Cytosolic protein binding to band-3 protein inhibits endocytosis of isolated human erythrocyte membranes

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Recent studies of haemoglobin binding to the cytoplasmic side of the erythrocyte membrane have shown that the predominant high-affinity interaction occurs with the major integral membrane protein known as band-3 protein and that this interaction may occur within the intact erythrocyte in a manner regulated by cell pH. We report here that haemoglobin and glyceraldehyde 3-phosphate dehydrogenase binding to band-3 protein in isolated membranes can inhibit endocytosis during vesiculation *in vitro*. The specificity of this effect was demonstrated by showing that myoglobin, which has an affinity for the membrane fully one to two orders of magnitude lower than that for haemoglobin, does not inhibit endocytosis.

The factors that influence membrane shape and flexibility are also very probably important to membrane budding (endocytosis and exocytosis). Despite the relative stability of the erythrocyte membrane, there are several well-documented examples of erythrocyte membrane invagination in response to certain amphipathic drugs (Deuticke, 1968; Ginn *et al.*, 1969; Ben-Bassat *et al.*, 1972). Furthermore, membrane invagination can be induced in neonatal erythrocytes by antibody binding (Blanton *et al.*, 1968). Membrane evagination occurs with ATP depletion (Lutz, 1978) and in irreversibly sickled cells (Lessin *et al.*, 1978). It has been suggested that membrane budding is a normal occurrence, perhaps related to erythrocyte age, and that these changes may signal splenic sequestration (Holroyde *et al.*, 1970).

The cytoskeletal protein meshwork is thought to determine membrane stability in isolated erythrocyte 'ghosts' (Lux, 1979; Branton *et al.*, 1981; Gratzer, 1981). These proteins tend to remain associated in medium of high ionic strength or in the presence of 0.1 mM-Mg²⁺. Homogenization under these conditions leads to the formation of right-side-out vesicles. Ion depletion and homogenization, on the other hand, lead to formation of inside-out vesicles. Lange *et al.* (1980) have shown that increases in erythrocyte membrane cholesterol content inhibit endocytosis when isolated membranes are homogenized in media of low ionic strength after a 37°C

incubation. These results and others (Schrier *et al.*, 1979) suggest that membrane budding is a multifactorial phenomenon. They point to the need for studies of other potential factors that might influence budding.

In the present paper the effect of protein binding on endocytosis is studied. Haemoglobin and glyceraldehyde 3-phosphate dehydrogenase are used, since they have been shown to interact with the 40000-dalton cytoplasmic portion of band-3 protein in solution (Yu & Steck, 1975; Salhany *et al.*, 1980), and probably within the intact cell under physiological conditions (Kliman & Steck, 1980; Eisinger *et al.*, 1982). Myoglobin was used as a control for a non-specific binding protein on the basis of its comparatively very low affinity for the membrane, as suggested earlier (Salhany *et al.*, 1980) and shown in the present study.

Materials and methods

Recently out-dated human whole blood or packed erythrocytes were obtained from the Omaha Chapter of the American Red Cross. Fresh blood was obtained from a healthy donor. White erythrocyte 'ghosts' were prepared as previously described (Salhany *et al.*, 1978). After the fourth wash in 5 mM-sodium phosphate buffer, pH 8, the 'ghosts' were salt-stripped in 200 mM-NaCl/5 mM-sodium phosphate buffer, pH 8, and washed twice in 5 mM-phosphate buffer, pH 8, and once in 5 mM-phosphate buffer, pH 6.

Haemoglobin was prepared essentially as described previously (Salhany *et al.*, 1972), except

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that the erythrocytes were washed in 150 mM-NaCl/5 mM-phosphate buffer, pH 8, lysed in 5 mM-phosphate buffer, pH 8, and dialysed against 5 mM-phosphate buffer, pH 6, at 4°C, after chromatography in 0.1 M-NaCl/20 mM-Tris/HCl buffer, pH 8. Glyceraldehyde 3-phosphate dehydrogenase (from yeast) was obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.) and dialysed against 5 mM-phosphate buffer, pH 6.

Vesiculation of the unsealed erythrocyte 'ghosts' as a function of bound haemoglobin was studied by using Steck's (1974) method with the following variations. Portions (2 ml) of packed erythrocyte 'ghosts' were mixed 1:1 (v/v) with various haemoglobin concentrations (0 to 300 μ M) at pH 6, which led to virtually complete haemoglobin binding. The samples were diluted with 5 mM-phosphate buffer, pH 6, to about 40 ml and centrifuged at 29 000 *g* (15 000 rev./min) in a Sorvall centrifuge with rotor SS-34 for 10 min. The supernatant fractions were colourless. The tubes were placed on ice, and filled to 40 ml with 0.5 mM-phosphate buffer, pH 6, and incubated for 60 min. They were then centrifuged at 29 000 *g* for 30 min, then incubated at 37°C for 35 min and homogenized with four passes through a 27-gauge needle. After the homogenization, vesicles were washed in 0.5 mM-Tris/HCl buffer, pH 8, and suspended in 10 ml of the column buffer (0.5 mM-Tris/HCl buffer, pH 8, containing 0.5 mM-CaCl₂ and 0.5 mM-MnCl₂).

Preparations of inside-out vesicles were characterized on a concanavalin A-cellulose column. The method is adapted from the procedure of Kondo *et al.* (1980) and is based on the principle that erythrocyte-membrane sugars are exclusively located on the outer surface. Details of concanavalin A-cellulose preparation were as described by Kondo *et al.* (1980). Concanavalin A-cellulose columns in 0.5 mM-Tris/HCl buffer, pH 8, containing 0.5 mM-CaCl₂ and 0.5 mM-MnCl₂, were packed, and vesicles (0.3 mg of protein/ml) were gently stirred into the top layer. The column was left to stand for 10 min and then eluted with the same buffer, and 2 ml samples were collected. Measurements of the absorbance at 495 nm (where there is virtually no haemoglobin absorbance; Antonini & Brunori, 1971) were used to monitor vesicle turbidity. After the peak of inside-out vesicles appeared, 9 ml of methyl mannoside (Sigma Chemical Co.) in 0.5 mM-Tris/HCl buffer, pH 8 (0.1 M final concentration), was applied to the column. The column was left to stand overnight to assure complete release of right-side-out vesicles, filled with more methyl mannoside solution, and additional fractions were collected. Confirmation that peak turbidities corresponded to the presence of vesicles was made by protein determinations by the method of Lowry *et al.* (1951), with bovine serum albumin as standard.

Routine transmission electron microscopy was performed with a Zeiss M-9 microscope.

Results and discussion

The breakdown of erythrocyte 'ghosts' into inside-out vesicles occurs by one of two pathways; which one is followed depends on temperature when other conditions are the same (Gratzer, 1981; Steck, 1974). When unsealed erythrocyte 'ghosts' are warmed to 37°C in low-ionic-strength buffer, the 'ghosts' swell and release a network consisting of cytoskeletal proteins (Gratzer, 1981). Vesicles then probably form from the reticulum-free section of the bilayer, since the kinetics of breakdown of the erythrocyte 'ghosts' and the amount of free reticula parallel each other (Yu *et al.*, 1973). The effect of haemoglobin binding on endocytosis by this pathway is illustrated by the chromatograms in Fig. 1. Haemoglobin-free salt-stripped membranes (which also lack glyceraldehyde 3-phosphate dehydrogenase; see Salhany & Gaines, 1981) produced predominantly inside-out vesicles (i.e. the first peak that does not bind to the matrix) in 0.5 mM-phosphate buffer, pH 6, on being warmed to 37°C and homogenized as described above. Addition of chromatographed dialysed haemoglobin to unsealed erythrocyte 'ghosts' under conditions where all of the protein binds (Salhany *et al.*, 1980) caused the ratio of inside-out vesicles to right-side-out vesicles to change markedly when vesiculation was induced. Addition of methyl mannoside to the column was required to elute the right-side-out vesicles. Routine transmission electron microscopy was used to confirm the presence of vesicles in the peaks. If methyl mannoside was not added, no peaks appeared in the positions of the right-side-out vesicles, showing that specific chemical displacement of these vesicles by a sugar is required, thus confirming their right-side-out orientation. Multiple peaks of various intensities are usually observed as the right-side-out vesicles are eluted from the column; the factors that distinguish these various populations have not been investigated in detail in the present study. Fig. 2 shows the ratio of inside-out vesicles to right-side-out vesicles in the presence of different amounts of added haemoglobin.

To determine how specific the haemoglobin effect is and to test whether it is related to binding to band-3 protein, we sought two types of control experiments. One would be addition of a protein that binds specifically to band-3 protein, and a second would be addition of a protein that binds to the low-affinity secondary (i.e. non-specific) sites. We chose glyceraldehyde 3-phosphate dehydrogenase as an example of a protein that binds specifically to band-3 protein (Yu & Steck, 1975). Myoglobin was selected as an example of a non-specific binding protein.

Fig. 1. Chromatograms of human erythrocyte-membrane vesicle preparations on concanavalin A-cellulose columns

Turbidity measurements at 495 nm (1 cm cell) are plotted versus tube number for control vesicles prepared as described in the text (●) and for vesicles prepared in an identical manner except that the starting unsealed erythrocyte 'ghosts' had 180 μ M-haemoglobin (haem basis) bound (○). The conditions for adding the haemoglobin, described more fully in the text, were such that all the haemoglobin was bound. After vesiculation, the material was washed free of haemoglobin and suspended in column buffer as described in the text. The first peak contains the inside-out-vesicle fraction, which does not bind to concanavalin A-cellulose (Kondo *et al.*, 1980). The arrow indicates the point at which methyl mannoside was added. The additional peaks represent methyl mannoside-displaced right-side-out vesicles (Kondo *et al.*, 1980).

The fact that myoglobin is a very weak non-specific binding protein is vividly illustrated in Fig. 3. We have previously measured haemoglobin's affinity to be about 0.2 μ M under the present conditions (Salhany *et al.*, 1980). The conditions used for Fig. 3 were such that the concentration of membrane sites was greater than the concentration of haemoglobin, and, as a consequence of its high affinity, we observed stoichiometric haemoglobin binding, as expected. When the same membrane site concentration per unit sample volume was used for myoglobin, non-stoichiometric hyperbolic binding was observed. This indicates that myoglobin has a very low affinity. The inset to Fig. 3 shows a double-reciprocal plot, from which we find that myoglobin's dissociation constant was about 53 μ M.

Fig. 2. Ratio of integrated peak intensities from chromatograms similar to those in Fig. 1 plotted versus the final concentration of haemoglobin (●), glyceraldehyde 3-phosphate dehydrogenase (○) or myoglobin (□) added per mg of unsealed erythrocyte 'ghost' protein

For experimental details see the text. The vertical bars show the standard deviations for at least three separate determinations.

This value is fully one to two orders of magnitude lower than that for haemoglobin under the same conditions (Salhany *et al.*, 1980). The value is close to that previously estimated for haemoglobin binding to the low-affinity sites (Salhany *et al.*, 1980).

When enough glyceraldehyde 3-phosphate dehydrogenase was added to saturate all band-3-protein sites on the membrane, we see that it also inhibited membrane endocytosis (Fig. 2). Addition of myoglobin did not inhibit endocytosis (Fig. 2). The presence of myoglobin does seem to eliminate completely those few right-side-out vesicles that form under control conditions, thus explaining the slightly higher ratio of inside-out vesicles to right-side-out vesicles. These results suggest that, when a protein binds to band-3 protein, membrane endocytosis caused by ion depletion at 37°C is inhibited, whereas non-specific protein binding to the membrane does not have a similar effect. The mechanism by which protein binding to band-3 protein inhibits endocytosis is unknown.

We must point out one potentially important consequence of our present finding to the problem of sickle-cell disease. It is now quite well established that sickle-cell haemoglobin has a higher affinity for the membrane than has normal haemoglobin (Fischer *et al.*, 1975; Shaklai *et al.*, 1981). There is an increase in membrane-bound haemoglobin in erythrocytes from individuals with sickle-cell

Fig. 3. Centrifugation measurements of the fractional saturation ($\bar{Y}$) plotted versus the final total concentration of haemoglobin tetramer (Hb_4) (●) or myoglobin (Mb) (○) added

The concentration of unsealed erythrocyte 'ghosts' was 1.3 mg/ml. The conditions are otherwise as stated in the text. The inset shows a double-reciprocal plot of the myoglobin data ($K_D = 53 \mu M$).

anaemia (Bank *et al.*, 1974). Bookchin *et al.* (1981) have reported that endocytosis is markedly inhibited in these membranes. Furthermore, Lessin *et al.* (1978) have shown exocytotic budding in irreversibly sickled cells with membrane-associated microbodies that contain haemoglobin. Although there are several other factors that could be responsible for these effects, our finding, combined with the results showing increased membrane-bound haemoglobin, could suggest that haemoglobin binding may contribute to this behaviour. Further research along these lines may prove rewarding and could help to answer some questions concerning cell viability in this disease.

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References

- Antonini, E. & Brunori, M. (1971) *Front. Biol.* **21**, 18–19
- Bank, A., Mears, G., Weiss, R., Odonnell, J. V. & Natta, C. (1974) *J. Clin. Invest.* **54**, 805–809
- Ben-Bassat, I., Bensch, K. G. & Schrier, S. L. (1972) *J. Clin. Invest.* **51**, 1833–1844
- Blanton, P. L., Martin, J. & Haberman, S. (1968) *J. Cell Biol.* **37**, 716–728
- Bookchin, R. M., Raventos, C. & Lew, V. L. (1981) *Ann Arbor Conf. 5th: Red Cell* 163–182
- Branton, D., Cohen, C. M. & Tyler, J. (1981) *Cell* **24**, 24–32
- Deuticke, B. (1968) *Biochim. Biophys. Acta* **163**, 494–500
- Eisinger, J., Flores, J. & Salhany, J. M. (1982) *Proc. Natl. Acad. Sci. U.S.A.* **79**, 408–412
- Fischer, S., Nagel, R. L., Bookchin, R. M., Roth, E. F., Jr. & Tellez-Nagel, I. (1975) *Biochim. Biophys. Acta* **375**, 422–433
- Ginn, F. L., Hochstein, P. & Trump, B. F. (1969) *Science* **164**, 843–845
- Gratzner, W. B. (1981) *Biochem. J.* **198**, 1–8
- Holroyde, C. P. & Gardner, F. H. (1970) *Blood* **36**, 566–572
- Kliman, H. J. & Steck, T. L. (1980) *J. Biol. Chem.* **255**, 6314–6321
- Kondo, T., Dale, G. L. & Beutler, E. (1980) *Biochim. Biophys. Acta* **602**, 127–130
- Lange, Y., Cutler, H. B. & Steck, T. L. (1980) *J. Biol. Chem.* **255**, 9331–9337
- Lessin, L. S., Kurantsin-Mills, J., Wallas, C. & Weems, H. (1978) *J. Supramol. Struct.* **9**, 537–554
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. & Randall, R. J. (1951) *J. Biol. Chem.* **193**, 265–275
- Lutz, H. U. (1978) *J. Supramol. Struct.* **8**, 375–389
- Lux, S. E. (1979) *Semin. Hematol.* **16**, 21–51
- Salhany, J. M. & Gaines, K. C. (1981) *Trends Biochem. Sci.* **6**, 13–15
- Salhany, J. M., Mathers, D. H. & Eliot, R. S. (1972) *J. Biol. Chem.* **247**, 6985–6990
- Salhany, J. M., Swanson, J. C., Cordes, K. A., Gaines, S. B. & Gaines, K. C. (1978) *Biochem. Biophys. Res. Commun.* **82**, 1294–1299
- Salhany, J. M., Cordes, K. A. & Gaines, E. D. (1980) *Biochemistry* **19**, 1447–1454
- Schrier, S. L., Hardy, B. & Bensch, K. (1979) in *Normal and Abnormal Red Cell Membranes* (Lux, S. E., Marchesi, V. T. & Fox, C. F., eds.), pp. 437–449, Alan R. Liss, New York
- Shaklai, N., Sharma, V. S. & Ranney, H. M. (1981) *Proc. Natl. Acad. Sci. U.S.A.* **78**, 65–68
- Steck, T. L. (1974) *Methods Membr. Biol.* **2**, 245–281
- Yu, J. & Steck, T. L. (1975) *J. Biol. Chem.* **250**, 9176–9184
- Yu, T., Fischman, D. & Steck, T. C. (1973) *J. Supramol. Struct.* **1**, 233–248