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Clathrin: A unique protein associated with intracellular transfer of membrane by coated vesicles

(brain/adrenal medulla/lymphoma cells/peptide mapping)

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ABSTRACT **Coated vesicles have been purified from brain, adrenal medulla, and a nonsecreting lymphoma cell line. A single major protein species, clathrin, with an apparent molecular weight of 180,000, forms the coat of all these vesicles. Peptide mapping suggests that the amino acid sequence of clathrin is conserved, irrespective of tissue or species studied. Coated vesicles of different sizes are found. The coats are constructed with variable numbers of clathrin subunits, arranged in closed networks of hexagons and pentagons. The amount of clathrin in lymphoma cells suggests that coated vesicles transfer substantial amounts of membrane within cells, not necessarily in association with a secretory process.**

Transfer of material between the different membranous organelles of cells—the endoplasmic reticulum, Golgi apparatus, specialized vacuoles, and plasma membrane—is believed to be mediated by various type of vesicles (1–3). One such class, known as "coated vesicles," has been observed by electron microscopy in many different cells. These vesicles are remarkable for the lattice-like coats they have on their cytoplasmic surfaces. The biological role of coated vesicles seems to vary depending on the functions of the cell type. In the frog neuromuscular junction, the vesicles have been shown to resorb membrane from the presynaptic membrane for re-use, after secretion of acetylcholine by synaptic vesicles (4). In other cells, whose function is to absorb material, coated vesicles are believed to form from the plasma membrane, thereby endocytosing extracellular protein (5, 6). This type of vesicle also appears to transport noncytoplasmic enzymes from the Golgi apparatus to their sites of action (6, 7). Having developed a procedure for the purification of coated vesicles from pig brain (8), I found that their coats consisted essentially of a single protein with an apparent molecular weight of 180,000 on sodium dodecyl sulfate gel electrophoresis. I named the protein "clathrin" to indicate the lattice-like structures which it forms. I have now purified coated vesicles from a different source, the adrenal medulla (a secretory tissue) of bullocks, and compared the major protein of these vesicles with clathrins from coated vesicles of bullock brain and pig brain. I have also isolated coated vesicles from a nonsecreting mouse lymphoma cell line, E.L.4. (9). The major proteins from each of these different coated vesicle preparations are similar: this is shown by one-dimensional fractionation of peptide fragments, obtained by chemical cleavage of these proteins, on 15% sodium dodecyl sulfate/polyacrylamide gels.

MATERIALS AND METHODS

Isolation of Coated Vesicles from Different Sources. Pig and bullock brains and bullock adrenal medullas were obtained from Garnham and Sons, Coldhams Lane, Cambridge, England. Homogenates of the different tissues were prepared essentially as described for pig brains (8), using buffer solution containing 0.1 M MES [2-(*N*-morpholino)ethane sulfonic acid] pH 6.5, 1 mM ethylene glycol-*bis*(β-aminoethyl ether)-*N,N'*-tetraacetic acid, 0.5 mM $MgCl_2$, and 0.02% sodium azide. Cells of a lymphoma line, E.L.4 (9), were grown as ascites tumors in 20 mice [strain B10(H-2^b)] for 12 days. This line was shown to be a nonsecretor since, when grown for one generation *in vitro* on L-[^{14}C]phenylalanine, less than 1% of the total labelled protein was found in the medium. Ascites cells were harvested by centrifugation, resuspended in an equal volume of ice-cold preparation buffer, and lysed and homogenized in a Sorvall Omnimixer at full speed for 3 × 30 sec intervals.

Homogenates were centrifuged at 20,000 × *g* for 30 min to remove debris, and then at 55,000 × *g* for 1 hr to obtain crude coated vesicles. These were further purified by three sucrose gradient centrifugation steps (8). Purification was monitored with the electron microscope. A drop of sample was placed on a carbon coated grid, negatively stained with 1% uranyl acetate (10), air dried, and examined in a Philips EM 300 microscope. Particles shown here were photographed either on the carbon film or suspended in stain over holes in the carbon film.

Sodium dodecyl sulfate ($NaDodSO_4$)/polyacrylamide gel electrophoresis was carried out as before (8, 11). For speed of monitoring samples during the purification, a micro-slab gel electrophoresis apparatus was used (12).

Protein Chemistry. For the following procedures protein from the coated vesicles was first separated from lipid by precipitation in chloroform:methanol:0.1 M sodium acetate, at pH 4.0 (1:2.2:1, vol/vol) and pelleted by centrifugation (13).

Carboxymethylation of the reduced protein was carried out, using iodoacetate, in 6.0 M guanidinium hydrochloride, at pH 8.0. Samples of the carboxymethylated protein were hydrolyzed in 6 M HCl containing 0.1% phenol at 105°, for 24, 48, and 72 hr, respectively, and analyzed for amino acids and amino sugars on a Durrum amino acid analyzer, D-500. Average values over the time course were taken, except for serine and threonine—which were extrapolated to zero time, and for isoleucine and leucine—where the averages of the 48 hr and 72 hr values were used. Tryptophan was estimated by the method of Edelhoch (14).

For cyanogen bromide cleavage, protein was dissolved in 70% formic acid and reacted with excess cyanogen bromide, overnight at room temperature. The resultant solution was diluted 5- to 10-fold with water and freeze dried. Samples were then examined by electrophoresis on 15% $NaDodSO_4$/polyacrylamide gels.

Abbreviation: $NaDodSO_4$, sodium dodecyl sulfate.

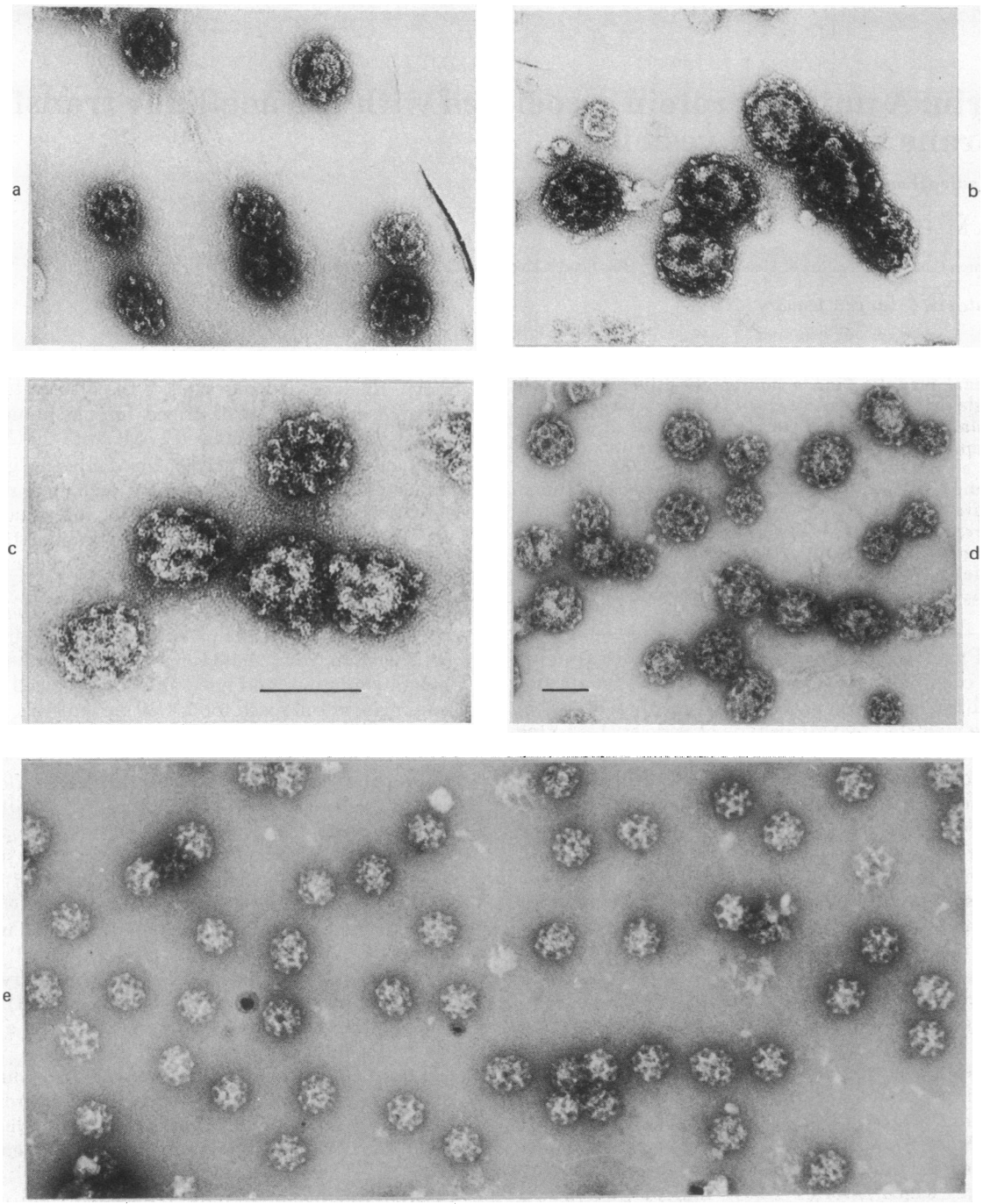


FIG. 1. Coated vesicles purified from different sources: (a) bullock brain (×148,500); (b) lymphoma cells (×148,500); (c) adrenal medulla (×148,500); (d) adrenal medulla fraction containing large coated vesicles (×67,500); (e) adrenal medulla fraction containing small coated vesicles (×67,500). The horizontal lines represent 1000 Å for magnifications of 148,500 and 67,500 respectively. All the specimens were negatively stained with 1% uranyl acetate. (a) and (b) are photographs of particles suspended in stain over holes in the carbon grid, where some contraction of the specimen occurs.

Cleavage of protein at the amino peptide bonds of cysteine residues was carried out using excess 2-nitro-5-thiocyanobenzoic acid (15) in 0.2 M Tris-acetate at pH 8.2 containing 6 M guanidinium hydrochloride and 0.1 mM dithiothreitol. After desalting, samples were examined by electrophoresis on 15% $NaDodSO_4$/polyacrylamide gels.

Lipid Estimation. Phospholipids were extracted and estimated as before (8). Cholesterol content of extracted samples was determined by gas chromatography on a Beckman GC-65 by the method of Lowden (16).

RESULTS

The Structure of Coated Vesicles from Different Sources. Coated vesicles were isolated in yields of approximately 30% from pig and bullock brains, 3–4 mg being obtained from 100 g wet weight of brain. About 1.5 mg was purified from 100 g of adrenal medulla and 0.5 mg from 15 g of lymphoma cells. Electron micrographs of samples from these different sources all show the same striking lattice-coated structures but with different size distributions (Fig. 1). In the brain preparations most particles have diameters

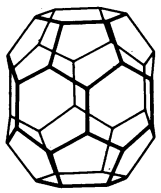


FIG. 2. The structure of a coated vesicle from pig brain. The network of hexagons and pentagons is built from 108 identical subunits.

of about 700 Å. The coated vesicles from lymphoma cells are large, with diameters of 1000–1200 Å. Both small and large particles were obtained from adrenal medulla; these were partially separated on the final sucrose density gradient during purification. Coated vesicles were also partially purified from pig liver and Krebs ascites cells of a single mouse, by the same procedure as described for brain and lymphoma cells, respectively.

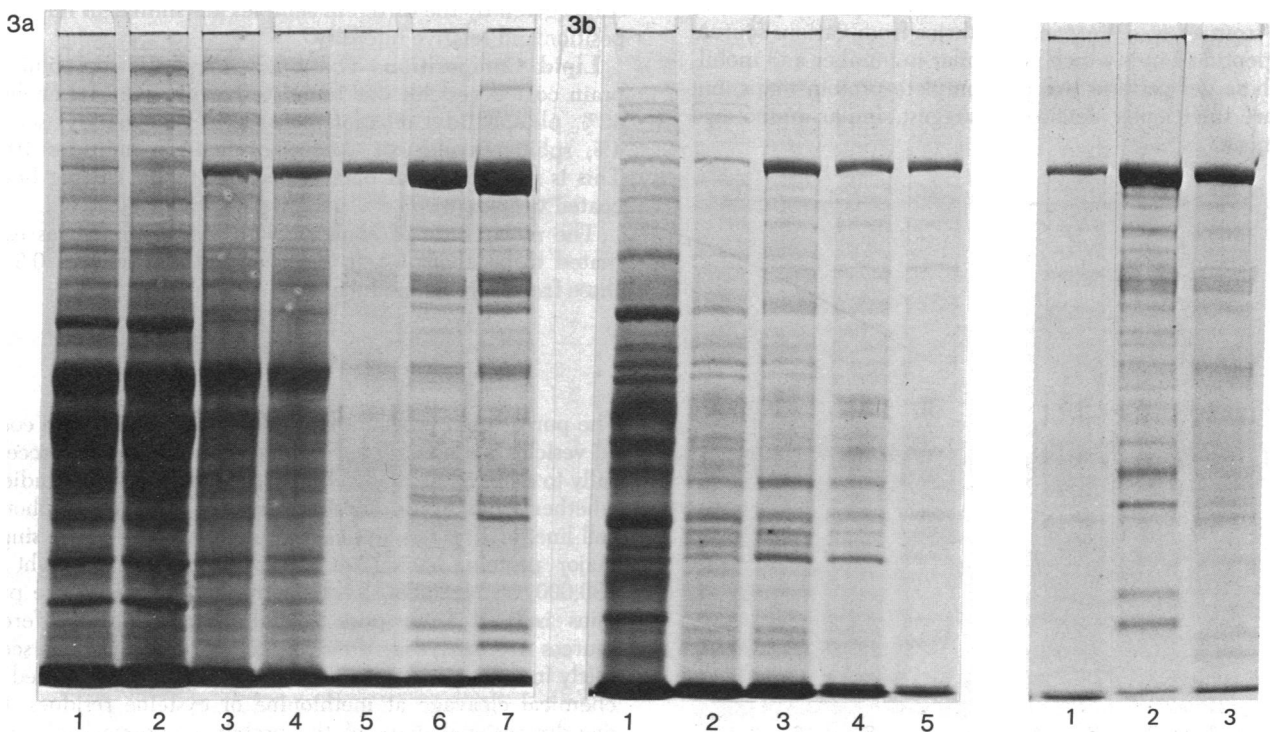
Coated vesicles from pig brain contain just one major protein species, clathrin, which covers the vesicles (8). Tilting specimens in the electron microscope shows that the coat is always a closed network of hexagons and 12 pentagons (23). One of the most obvious structures, built from 108 identical subunits, is shown in Fig. 2. Another one built from the same number of subunits, but with a different arrangement of hexagons and pentagons, has also been observed. Particles with 84 subunits and tetrahedral symmetry also occur. The larger coated vesicles are constructed on similar principles but contain a greater variety of structures.

My method of preparation yields particles frequently permeated by uranyl acetate during staining. This suggests that, whatever their source and size, these coated vesicle preparations contain many "empty" coats.

NaDodSO$_4$/Polyacrylamide Gel Electrophoresis. Various samples from the purification steps were kept at 0–4° and the protein in them later fractionated by NaDodSO$_4$/polyacrylamide gel electrophoresis. The resulting gel patterns for samples from adrenal medulla and lymphoma cells are shown in Fig. 3a and b. A high-molecular-weight protein copurifies with the coated vesicles from these sources, as previously shown with those from pig brain (8). Fig. 4 shows a gel on which samples of the final preparation of coated vesicles from different sources were electrophoresed side by side. Irrespective of source, the major protein forms 70–90% of the stained material and migrates the same distance. The minor proteins, which may be impurities or proteins associated with the vesicles, vary according to the source. The mobility of this major coated vesicle protein, clathrin, corresponds to an apparent molecular weight of 180,000 (8). A protein species of similar mobility was also observed in partially purified coated vesicles from pig liver and Krebs ascites cells.

Chemical Composition of the Protein of Coated Vesicles. (*A*) Amino acid composition. Table 1 shows the amino acid compositions of the clathrins extracted from samples from the different sources. Their compositions are very similar. The differences between them are mostly within experimental error, and some may be due to impurities. Less than 0.3 nmol of glucosamine or galactosamine was detected per nmol of protein in the separate determinations.

(*B*) Cyanogen bromide cleavage. Fig. 5 shows the results of NaDodSO$_4$/gel electrophoresis of polypeptide fragments obtained by cyanogen bromide cleavage of clathrins from the different sources. The fragments observed range be-



FIGS. 3 AND 4. *Fig. 3.* Electrophoresis on 7.5% NaDodSO$_4$/polyacrylamide gels of samples retained during purification of coated vesicles from two sources. (a) Adrenal medulla: (1) extract; (2) supernatant; (3) crude coated vesicle pellet; (4) first sucrose gradient fraction; (5) second gradient fraction; (6) final pellet of smaller coated vesicles; (7) pellet containing larger coated vesicles. (b) Lymphoma cells: (1) supernatant; (2) crude coated vesicle pellet; (3) first sucrose gradient fraction; (4) second gradient fraction; (5) final pellet of coated vesicles. *Fig. 4.* Coated vesicle protein from: (1) lymphoma cells; (2) bullock brain; and (3) adrenal medulla. (Electrophoresis on a 7.5% NaDodSO$_4$/polyacrylamide gel.)

Table 1. The number of amino acid residues per subunit molecular weight of 180,000 for clathrins from different sources

	Pig brain	Bullock brain	Adrenal medulla
Cysteine	16	14	nd*
Aspartic acid	160	163	165
Threonine	74	75	77
Serine	104	106	112
Glutamic acid	218	220	216
Proline	95	93	85
Glycine	98	96	116
Alanine	141	139	135
Valine	106	105	111
Methionine	38	38	38
Isoleucine	80	80	83
Leucine	149	150	160
Tyrosine	60	60	42
Phenylalanine	77	73	72
Histidine	36	38	35
Lysine	94	94	95
Arginine	71	72	85
Tryptophan	22	nd	nd

* Not determined.

tween molecular weights of about 10,000–30,000. About 12 distinct bands are visible for each sample, while 39 peptides would be expected from cleavage at the 38 methionine residues in the coated vesicle proteins. However, short peptides would run at the buffer front and therefore not contribute to the number observed. Some of the larger fragments may represent partially cleaved products. The uncleaved protein does not enter the 15% gels used for these comparisons. Apart from a few differences, clathrins from various sources show peptide bands which are similar in number and mobility. These gel patterns are not complete protein maps, but they are sufficiently detailed to suggest similar amino acid sequences.

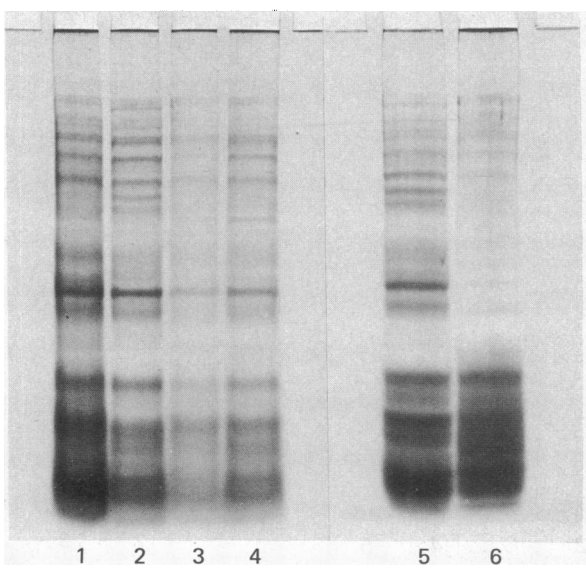


FIG. 5. Maps of polypeptide fragments obtained by cyanogen bromide cleavage of clathrins from: (1) pig brain; (2) bullock brain; (3) adrenal medulla (fraction containing smaller vesicles); (4) adrenal medulla (fraction containing larger vesicles); (5) bullock brain; (6) lymphoma cells. (15% $NaDodSO_4$/polyacrylamide gel.)

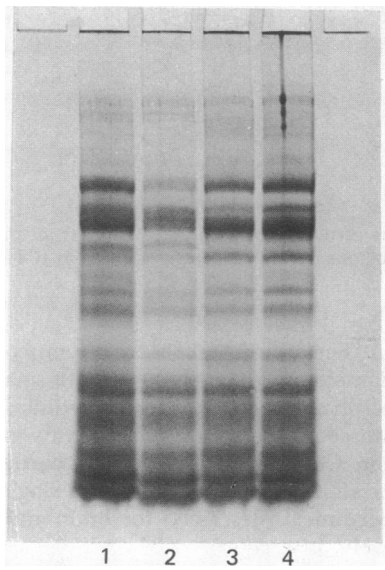


FIG. 6. Maps of polypeptide fragments obtained after cleavage at cysteine residues of clathrins from: (1) pig brain; (2) bullock brain; (3) adrenal medulla (fraction containing smaller vesicles); (4) adrenal medulla (fraction containing larger vesicles). (15% $NaDodSO_4$/polyacrylamide gel.)

(*C*) Cleavage at cysteine residues. Fig. 6 shows the gel patterns obtained from clathrins cleaved at cysteine residues, using 2-nitro-5-thiocyanobenzoic acid. The maps are more complete and confirm the similarity of the proteins revealed by cyanogen bromide cleavage. About the correct number of peptides expected from the number of cysteine residues in the brain proteins was obtained. The patterns of bands given by the different samples are similar in number, position and relative intensity.

Lipid Composition. The lipid composition of bullock brain coated vesicles was found to be phosphatidyl choline 42%, phosphatidyl ethanolamine 33%, phosphatidyl serine 4%, sphingomyelin 8%, and phosphatidyl inositides 10%. This is similar to that determined previously for pig brain coated vesicles (8).

The molar ratio of cholesterol to phospholipid was estimated to be about 0.1 in brain coated vesicles and 0.3 in those from adrenal medulla.

DISCUSSION

The purification procedure described (8) for pig brain coated vesicles is a general one which has been applied successfully to other tissues and cells. All the coated vesicles studied, whether from brain, adrenal medulla, liver, a lymphoma cell line, or Krebs ascites cells, are characterized by a single major protein, having an apparent molecular weight of 180,000, on $NaDodSO_4$/gel electrophoresis. When the proteins isolated from pure coated vesicles from different sources are analyzed, their amino acid compositions seem fairly invariant. More striking are the fragments obtained by chemical cleavage at methionine or cysteine residues: the one-dimensional maps of the protein are remarkably constant, irrespective of either tissue or species, which indicates that the major protein of all these coated vesicles, clathrin, may contain a highly conserved amino acid sequence. By contrast, similar techniques applied to myosin heavy chains (which have a similar molecular weight, 200,000) give pat-

terns characteristic of the tissues from which they were isolated (17, 18). The absence of amino sugars in clathrin suggests that carbohydrate is lacking. This would be expected of a cytoplasmic protein which can associate with, and dissociate from, membranes in a cyclic fashion, if one function of covalently bound carbohydrate is to "lock" protein irreversibly into a membrane (19). The low level of cholesterol associated with the phospholipids is closer to that of internal membranes than that of the plasma membrane (20). Coated vesicles transfer membrane from one membranous organelle to another within the cell. Since different organelles are believed to contain different proteins and different proportions of lipids, some form of control must exist to ensure that the compositions of the donor and receptor membranes are not randomized during transfer. The specificity involved here is likely to occur during the formation of the coated vesicles.

The coated vesicles from a given tissue are not homogeneous: the size of the particles is not uniform and some of the coats appear to be empty. In those from pig brain (23) the clathrin subunits form a closed network of hexagons and pentagons. The arrangement of clathrin in the most usual size of coat (having 108 subunits) is also not unique—at least two different species exist. Larger coats, which again are closed networks, are found in pig brain preparations but are more abundant in those from adrenal medulla and lymphoma cells. The finding that the same protein covers large and small vesicles, coupled with the observation that large coated vesicles have a different function from smaller ones in rat vas deferens (6), suggests that the size of coat around a vesicle is not determined by clathrin itself. Specific transfer of membrane from one location to another within a cell must involve specific recognition and therefore other proteins: it is presumably these other factors which determine what size the coated vesicle should be.

Coated vesicles do not appear to have one simple function common to all cells. They have been seen most frequently in cells which are involved in secretory or absorption processes—for example, in nerve cells (21, 22), pancreatic exocrine cells (7), the developing oocyte of the mosquito (5), and rat vas deferens epithelium (6). They are involved in resorption of plasma membrane (4), ingestion of extracellular protein, which is accompanied by resorption of plasma membrane (5, 6) and in less clear functions with the Golgi apparatus (6, 7). The finding that coated vesicles can be isolated from nonsecreting lymphoma cells in amounts comparable to those found in brain or adrenal medulla, suggests that their existence may be much more widespread than suspected. From the quantity of clathrin isolated from lymphoma cells, the number of coated vesicles in each cell can be estimated. Assuming a recovery of about one third of the vesicles during purification, there must be a few thousand of the larger ones per lymphoma cell, enough to transfer substantial amounts of membrane within the cell.

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