH 8.0) containing 30 μM phenylmethanesulfonyl fluoride. After 15 min gentle agitation, the beads were left to settle and washed twice with the lysing buffer. Phase-contrast microscopy revealed beads covered with ghosts (fig.3), while free ghosts could not be observed in the supernatant. The bound membranes were released by suspending the coated beads in 5 vol. lysing buffer containing 40 mM dithiothreitol. After 4 h gentle agitation, all ghosts were detached from the beads. Such detachment did not occur when the beads were agitated in the absence of dithiothreitol, implying that the membranes were covalently bound to the solid support via disulfide bonds (this conclusion was further substantiated by the observation that no membranes could be obtained by mixing cells with beads that had been inactivated by treatment with dithiothreitol or ethylamine). After removing the beads by centrifugation at 100 × g for 2 min, the detached ghosts were collected from the supernatant by centrifugation at 48 000 × g for 30 min. As many as 2×10^8 ghosts were obtained from 1 ml

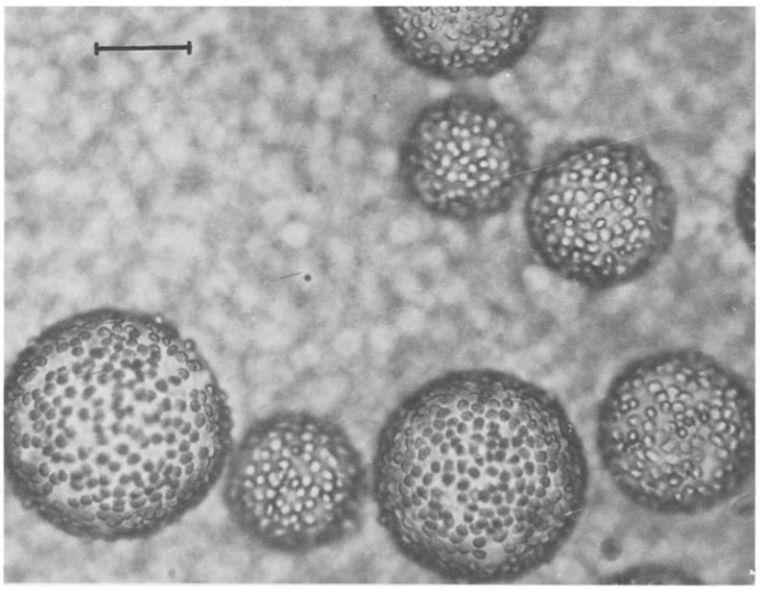

Fig.2. Sepharose 4B beads covered with covalently attached red blood cells. Bar = 50 μm.

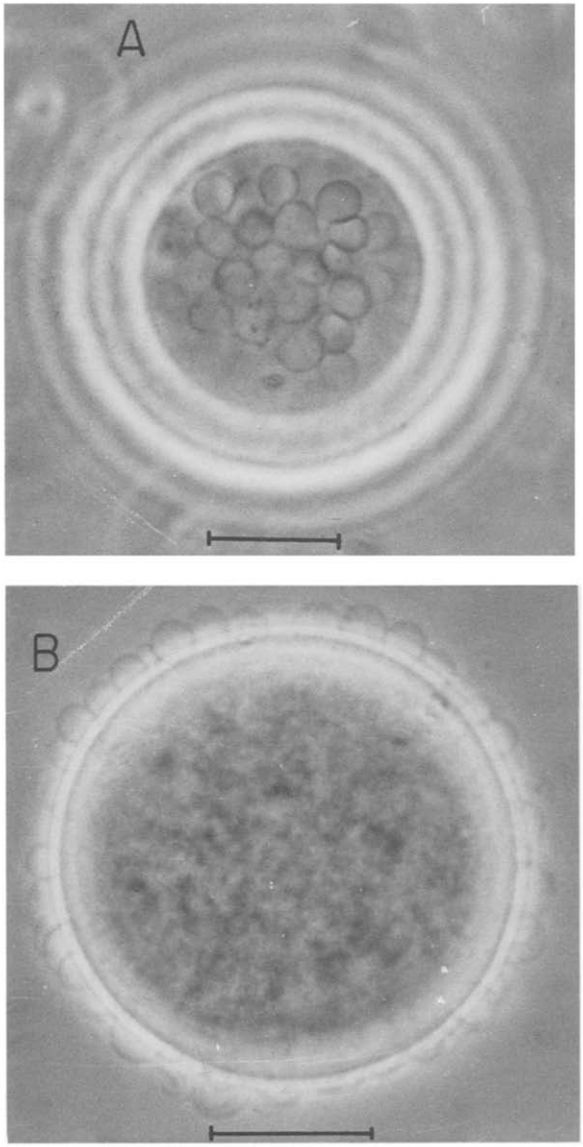

Fig.3. Red blood cell ghosts covalently attached to a Sepharose 4B bead: (A) focus on the top of the bead; (B) focus on the circumference of the bead. Bar = 20 μm.

activated beads. The ghosts were found to be identical to those obtained by the conventional methods [2–5], both by phase-contrast microscopy and by SDS–gel electrophoresis of their protein constituents (fig.4A).

The covalent binding of the erythrocytes to the solid support involves proteins which are located at the outer surface of the cells. We therefore modified the above procedure to isolate and identify these cell surface proteins. Membrane-coated beads were incubated at 37°C for 30 min in 2% SDS rather than dithiothreitol. This treatment solubilized the bulk of the membranes and their protein constituents. The remaining covalently bound proteins, containing ~3%

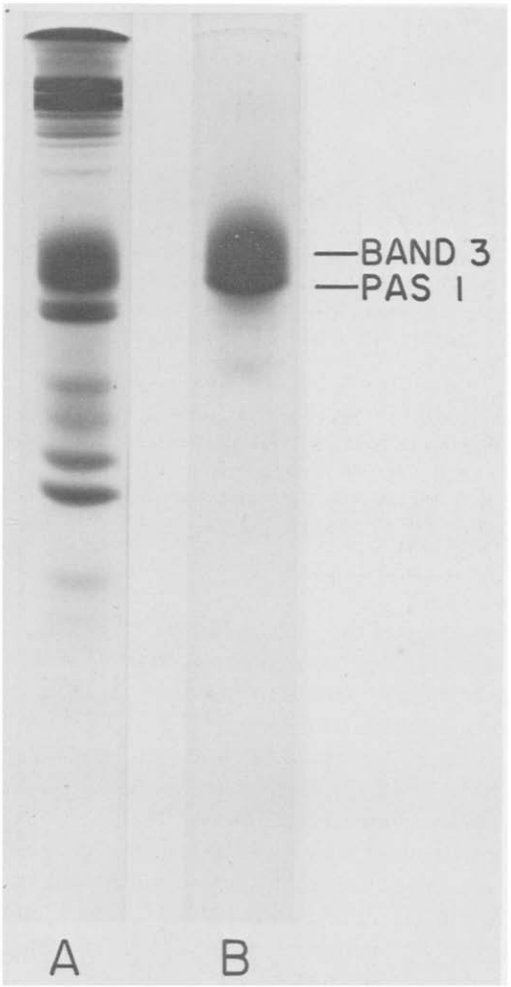

Fig.4. Gel electrophoresis in SDS, detected by staining with Coomassie brilliant blue R-250: (A) ghosts obtained by covalent fractionation on solid support; (B) membrane proteins covalently bound to the solid support during attachment of the cells; the position of band 3 and the major fraction of glycophorin (PAS 1) is indicated. Staining of the two gels with periodic-Schiff reagent resulted in patterns indicating the presence of glycophorin in both samples.

of the total protein content of the bound ghosts, were released when the treated beads were washed and then incubated with 40 mM dithiothreitol in 0.2% SDS for 2 h at 37°C. Thus, only a minor fraction of the protein constituents of the membranes isolated by our method were chemically modified. Gel electrophoresis of this protein fraction indicated that it contains the two major glycoproteins of the erythrocyte membrane, band 3 glycophorin (fig.4B). These two proteins were shown to be located on the outer surface of the human red cell by various labeling techniques [4,5]. However, while these techniques only allow the identification of the surface proteins, our method also permits the isolation of the membrane constituents through which the cells bind to the solid support.

Attachment of cells to solid supports via specific ligands or by different kinds of nonspecific interactions, such as hydrophobic or electrostatic, has been reported (e.g., [6–8]). Preparation of membranes from various cells on a solid support covered with polylysine was recently described, and is based on electrostatic interaction between the positively charged beads and the negatively charged cells [9–12]. The main advantage of our method is that the attachment of the cells to the solid support is via strong covalent bonds which are independent of conditions such as pH, ionic strength, buffer type and temperature of the medium. As a result of the covalent binding, we were able to lyse the attached cells by mild hypotonic pressure and to obtain whole ghosts attached to the beads rather than just pieces of membranes. A practical limitation of the new method is the large volumes of the resin needed for large scale membrane preparation. However, the deactivated beads, obtained after the detachment of the membranes, can be regenerated by treatment with a 1.5 M excess of 2,2′-dithiopyridine at pH 8.0 for 30 min, followed by treatment with methyl 4-mercaptobutyrimidate HCl under the same conditions.

The primary objective for the isolation of red cell ghosts, as described here, was the examination of the basic principles of the method. The human red cell ghosts were selected as a model system for the present study because they are well characterized. The method described offers considerable promise for obtaining highly purified membrane since all undesirable cell components can be removed from the bound membranes more easily and efficiently than currently possible with other purification techniques. The isolation of other cell membranes by covalent fractionation on solid supports is now in progress. Furthermore, the potential use of this new method is not limited to the isolation of membranes from cells or their organelles as evidenced by its application here to the isolation and the identification of cell surface proteins.

Acknowledgements

We thank Dr Halina Lis and Dr Nathan Sharon for their critical reading of the manuscript.

References

[1] Graham, J. M. (1975) in: New Techniques in Biophysics and Cell Biology (Pain, R. H. and Smith, B. J. eds) vol. 2, pp. 1–42, John Wiley and Sons, London.
[2] Furthmayr, H. and Marchesi, V. T. (1976) Biochemistry 15, 1137–1144.
[3] Fairbanks, G., Steck, T. L. and Wallach, D. F. H. (1971) Biochemistry 10, 2606–2617.
[4] Marchesi, V. T., Furthmayr, H. and Tomita, M. (1976) Ann. Rev. Biochem. 45, 667–698.
[5] Furthmayr, H. (1977) in: Receptors and Recognition (Cuatrecasas, P. and Greaves, M. F. eds) ser. A, vol. 3, pp. 101–132, Chapman and Hall, London.
[6] Halperin, G. and Shaltiel, S. (1976) Biochem. Biophys. Res. Commun. 72, 1497–1503.
[7] Mazia, D., Schatten, G. and Sale, W. (1975) J. Cell. Biol. 66, 198–200.
[8] Edelman, G. M. and Rutishauser, U. (1974) Methods Enzymol. 34, 195–225.
[9] Jacobson, B. S. and Branton, D. (1977) Science 195, 302–304.
[10] Cohen, C. M., Kalish, D. I., Jacobson, B. S. and Branton, D. (1977) J. Cell. Biol. 75, 119–134.
[11] Jacobson, B. S. (1977) Biochim. Biophys. Acta 471, 331–335.
[12] Kalish, D. I., Cohen, C. M., Jacobson, B. S. and Branton, D. (1978) Biochim. Biophys. Acta 506, 97–110.