Assembled Clathrin in Erythrocytes*

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Clathrin cages were isolated from rat erythrocytes. These structures exist in the intact cell as demonstrated by immunofluorescence and were not formed during the isolation procedure. The cages were largely devoid of membrane but contained the assembly protein complex and both the 50-kDa kinase (pp50) and casein kinase II activities found previously in clathrin-coated vesicles.

Coated vesicles have been described in a wide variety of nucleated cells of vertebrate and invertebrate animals (see Willingham and Pastan, 1985 and Keen, 1985, for reviews) and in yeast (Mueller and Branton, 1984), protozoa (Bowers and Korn, 1968), algae, and higher plants (see Newcomb, 1980, for review). These intracellular organelles participate in a variety of cellular processes involving membrane transport, including receptor-mediated endocytosis, biosynthesis of membrane proteins, and transcytosis (for review, see Willingham and Pastan, 1985). Because erythrocytes lack intracellular organelles and are not known to carry out endocytosis or other processes in which clathrin-coated membranes are known to participate, clathrin has been thought to be absent from these cells (Goud et al., 1985). Clathrin, active in the reticulocyte, is presumably degraded during reticulocyte maturation. Davis and Bennett (1985), on the other hand, reported the presence of clathrin skelletions in human erythrocytes but suggested that such clathrin was not in an assembled state (i.e. in coated vesicles or cages), given the excess of uncoating protein in the cytosol.

During our studies on the in vivo phosphorylation of coated vesicle proteins,1 we discovered that mature erythrocytes contain clathrin, the assembly protein complex composed of 100–110- and 50-kDa polypeptides (Keen et al., 1979). Unexpectedly, these two coated vesicle components were found to be assembled into cages devoid of membrane. The erythrocyte cages also possessed the two protein kinase activities which we have shown to be associated with coated vesicles (Bar-Zvi and Branton, 1986). Further evidence for the existence of assembled clathrin in erythrocytes was provided by immunofluorescent staining of erythrocyte ghosts with either antibodies to the clathrin light chains or antibodies to the assembly protein.

**EXPERIMENTAL PROCEDURES**

Materials—Poly-DL-lysine and ATP were obtained from Sigma. Carrier-free 32P, was obtained from Du Pont-New England Nuclear. [γ-32P]ATP was prepared as described previously (Bar-Zvi and Branton, 1986). Bicinchoninic acid protein reagent was purchased from Pierce Chemical Co. Calf brain coated vesicles were prepared as described previously (Bar-Zvi and Branton, 1986).

Antibodies—Anti-clathrin light chain antibodies were obtained from a rabbit injected with native clathrin skelletions purified from bovine brain coated vesicles. The antibodies were affinity-purified on a column containing native clathrin light chains coupled to Sepharose. Anti-clathrin antibodies were raised against polypeptides eluted from the 100–110-kDa region of an SDS-polyacrylamide gel on which highly purified bovine brain coated vesicles had been resolved. These antibodies were likewise purified on an affinity column containing 100–110-kDa proteins coupled to Sepharose. Both anti-clathrin light chain and anti-100–110-kDa antibodies specifically immunoprecipitated the corresponding antigens and identified them on immunoblots of coated vesicles.

Erythrocyte Preparation—The following steps were performed at 4°C. Blood was obtained from male white rats (250–400 g) by heart puncture. Approximately 10 ml of fresh blood was mixed with 3 ml of ice-cold anticoagulant solution containing 75 mM sodium citrate and 38 mM citric acid. Cells were washed by brief centrifugation in Hepes-buffered saline (20 mM Hepes-NaOH, pH 7.4, 130 mM NaCl), and leukocytes and platelets were removed by adsorption with a mixture of a-cellulose and Simagell (1:1). Erythrocytes were separated from reticulocytes and remaining white blood cells by centrifugation through a 45/70% Percoll step gradient for 10 min at 2500 rpm in a Sorvall SS34 rotor (without braking). Erythrocytes were recovered in the pellet, while reticulocytes banded at the 45/70% Percoll interface, and a mixture of lysed cells and remaining leukocytes banded at the 0/45% Percoll interface. Percoll was removed by washing the cells twice in Hepes-buffered saline. Human blood from healthy donors was obtained from the Red Cross and treated as above, with omission of the Percoll gradient step.

Clathrin Cage Preparation—Washed erythrocytes were resuspended in KCl isolation buffer containing 1 mg/ml phenylmethylsulfonyl fluoride (Bar-Zvi and Branton, 1986) and lysed by freezing in liquid nitrogen and thawing or, alternatively, by addition of 0.5% Triton X-100. Lysed cells were centrifuged at 12,000 × g for 20 min, and the supernatant was recentrifuged at 100,000 × g for 30 min. The pellet from this step was homogenized in the isolation buffer and centrifuged at both low and high speeds as above. The resulting pellet was centrifuged at 12,000 × g for 5 min to remove aggregated material. The supernatant was applied to a 7.5–30% glycerol gradient in KCl isolation buffer and centrifuged at 24,000 rpm for 2 h (brake off) in a Beckman SW 27.1 rotor. Fractions of 1 ml each were obtained from gradient tubes by piercing the bottom. Fractions containing clathrin cages were identified by SDS-PAGE. Cages were sedimented from diluted fractions by centrifugation at 100,000 × g for 1 h. This procedure routinely yielded approximately 5 μg of clathrin cages/ml of packed cells.

Immunofluorescence—Fresh rat blood cells were washed in phosphate-buffered saline and applied to glass coverslips previously coated with 1 mg/ml poly-L-lysine. After a 30-min incubation, attached cells were fixed in 3% paraformaldehyde for 15 min, permeabilized in 0.05% Triton X-100, and stained with rabbit antibodies, followed by fluorescein isothiocyanate-labeled goat anti-rabbit IgG. Fluorescence images from stained and fixed reticulocytes and reticulocyte pellets were acquired with a Zeiss Axioskop microscope equipped with an HBO 100-W mercury arc lamp and a Leica cooled image intensified charge-coupled device camera.
were fixed overnight at 4 °C in 3% HCHO in Small’s cytoskeleton buffer (Small, 1981). Cells were made permeable by incubation for 3 min at room temperature with 0.2% Triton X-100 in Tris-buffered saline containing 10 mM Tris-HCl, pH 7.5, 150 mM NaCl and then incubated in the same buffer containing 10% neonatal calf serum for 1 h. Individual coverslips were then incubated with diluted, affinity-purified rabbit antibodies for 3 h at room temperature. Coverslips were rinsed three times with Tris-buffered saline containing 0.1% Tween 20 and then incubated with affinity-purified fluorescein isothiocyanate-conjugated goat anti-rabbit antibody (Boehringer Mannheim) for 1 h. After rinsing in three changes of Tris-buffered saline containing 0.1% Tween 20, coverslips were mounted in polyvinyl alcohol containing 1 mg/ml p-phenylenediamine and observed with an epifluorescence-equipped Zeiss microscope.

Other Procedures—In vitro phosphorylations were carried out as described previously (Bar-Zvi and Branton, 1986). Immunoprecipitations and in vivo phosphorylations were performed as described elsewhere, with the exception that the washing solution was made with KCl isolation buffer in place of NaF isolation buffer. Immunoprecipitated proteins were quantified in Coomassie Blue-stained gels with the aid of a Hoefer GS 300 scanning densitometer. SDS-PAGE was carried out in 10% polyacrylamide gels as described by Laemmli (1970). Immunoblots were performed as described by Olmsted (1981). Protein was determined according to Smith et al. (1985) using bovine serum albumin as standard. Relative phosphoprotein concentrations were determined by diphenylhexatriene fluorescence (Prasad et al., 1985).

RESULTS AND DISCUSSION

A polypeptide of the same electrophoretic mobility as the 180-kDa clathrin heavy chain was sedimented when an erythrocyte lysate was centrifuged at 100,000 × g for 30 min. Further fractionation of this high speed pellet in a glycerol gradient produced a peak which cosedimented with purified coated vesicles isolated from either rat reticulocytes or bovine brain (Fig. 1). The protein components of the peak fractions resembled those of rat reticuloctye coated vesicles and included major polypeptides of 180 kDa (clathrin heavy chain), 100–110 and 50 kDa (assembly protein), and 33 kDa (clathrin α-light chain) and a minor 30-kDa polypeptide (clathrin β-light chain) (Fig. 1). The identities of the 180-, 100–110-, and 33-kDa polypeptides were confirmed by their cross-reaction on immunoblots with antibodies raised against bovine brain clathrin heavy chain, assembly protein 100–110-kDa polypeptides, and clathrin light chains, respectively (Fig. 2).

The principal light chain of clathrin shown to be present in erythrocytes was the α-light chain, although traces of the β-light chain were also detected. Analysis by densitometry of Coomassie Blue-stained reticulocyte coated vesicle proteins indicated a molar ratio of at least 10 clathrin α-light chains per β-light chain, in agreement with Davis and Bennett (1985), who reported one type of clathrin light chain in erythrocyte triskelions. The molar ratio of clathrin monomer to assembly protein, calculated from densitometric scans of the 180- and 100–110-kDa gel band regions, is 5.5 ± 1.0 (n = 5); by comparison, we obtained a ratio of 1.9 ± 0.4 (n = 5) for these proteins in brain coated vesicles, similar to the stoichiometries reported by Zaremba and Keen (1983) and Pearse and Robinson (1984). Davis and Bennett (1985) suggested that all erythrocyte triskelions should be soluble, given the presence of an excess of uncoating protein. This is not in agreement with our findings (Fig. 1). Davis and Bennett (1985) purified clathrin from human erythrocytes lysed in 7.5 mM NaF, 0.5 mM EDTA. However, we were able to isolate cages successfully from both human and rat erythrocytes lysed in Davis and Bennett’s buffer (not shown), suggesting that differences in the cell source or lysis buffer do not account for the difference in results. We suspect that clathrin cages existed in the erythrocyte lysate of Davis and Bennett but probably disassembled during the subsequent chromatography steps which our isolation scheme avoids.

Negative staining of peak fractions from the glycerol gradient revealed a multitude of assembled clathrin structures (Fig. 3). Most of these structures appeared to be cages, while about 20% of them (n = 128) appeared to contain material within the cage (Fig. 3, arrowheads). Because both coated vesicles and cages composed of clathrin and assembly protein may show an electron-dense core region (Vigers et al., 1986 and footnote 3), it was not possible to distinguish between these two types of structures based on their appearance. The lack of membrane in the erythrocyte clathrin cages was confirmed by the fractionation of brain coated vesicles and erythrocyte cages, preincubated with the fluorescent lipid probe diphenylhexatriene, on a glycerol gradient. Although the diphenylhexatriene fluorescence cosedimented with the brain

![Fig. 1. Assembled clathrin resolved on a glycerol gradient.](image)

![Fig. 2. Immunoblot of erythrocyte coat proteins.](image)

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3 W. Bazari and D. Branton, manuscript in preparation.
coated vesicles, no fluorescence peak was detected in association with erythrocyte clathrin cages (Fig. 4), suggesting that the majority of these structures lack membrane.

To rule out the possibility that the clathrin cages which we isolated originated in contaminating non-erythrocyte blood cells, we compared the quantity of clathrin and assembly protein immunoprecipitated from the purified erythrocyte fraction with that immunoprecipitated from the crude fraction which contained lymphocytes and reticulocytes, in addition to erythrocytes (Table I). Whereas greater than 99% of the white blood cells and reticulocytes were removed from whole blood by the erythrocyte purification procedure (Beutler et al., 1976; Rennie et al., 1979), such purification reduced the amount of immunoprecipitated coat protein only slightly, suggesting that the clathrin and assembly protein indeed originated in erythrocytes and not in contaminating cells. Furthermore, unlike the high speed pellet prepared from reticulocytes, the erythrocyte pellet did not contain ribosomes, confirming that the pellet was not derived from a cell containing intracellular organelles (not shown).

About one-half of the total clathrin and one-third of the assembly protein were found to be in the assembled form by immunoprecipitation of these protein complexes from erythrocytes following cell fractionation (not shown). To test for possible post-homogenization assembly of the erythrocyte coat proteins, 125I-triskelions from rat liver were mixed with erythrocytes following cell fractionation (not shown). To test for possible post-homogenization assembly of the erythrocyte coat proteins, 125I-triskelions from rat liver were mixed with the erythrocyte lysate. Immediate contact with erythrocyte contents was ensured in this experiment by addition of labeled triskelions to the lysis buffer prior to the addition of erythrocytes. Following lysis, the incorporation of the label into assembled structures was analyzed (Table II). Essentially no incorporation of labeled triskelions was observed, indicating that no assembly occurred during lysis and subsequent steps. This conclusion is in agreement with Goud et al. (1985), who showed that under similar conditions there is no assembly of clathrin during the lysis and fractionation of rat brain cortex. However, the added clathrin assembled as expected, following dialysis against 20 mM Mes-NaOH, 2 mM CaC12 buffer (Table II). The possibility that assembled clathrin is disassembled during the steps subsequent to erythrocyte lysis would not be consistent with the stability of coated vesicles in the lysis buffer; coated vesicles isolated in this buffer remained in an assembled state for at least 2 months at 4 °C.

The existence of clathrin cages in the intact erythrocyte was further supported by immunofluorescent staining using affinity-purified antibodies to the light chains of clathrin or to the assembly protein. Staining with either antibody produced a punctate distribution of fluorescence (Fig. 5), in agreement with the typical punctate patterns shown for the localization of clathrin heavy chain (Anderson et al., 1978), clathrin light chains (Lisanti et al., 1982), and the 100-kDa polypeptides of the assembly protein (Robinson and Pearse, 1986) in other cells. After staining with either anti-clathrin light chain or anti-100–110-kDa antibodies, the number of fluorescent dots per cell varied from cell to cell. Furthermore, more than one-half of the cells in a typical microscope field were stained with the anti-clathrin light chain antibody (panel A), while only about one-eighth were stained by the anti-100–110 kDa antibody (panel B). Although the erythrocyte ghosts were difficult to see by phase contrast, staining of ghosts on

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Table I

| Clathrin | Assembly protein |
|---------|-----------------|
| Unfractionated | Unfractionated |
| Erythrocytes | Erythrocytes |
| arbitrary units | arbitrary units |
| 0.35 ml | 4380 | 4190 | 2750 | 1940 |
| 0.70 ml | 7440 | 6530 | 3780 | 2660 |

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**Fig. 3.** Electron microscopy of erythrocyte cages. Erythrocyte cages were prepared as described in Fig. 1. A peak clathrin-containing fraction was negatively stained with 3% uranyl acetate. Bars, 100 nm.

**Fig. 4.** Assembled cages in erythrocytes lack an internal membrane. Either the high speed pellet of the erythrocyte lysate (○) (obtained as in Fig. 1) or purified bovine brain coated vesicles (△) were incubated for 1 h at room temperature with diphenylhexatriene and then resolved on a glycerol gradient. Fractions of 0.5 ml each were collected and analyzed for diphenylhexatriene fluorescence (○, △). Aliquots of 35 μl of each fraction were resolved by SDS-PAGE. The Coomassie Blue-stained clathrin heavy chain band was quantified by densitometry using a Hoefer GS 300 scanning densitometer (○).

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4 D. Bar-Zvi, unpublished observation.
identically prepared coverslips with antibodies to spectrin showed that ghosts were distributed at a similar density on all coverslips and were made equally permeable to antibodies by detergent extraction (not shown). Thus, the disparity in the proportion of cells stained by our two antibodies appears to be real and accords with the measured excess of clathrin over assembly protein in erythrocytes (see above).

Erythrocyte clathrin cages possess both of the protein kinase activities (50-kDa kinase and casein kinase II) shown to be associated with clathrin-coated vesicles. Incubation of erythrocyte cages with Mg\(^{2+}\)–γ\(^{32}\)P]ATP resulted in the labeling of the 50-kDa polypeptide (Fig. 6, lane 1), which is consistent with the previously described autophosphorylation of the assembly protein (Pauloin et al., 1982; Campbell et al., 1984; Keen et al., 1987). When phosphorylation was assayed in the presence of polylysine, a stimulator of casein kinase I\(\text{II}\) activity (Fig. 6, lane 2), casein kinase was shown to preferentially phosphorylate the clathrin β-light chain (Usami et al., 1985; Schook and Puszkin, 1985; Bar-Zvi and Branton, 1986). Although both erythrocyte clathrin light chains were labeled in the presence of polylysine, when the higher abundance of the clathrin α-light chain (Fig. 1) was taken into account, the specific activity of the phosphorylated β-light chain was at least 10 times higher than that of the α-light chain. Because the clathrin cages lack membrane (Figs. 19 and 20) but possess casein kinase II activity (Fig. 6), we conclude that casein kinase II is associated with the coat proteins and not with the membranes of coated vesicles.

### Table II

Clathrin is not assembled during the handling of erythrocyte cell lysate

|  | Without dialysis | With dialysis |
|---|-----------------|---------------|
| \[^{32}\text{P}\]Clathrin sedimented |  |  |
| \[^{32}\text{P}\]Clathrin added to |  |  |
| Erythrocyte lysates | <2 | 54 |
| Lysis buffer |  | 78 |

The presence in erythrocytes of cages composed of clathrin and assembly protein is unexpected, and the role of clathrin in mature erythrocytes is not clear. Two possibilities may be suggested: that clathrin is nonfunctional while gradually disappearing from the cell or that it maintains a residual function. In agreement with the first possibility, erythrocytes do not possess intracellular organelles and are not believed to carry out membrane transport processes or receptor-mediated endocytosis. Reticulocytes, by contrast, are very active in the endocytosis of transferrin (for hemoglobin synthesis) and other ligands. Maturation of reticulocytes to erythrocytes involves degradation of many proteins and cell components (Rappoport et al., 1974; Van Bockxmeer and Morgan, 1979). Further changes occur during the aging of mature erythrocytes: cells become more dense (Rennie et al., 1979), and the plasma membrane becomes more rigid (Shiga et al., 1979). ATP-dependent proteolysis activity declines rapidly upon reticulocyte maturation and decreases further with the age of the erythrocytes (Speiser and Etilinger, 1982). The activities of several metabolic enzymes, including pyruvate kinase, acetylcholinesterase and phosphoglycerate kinase decrease as a function of cell age (Kadluboski and Agutter, 1977; Cohen et al., 1976; Rennie et al., 1979). Consistent with these changes,
the quantity of assembled clathrin in rat erythrocytes is less than 10% of that found in rat reticulocytes. It is thus possible that the disparity in the proportion of cells stained by our two antibodies (Fig. 5) results from a difference in the rate of degradation of the two proteins during reticulocyte maturation and erythrocyte aging.

On the other hand, a possible role for clathrin is suggested by observations of recycling and down-regulation of insulin receptors in erythrocytes (Peterson et al., 1983). The number of insulin receptors per cell has been shown to decrease exponentially with erythrocyte age (Dons et al., 1981; Wilson and Peterson, 1986). In contrast, the maximal extent of insulin receptor down-regulation decreases linearly with age (Wilson and Peterson, 1986), which suggests that some factor other than the copy number of the receptor itself participates in its regulation. Since receptor recycling and down-regulation have been shown to involve clathrin-coated membranes in other cell types (Willingham and Pastan, 1985), it is likely that some of the coat proteins are involved in these processes in erythrocytes.

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REFERENCES

Anderson, R. G. W., Vasile, E., Mello, R. J., Brown, M. S., and Goldstein, J. L. (1978) Cell 15, 919–933
Bar-Zvi, D., and Braxton, D. (1986) J. Biol. Chem. 261, 9614–9621
Beutler, E., West, C., and Blume, K.-G. (1984) J. Biol. Chem. 259, 9614–9621
Cohen, N. S., Ekholm, J. E., Luthra, M. G., and Hanahan, D. (1976) J. Lab. Clin. Med. 88, 328–333
Campbell, C., Squicciarini, J., Shia, M., Pilch, P., and Fine, R. E. (1984) Biochemistry 23, 4420–4426
Cohen, N. S., Ekholm, J. E., Luthra, M. G., and Hanahan, D. J. (1976) Biochim. Biophys. Acta 419, 229–242
Davis, J. Q., and Bennett, V. (1985) J. Biol. Chem. 260, 14850–14856
Dons, R. F., Corash, L. M., and Gorden, P. (1981) J. Biol. Chem. 256, 2982–2987
Goud, B., Huet, C., and Louvard, D. (1985) J. Biol. Chem. 100, 521–527
Hanpal, M., Luna, E., and Brannon, D. (1984) J. Biol. Chem. 259, 11075–11082
Kadlubowski, M., and Agutter, P. S. (1977) Br. J. Haematol. 37, 111–125
Keen, J. H. (1985) in Endocytosis (Pastan, I., and Willingham, M. C., eds) pp. 85–130, Plenum Publishing Corp., New York
Keen, J. H., Willingham, M. C., and Pastan, I. H. (1979) Cell 16, 303–312
Keen, J. H., Chestnut, M. H., and Beck, K. A. (1987) J. Biol. Chem. 262, 3864–3871
Laemmli, U. K. (1970) Nature 227, 680–685
Lisanti, M. P., Shapiro, L. S., Moskowitz, N., Hua, E. L., Puskin, S., and Schoo, W. (1982) Eur. J. Biochem. 125, 463–470
Mueller, S. C., and Branton, D. (1984) J. Cell Biol. 98, 341–346
Newcomb, E. H. (1980) in Coated Vesicles (Ockleford, C. D., and Whyte, A., eds) pp. 55–88, Cambridge University Press, Cambridge
Paukowiec, A., Bernier, J., and Pelletier, S. (1984) J. Cell. Biol. 98, 11955–11957
Pothulo, A., Bernier, J., and Pelletier, S. (1982) Nature 298, 574–576
Pearse, B. M. F., and Robinson, M. S. (1984) EMBO J. 3, 1951–1957
Peter, S. W., Miller, A. L., Kelleher, R. S., and Murray, E. F. (1983) J. Biol. Chem. 258, 9605–9607
Prasad, K., Lippoldt, R. E., and Edelhoch, H. (1985) Biochemistry 24, 6421–6427
Rapoport, S., Hartwig, A., and Gross, J. (1974) Acta Biol. Med. Ger. 32, 601–608
Rennie, C. M., Thompson, S., Parker, A. C., and Maddy, A. (1979) Clin. Chim. Acta 98, 119–125
Robinson, M. S., and Pearse, B. M. F. (1986) J. Cell Biol. 102, 48–54
Schook, W. J., and Puskin, S. (1985) Proc. Natl. Acad. Sci. U. S. A. 82, 8039–8043
Shiga, T., Maeda, N., Suda, T., Kon, K., and Sekiya, M. (1979) Biochim. Biophys. Acta 553, 84–96
Small, J. V. (1981) J. Cell Biol. 91, 695–705
Smith, P. K., Krohn, R., Hermanson, G. T., Mallia, A. K., Gartner, F. H., Provenzano, M. D., Fujimoto, E. K., Goeke, N. M., Olson, B. J., and Klenk, D. C. (1985) Anal. Biochem. 150, 76–85
Speiser, S., and Ethington, J. D. (1982) J. Biol. Chem. 257, 14122–14127
Usami, M., Takahashi, A., Katoda, K., and Katoda, K. (1985) J. Biochem. (Tokyo) 97, 1819–1822
Van Bockxmeer, F. M., and Morgan, E. H. (1979) Biochim. Biophys. Acta 584, 76–83
Vigers, G. P. A., Crowther, R. A., and Pearse, B. M. F. (1986) EMBO J. 5, 2079–2085
Willingham, M. C., and Pastan, I. (1985) in Endocytosis (Pastan, I., and Willingham, M. C., eds) pp. 1–44, Plenum Publishing Corp., New York
Wilson, C., and Peterson, S. W. (1986) J. Biol. Chem. 261, 2123–2128
Zaremba, S., and Keen, J. H. (1983) J. Cell Biol. 97, 1339–1347