Transport of the Membrane Glycoprotein of Vesicular Stomatitis Virus to the Cell Surface in Two Stages by Clathrin-coated Vesicles

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ABSTRACT The G protein of vesicular stomatitis virus is a transmembrane glycoprotein that is transported from its site of synthesis in the rough endoplasmic reticulum to the plasma membrane via the Golgi apparatus. Pulse-chase experiments suggest that G is transported to the cell surface in two successive waves of clathrin-coated vesicles. The oligosaccharides of G protein carried in the early wave are of the "high-mannose" (G_1) form, whereas the oligosaccharides in the second, later wave are of the mature "complex" (G_2) form. The early wave is therefore proposed to correspond to transport of G in coated vesicles from the endoplasmic reticulum to the Golgi apparatus, and the succeeding wave to transport from the Golgi apparatus to the plasma membrane. The G_1- and G_2-containing coated vesicles appear to be structurally distinct, as judged by their differential precipitation by anticoated vesicle serum.

The intracellular transport of proteins is a key aspect of the assembly of the various subcellular membranes and the organelles that these membranes define. Proteins destined for secretion, as well as those that will ultimately reside in such cellular locations as the plasma membrane, the Golgi apparatus, and lysosomes, all appear to be found at an earlier stage of their maturation in the endoplasmic reticulum. This would imply the existence of a mechanism to transport these proteins from the endoplasmic reticulum to their final destination, as well as a means to achieve the specific "sorting" of the distinct sets of proteins that need to be delivered to the different organelles.

While it is generally believed that transport between organelles is mediated by small vesicles that bud off from one membrane and fuse with another (14, 22), the actual mechanisms by which transport and sorting are accomplished have remained mysterious. Coated vesicles (4, 8, 16, 23, 24, 28) are attractive candidates for the proposed transport vesicles, because their structure and properties make them seem well suited to act as a family of intracellular carriers of proteins and lipids, and suggest simple mechanisms for intracellular transport and sorting (29).

Coated vesicles (typically 50–150 nm Diam) are unique in that they are encaged in an icosahedral basket-like "coat" (4, 16), the principal protein of which is "clathrin" (24). Morphological and cytochemical observations are consistent with the notion that coated vesicles mediate a wide variety of intracellular transport events (29). Of these, receptor-mediated endocytosis of macromolecules from the cell surface (10) was the first appreciated (28) and remains the best established function of coated vesicles (10) as the result of kinetic studies (1, 10) that have shown the coated vesicles to be transient intermediates (whose lifetime is only a few minutes) in the endocytic pathway.

For our investigations we have employed vesicular stomatitis virus (VSV), the simplest of the enveloped viruses from the standpoint of membrane biogenesis, since the virion contains only one species of integral membrane protein, the G protein. Because VSV shuts off host protein synthesis, G becomes the only glycoprotein synthesized, making the study of the synthesis and transport of a membrane protein as straightforward as possible. G is first inserted into the rough endoplasmic reticulum membrane (2, 5, 12, 13, 17, 18, 31), where it is also glycosylated as a nascent polypeptide chain. G is then transported to the plasma membrane via the Golgi apparatus,[1] in

[1] After its synthesis on membrane-bound ribosomes (31), G moves from a rough endoplasmic reticulum-enriched fraction to a less dense, internal membrane-enriched fraction, and then to the plasma membrane (2, 12, 13, 17, 18). It is in this light internal membrane fraction that G receives the "terminal" sugars including galactose and sialic acid (12, 13). This distinct intracellular site of terminal glycosylation, through which G passes while in transit from the rough endoplasmic reticulum to the plasma membrane, is operationally defined as the

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which the two Asn-linked oligosaccharides are modified by removal of mannose and addition of *N*-acetylglucosamine, galactose, and sialic acid residues (12, 13).

As a result of these two stages of intracellular processing of G, two principal intracellular forms of G, G_1 and G_2, are distinguishable by gel electrophoresis (17, 18, 27). G_1 has a "high mannose" content, represents the pre-Golgi form, and possesses oligosaccharides that can be cleaved by endoglycosidase H. G_2, the product of terminal glycosylation of G_1 in the Golgi apparatus, possesses oligosaccharides resistant to cleavage by Endo H. Subsequently, G is incorporated into the viral envelope after the budding out of the progeny viral nucleocapsid through the cell surface membrane. Since VSV encodes for only five proteins (G, M, N, NS, and L), all components of virions, it is likely that the maturation of G protein follows only host-specific pathways.

In a previous communication (30) we reported preliminary biochemical and kinetic evidence that G protein is transported from its site of synthesis in the endoplasmic reticulum to the cell surface within small vesicles, and have identified these as coated vesicles. Two successive rounds of transport in coated vesicles are used, the first resulting in transfer of the G protein of VSV from the endoplasmic reticulum to the Golgi apparatus,[1] the second in transport from the Golgi apparatus to the plasma membrane. In this report we provide a more complete account of these findings and extend them by demonstrating differences between the two classes of coated vesicles involved in transport of G protein to the cell surface.

MATERIALS AND METHODS

Cells and Virus

Chinese hamster ovary (CHO) cells were maintained in suspension in Joklik's minimum essential medium (MEM) and infected with VSV as described (18). A temperature-sensitive group III mutant of VSV, tsG31 (25), was kindly provided by Dr. J. Lenard of Rutgers University, and used for these studies. At the restrictive temperature of 39.5°C the defect in M protein in this strain results in a loss of virion production (33). Synthesis, glycosylation, and transport of G to the cell surface are not affected by this mutation (Fig. 1). When wild-type VSV was used in initial experiments, extensive contamination of coated vesicle preparations with virions was observed. The use of tsG31 eliminated this source of contamination but only at the restrictive temperature.

Pulse-Chase Experiments

2×10^8 CHO cells were infected with 5 plaque-forming units/cell of VSV tsG31 as described (18) at 31°C. At 5 h after infection, cells were washed in Earle's saline and suspended in 100 ml of infection medium that lacked any Met and contained dialyzed fetal calf serum (18). After a 30-min equilibration at 39.5°C, [^{35}S]Met (typically 1–5 mCi, >1,000 Ci/mmol; Amersham Corp., Arlington Heights, Ill.) was added. After a pulse of 5-min duration, unlabeled Met (2.5 mM) was added and 15-ml samples (3×10^7 cells) were taken at various times of chase. These cells were immediately mixed with 35 ml of ice-cold Earle's solution, centrifuged within 2 min, and the pellets were quickly frozen in liquid nitrogen for later use. Zero time refers to the initiation of chase. Incorporation of ^{35}S into protein stopped immediately upon the initiation of chase.

Golgi apparatus. This identification is made because electron microscopic autoradiographic (20) and careful cell fractionation studies (3, 7, 32) have shown that terminal glycosylation in other cell types takes place in the Golgi apparatus. Recent studies (35) of the mannose-trimming enzymes have shown that these also are found in the Golgi apparatus of liver. Whereas it seems highly likely that the Golgi apparatus, as defined operationally in the context of VSV-maturation, is in fact the morphologically defined Golgi apparatus, this presumption is only by analogy, and needs to be directly established.

Copurification of Coated Vesicles with Brain Carrier

The procedure of Pearse (23, 24) was adapted for the purification of coated vesicles from small quantities of radioactive CHO cells by using large quantities of brain extract as carrier. Typically, 3×10^7 VSV-infected CHO cells (^{35}S-labeled, from pulse-chase experiments, 0.1 g wet wt) were thawed at 37°C, swollen for 5 min on ice in 2 ml of 1 mM EDTA-10 mM Tris-HCl pH 7.5 containing 0.1 mM phenylmethylsulphonyl fluoride (PMSF) (tris buffer), and then disrupted with 20 strokes of a tight-fitting Dounce homogenizer (Kontes Co., Vineland, N. J.). Then the homogenate was adjusted to 2-(*N*-morpholine) propane sulfonic acid (MES buffer) (23) by adding 0.2 ml of a 10 times concentrate of 0.1 M MES, 1 mM EGTA, 0.5 mM $MgCl_2$ 0.02% NaN_3, 0.1 mM PMSF. This homogenate was centrifuged at 600 *g* for 10 min.

The resulting post-nuclear supernate was sonicated at 0°C with a microprobe (Branson Sonic Power Co., Danbury, Conn.) for 60 s in four 15-s bursts at the cavitation point. The sonicate was combined with a 20,000 *g*-20 min supernate obtained from 10 g of calf brain (23), and this mixture was incubated with 20 μg/ml pancreatic ribonuclease A (Worthington Biochemical Corp., Freehold, N. J.) for 15 min on ice. The sonication step greatly reduced the amount of N and L proteins in the final coated vesicle preparation, but did not affect the yield of G protein or clathrin. Coated vesicles remain intact when subject to a similar sonication, as judged by electron microscopy (unpublished observation). Sonication is believed to disrupt nucleocapsids, which contain N and L. Ribonuclease treatment served to remove a major contaminant of coated vesicle preparations that appeared in the electron microscope to consist of aggregated ribosomes.

The pellet, following centrifugation for 90 min in the Beckman JA-17 rotor at 17,000 rpm, was used as the source of crude coated vesicles in the Pearse (23) procedure. Either two or three successive sucrose gradients were used, exactly as described by Pearse (23), except that the SW 27.1 rotor was employed, with one gradient per sample being processed. The middle third of the first gradient above

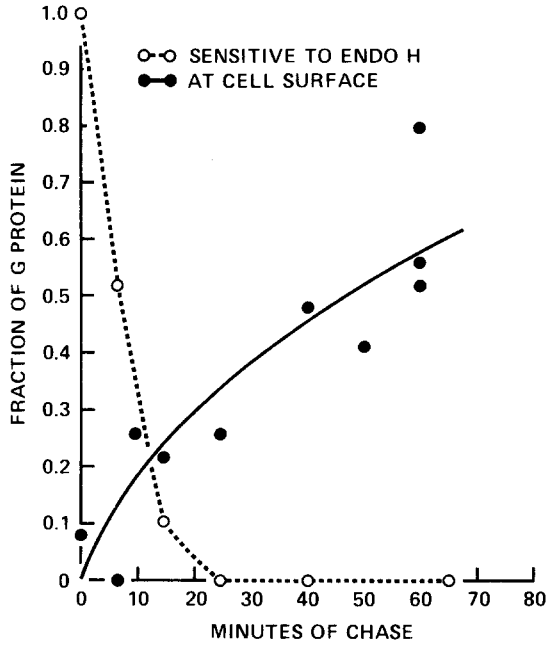
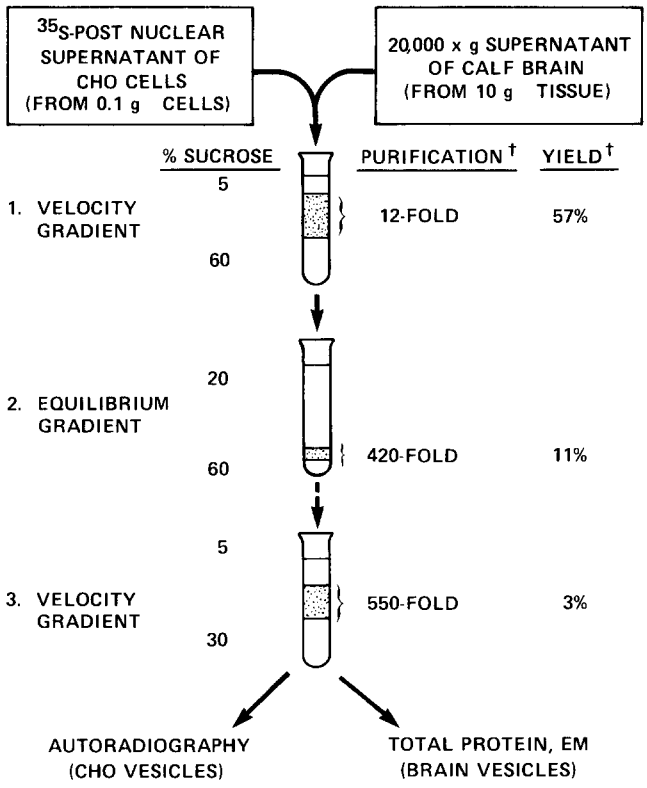


FIGURE 1 Kinetics of intracellular transport of G protein at 39.5°C in CHO cells infected with the tsG31 mutant of VSV. A culture of cells was pulse-labeled with [^{35}S]Met for 5 min and samples were taken at several times of chase. Half of each sample was then treated with chymotrypsin as described by Knipe et al. (17, 18), and the digested and control samples were subject to gel electrophoresis (19) and then autoradiography (17, 18). The fraction of [^{35}S]G hydrolyzed by external chymotrypsin (●) was determined by densitometry of the autoradiograph, and was taken to be the fraction of the [^{35}S]G that had reached the plasma membrane (17, 18). Samples of cells were also digested with endoglycosidase H in the presence of SDS as described (27). After electrophoresis and autoradiography, the fraction (○) of [^{35}S]G in the Endo H-sensitive band (Fig. 6, G^S and G^R bands) was determined from densitometer tracings of the autoradiograph (17, 18).



†RELATIVE TO 20,000 x g SUPERNATANT

FIGURE 2 Schema of the copurification procedure used to isolate coated vesicles from CHO cells in the presence of brain extract carrier. The method involves only slight modifications of that of Pearse (23). The shaded areas represent the approximate regions of the sucrose gradients that were harvested at each step. A detailed description of the appearance of the gradients is provided by Pearse (23). Date on the extent of purification refer to G protein in Table I, column II.

the thick white pad was collected, as shown in Fig. 2. This figure also shows the approximate regions of the second and third gradients collected, following Pearse's (23) descriptions. When the third gradient was to be used, the resuspended pellet of coated vesicles obtained from the second gradient was sonicated briefly (10 s) in MES buffer before loading the third sucrose gradient. This procedure increased the yield of G, presumably by disrupting aggregates of coated vesicles, which would otherwise sediment very rapidly in the third velocity gradient. Typically, 0.5–0.7 mg protein of coated vesicles was obtained from each sample after two gradients and 0.2–0.3 mg after three gradients. When examined in the electron microscope with uranyl acetate as negative stain, coated structures were essentially the only ones observed. Most coats appeared to contain a vesicle that excluded negative stain.

Quantitation of Pulse-Chase Experiments

The fraction of the [^{35}S]G pulse label in the whole cell ascribable to coated vesicles was determined by dividing the fraction (f) of the [^{35}S]G protein in the homogenate that was recovered in the copurified, coated vesicle preparation by the yield of coated vesicles in the purification. These yield-adjusted fractions are presented in Figs. 5 and 8 as percentages. The fraction f recovered in coated vesicles was determined from densitometry of autoradiographs of SDS gels (19) of coated vesicle preparations and of homogenates; the yield was determined from the fraction of ^{3}H recovered when coated vesicles containing [^{3}H]mannose-labeled G protein were added back to crude extracts and subjected to another round of purification (see Table I). The yield was 11 ± 1% after two sucrose gradients and 2.6 ± 0.1% after three.

It was also necessary to normalize the fraction of each time point to an internal standard as described (30) to eliminate "noise" generated by random variations in the yield from sample to sample in the same experiment. Both coated vesicle protein (representing mainly the carrier brain coated vesicles) and [^{3}H]Leu (derived from uniformly added [^{3}H]Leu-labeled CHO cells) have been used as the internal standards with identical results. When [^{3}H]Leu was to be used, the post-nuclear supernate from 7×10^{6} [^{3}H]Leu-labeled CHO cells was added to the post-nuclear supernate of each ^{35}S-labeled sample. Typically, 20,000 cpm of ^{3}H were recovered with the coated vesicles. ^{3}H cells were labeled by growth for four generations in growth medium that contained 20% of the normal level of leucine and 1 mCi of L-[4,5-^{3}H]leucine (Amersham Corp.) per liter.

RESULTS

Kinetics of Transport of G Protein from Its Site of Synthesis to the Cell Surface in VSV tsG31-Infected Cells

The procedures used to isolate coated vesicles are similar to those used to purify enveloped viruses; both types of particles

TABLE I

Copurification of CHO and Calf Brain Coated Vesicles

	I. Total protein*	II. [^{3}H]G-labeled CHO coated vesicles‡		III. ^{3}H-labeled Brain coated vesicles§		IV. ^{3}H-labeled CHO coated vesicles‖	
Step	Yield	Yield	Purity	Yield	Purity	Yield	Purity
20,000-*g* supernate	[100]%	[100] %	[1]-fold	[100] %	[1]-fold	[100] %	[1]-fold
Crude coated vesicles	8.3						
First sucrose gradient	4.7	57 ± 3	12				
Second sucrose gradient	0.026	11 ± 1	420	10	385	6.1	230
Third sucrose gradient	0.0047	2.6 ± .1	550			0.98	210
Anticoated vesicle precipitation after second gradient¶	0.0099	5.7	575				

Fold purification was calculated as percentage of ^{3}H recovered divided by percentage of total protein recovered.

* Determined by the Lowry method (21).

‡ Coated vesicles were copurified through the second sucrose gradient, using tsG31 VSV-infected CHO cells, which had been labeled for 30 min with [2-^{3}H]-mannose as described (30). All of the ^{3}H in the coated vesicle preparation was in G as judged by counting slices of an SDS gel. These coated vesicles were added back to unlabeled 20,000-*g* supernates, and the recovery of [^{3}H]G at each step of a subsequent purification was determined in independent triplicates. The mean ± SE are one standard deviation are shown.

§ Calf brain coated vesicles were tritiated by reductive methylation essentially as described (26) using $B^{3}H_{4}$ after formaldehyde. Labeling was carried out for 2 min at 4°C in a volume of 100 λ containing 75 μg coated vesicles, 10% (wt/vol) sucrose, 10 mM formaldehyde, 10 mCi of $NaB^{3}H_{4}$ (~10 Ci/mmol), 0.15 M tricine buffer, pH 8.5. The reaction was stopped by adding 5 μl of 1 M Tris-HCl, pH 6.8. The labeled coated vesicles were freed from unincorporated ^{3}H by gel filtration on Sephadex G-50 in the presence of 0.1 mg/ml of bovine serum albumin.

‖ Coated vesicles were copurified through the second sucrose gradient using CHO cells that had been uniformly labeled by growth on [^{3}H]leucine-containing medium. This preparation was added back to an unlabeled 20,000-*g* supernate, and the recovery of ^{3}H-protein after a subsequent purification was ascertained.

¶ As described in Fig. 9.

occupy the same "virus window" of subcellular fractionation, a region of small S value and high density (34). Indeed, the principal technical problem encountered in our initial experiments with VSV is an extensive contamination of coated vesicle preparations with VSV virions. To circumvent this, we have employed a temperature-sensitive mutant of VSV, tsG31 (30, 33). The defect in the M protein of this strain prevents budding of virions at the restrictive temperature (39.5°C), thereby eliminating by genetic means the principal source of contamination of coated vesicles.

Fig. 1 shows the kinetics of intracellular transport at 39.5°C of G in CHO cells infected with tsG31. Transit from ER to Golgi[1] was followed by the time-course of acquisition of resistance to endoglycosidase H in a pulse-chase experiment (27). G protein is initially completely sensitive to endoglycosidase H digestion but rapidly becomes resistant to its action with a 7-min half-time, as a result of the modification of its oligosaccharides, which occur in the Golgi. Thus, about half of the G protein has reached the Golgi apparatus by 7 min after synthesis. Transit to the cell surface was followed by determining the time-course with which newly synthesized G becomes susceptible to hydrolysis by extracellular chymotrypsin (17, 18). G is initially completely resistant to this enzyme, but gradually becomes sensitive over a fairly lengthy time period (50% at about 45 min). These results are almost identical to those obtained using wild-type VSV (17, 18). Thus, glycosylation and intracellular transport of G to the cell surface is not affected by the lesion of M protein in the ts31 strain, which prevents budding of virions from the cell surfaces.

Purification of Coated Vesicles from CHO Cells with Brain Extract as Carrier

We have previously provided evidence that G protein is a major constituent of coated vesicles purified from VSV-infected CHO cells (30). Such preparations require large amounts of starting material, not readily available with cultured cells, and are therefore not suitable for small-scale, pulse-chase experiments, such as are required to investigate the kinetics of the association of G with coated vesicles. We have therefore employed the same procedure to purify coated vesicles from small quantities of VSV-infected CHO cells, but use large quantities of a calf brain extract as carrier. The major steps in this copurification are diagramed in Fig. 2.

Because the same procedure (23) yields coated vesicles from both brain and CHO cells (30), it was anticipated that a copurification would result in a preparation of comparably pure coated vesicles, representing a mixture of brain- and CHO-derived coated vesicles. To check this we grew CHO cells for four generations in [^{35}S]methionine that contained medium to uniformly label the cells. We then copurified the coated vesicles using an 100-fold excess of brain extract as carrier. Fig. 3 shows an SDS polyacrylamide gel of the coated vesicle fraction obtained after two and three sucrose gradients. Fig. 3 *B* shows the Coomassie Blue-stained gel, representing the coated vesicles purified from the brain carrier. As shown, clathrin represents the major protein in the preparation, although other major groups (I, II, and III) of proteins are observed as has been reported (23, 37). The major change in comparing brain coated vesicles obtained after three sucrose gradients with those obtained after two is the enrichment in clathrin in groups I and III, and the depletion in a number of minor bands as well as those in group II.

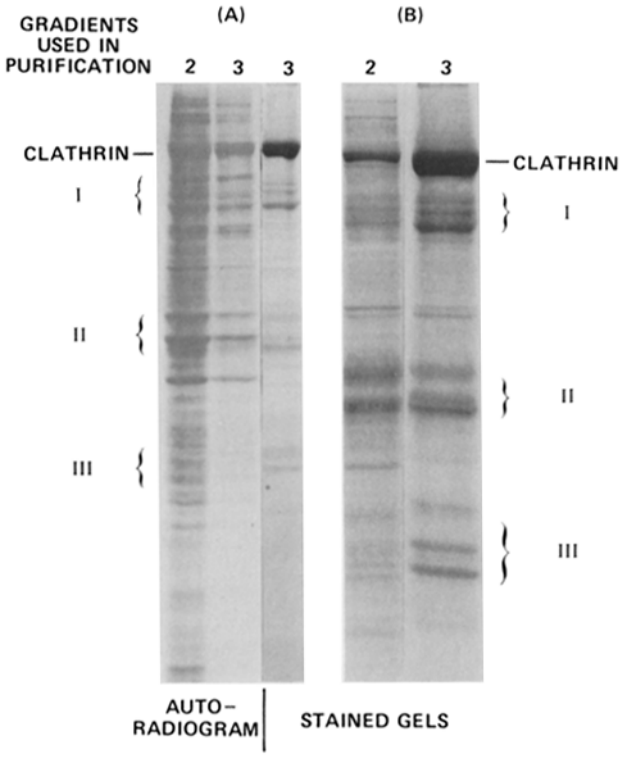


FIGURE 3 Copurification of coated vesicles from calf brain and CHO cells which had been uniformly labeled with [^{35}S]Methionine by growth for four generations in media containing one-fifth the level of Met of complete Joklik's medium, and 2 μCi/ml of [^{35}S]Met. A total of 3×10^8 cells were used for the copurification. Autoradiographs of an SDS-polyacrylamide gel (first two lanes on left) and the Coomassie Blue-stained gels (three lanes on right) of the coated vesicle fractions obtained after two and three successive sucrose gradients are shown, as indicated. Samples in part *A* were run on a different gel from those in part *B*.

Fig. 3 *A* presents an autoradiogram of a gel of the copurified preparations obtained after two and three gradients, thus revealing polypeptides of CHO cellular origin. In addition, the pattern of staining of the same gel is shown in Fig. 3 *A*. As shown, clathrin also represents a major polypeptide derived from CHO cells in this same, coated vesicle preparation. Clathrin is considerably enriched after passage through the third sucrose gradient, becoming the principal labeled protein. A similar result is obtained with uniformly ^{35}S-labeled, VSV-infected CHO cells, except for the presence of VSV proteins, as noted below.

Identification of Newly Synthesized G Protein in Coated Vesicles

In a previous communication (30) we have provided biochemical evidence that G protein is a major constituent of coated vesicles isolated from VSV-infected CHO cells. The molar ratio of G to clathrin in these coated vesicle preparations was found to be about 1:3. This finding has been further substantiated by the direct visualization of G in association with coated vesicles, using immunoelectron microscopy employing monospecific anti-G serum.[2] These results show that the bulk of G protein in such preparations is in coated vesicles. Because we wish to undertake kinetic, pulse-chase studies of

[2] Rubenstein, J. L., E. J. Patzer, R. E. Fine, and J. E. Rothman. Manuscript in preparation.

this association, it was also necessary to provide evidence that the newly synthesized pool of G protein found in coated vesicle fractions was also in coated vesicles. For this purpose, newly synthesized G protein was labeled by incubating VSV-infected CHO cells with [2-^{3}H]mannose for 30 min as described (30). Under these conditions all the [^{3}H]Man incorporated into protein is found in G, and all the ^{3}H in the coated vesicle preparation is in G protein. The coated vesicle fraction was obtained from these cells after two sucrose gradients and used as detailed below. Half of the [^{3}H]G protein in such coated vesicle preparations in Endo H-resistant (unpublished data).

A quantitative appraisal of the copurification of [^{3}H]Man-labeled G protein, putatively present in coated vesicles within the isolated coated vesicle fractions, and brain coated vesicles is presented in Table I. Column I shows the recovery of total protein derived from a crude extract of brain (the 20,000-*g* supernate) during the copurification of coated vesicles. To quantitate the purification of coated vesicles, brain coated vesicles that had been purified through three sucrose gradients (and that constitute our standard of purity), were labeled with ^{3}H by B(^{3}H)$_4$ treatment after formaldehyde. These ^{3}H-labeled brain vesicles were added to an unlabeled 20,000-*g* supernate, and the recovery of radioactivity and total protein were determined during a subsequent round of purification (Table I, column III). The increase in ^{3}H/protein ratio during the purification measures the extent of purification of coated vesicles from the crude extract. 10% of the total radioactivity was recovered in the coated vesicle fraction after two gradients, resulting in a 385-fold purification. An essentially identical recovery (11 ± 1%) and purification (420-fold) resulted when [^{3}H]Man-labeled G protein coated vesicles were added to the 20,000-*g* brain supernate and then copurified through the two sucrose gradients (Table I, column II). This result suggests that essentially all of the newly synthesized, pulse-labeled [^{3}H]G protein found in the coated vesicle fraction after two gradients is in fact associated with a particle that copurifies with authentic coated vesicles.

Very little further purification (550-fold total) is achieved by passing the [^{3}H]G-containing coated vesicles through a third sucrose gradient, and this at the expense of a much reduced yield (2.6 vs. 11%). This stands in contrast to the evidence shown in Fig. 3*A* that CHO coated vesicles themselves are purified significantly by a third sucrose gradient. Taken together these results indicate that virtually all the [^{3}H]mannose pulse-labeled G protein is found associated with coated vesicles after the second gradient even though the coated vesicles themselves are not pure.

This interpretation is further supported by two additional results summarized in Tables I and II. Anti-clathrin precipitation of coated vesicles (Table I, column II) purified through two gradients also results in only a small further purification of [^{3}H]G despite a marked increase in the abundance of G relative to other VSV-specific proteins (Table II). Finally when the coated vesicle fraction of uniformly [^{3}H]Leu-labeled CHO cells (which had been purified through two gradients), was added to the brain supernate (Table I, column IV), the yield and purification achieved after two gradients are both about one-half of those obtained with either ^{3}H-labeled brain coated vesicles or [^{3}H]G coated vesicles. A reasonable interpretation would be that the [^{3}H]Leu-labeled CHO coated vesicles used were actually only about 50% pure after two gradients, consistent with the improvement evident in Fig. 3 upon passage through the third gradient. A second passage through the two gradient purification removes the remaining impurities (about half of total proteins), and a subsequent passage through the third gradient results in no further purification, as is seen in column IV.

TABLE II

Enrichment of G Relative to Other VSV-encoded Proteins during the Purification of Coated Vesicles

Step	Ratio of G to: N	L	M	Percentage of total as G
				%
Homogenate	0.16	0.36	0.50	8.3
Second sucrose gradient	0.31	0.67	1.5	16.0
Third sucrose gradient	1.80	7.0	1.9	45.0
Anticoated vesicle precipitation after second gradient*	0.80	1.3	1.5	27.0

* As described in Fig. 9.

Copurification from the 25-min chase time point, containing the maximum amount of G_2, was quantitated as follows. Peak heights of G_2, N, L, and M were measured by densitometry of an autoradiograph of samples of the fractions. The relative peak heights G/N, G/L, G/M, as well as the percentage of the total peak height as G protein were calculated.

In a previous report (30), additional evidence was presented to indicate that [^{3}H]G protein found in the coated vesicle preparation after a two gradient purification is indeed completely contained within coated vesicles. Specifically, [^{3}H]G was quantitatively associated with smooth vesicles released from coated vesicles by treatment with metrizamide, and [^{3}H]G was coprecipitated with clathrin by anticoated vesicle antibody.

Pulse-Chase Experiments Show the Newly Synthesized G Protein in Coated Vesicles is in Transit to the Plasma Membrane

Because of the strong evidence that newly synthesized G protein is associated with coated vesicles obtained from VSV-infected CHO cells, pulse-chase experiments were carried out to determine whether this pool of G protein was in fact in transit from its site of synthesis in the endoplasmic reticulum to the plasma membrane.

VSV-infected CHO cells were pulse-labeled for 5 min with [^{35}S]Met, chase was initiated at time zero, and samples of the culture were taken after various times of chase. Coated vesicles were copurified through two sucrose gradients from those small quantities of ^{35}S-labeled cells, using extract from brain as carrier. The amount of ^{35}S-labeled G_1 and G_2 forms of G protein associated with coated vesicles were determined at each time point by densitometry of an autoradiograph (Fig. 4) and expressed as a percentage of the total radioactivity incorporated into G during the pulse (Fig. 5). The data shown in Fig. 5 have been corrected for the yield of the purification of 11 ± 1% after two gradients (Table I).

In such a pulse-chase experiment, a wave of labeled G protein is initiated, which moves from the site of incorporation of [^{35}S]Met in the rough endoplasmic reticulum, to the site of terminal glycosylation in the Golgi,[1] and then to the plasma membrane. If coated vesicles were to transport G from the reticulum to the Golgi apparatus, ^{35}S-labeled G protein should rapidly appear in the coated vesicles, and then disappear after transfer to the Golgi. This G should be in the G_1 (pre-Golgi) form characterized by a high mannose oligosaccharide that can

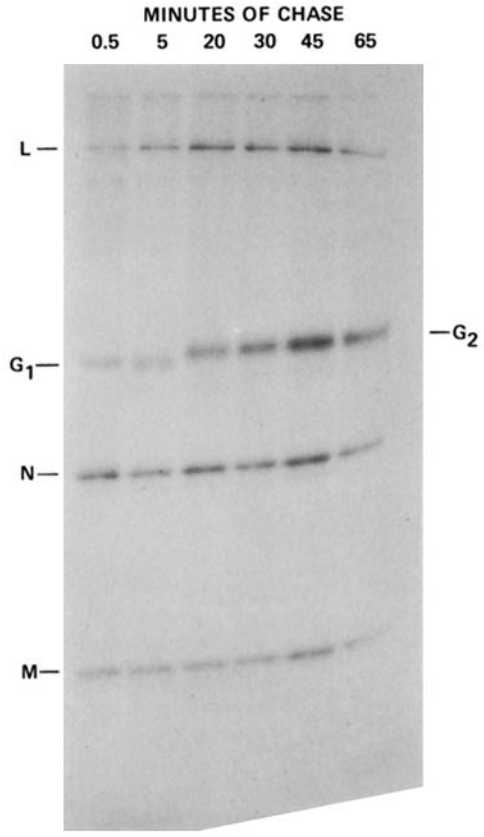


FIGURE 4 Autoradiographs of SDS-polyacrylamide gel of samples of the coated vesicle fraction obtained from pulse-labeled VSV-infected cells at the times of chase indicated. Only the first two sucrose gradients were employed. L, N, NS, and M refer to viral proteins other than the two forms of G, G_1, and G_2. Ten percent of the total coated vesicle fraction was electrophoresed.

be cleaved by Endo H. If coated vesicles were used in transport from the Golgi to the cell surface, a later wave of [^{35}S]G in the mature Endo H-resistant G_2 (post-Golgi) form would be expected instead. Only if coated vesicles transport G along both routes would two successive waves of labeled G in purified fractions be expected.

As shown in Fig. 5, two successive waves of G protein are in fact observed to pass through highly purified coated vesicle fractions. The first wave immediately follows the chase, consists of the G_1 form of G, and accounts at its peak for about 5% of the total [^{35}S]G in the cell. The second wave begins later, consists of the G_2 form of G, and accounts at its peak for about 13% of the pulse label. Fig. 6 shows that the G_1 protein of the first wave is almost all sensitive to Endo H, whereas the G_2 protein of the second wave is resistant to Endo H.

Figs. 7 and 8 show the results of an experiment performed identically to that in Fig. 5 except that the coated vesicle purification was carried through all three sucrose gradients. Quantitatively a smaller percentage of G_1-containing coated vesicles and a larger percentage of G_2-containing coated vesicles are recovered after three as compared to two sucrose gradients. However, the kinetics are almost identical when the recoveries are normalized to the reduced yield of 2.6 ± 0.1%. Two waves of G protein, the first containing G_1 and the second G_2, are again discernible.

The actual time-course of these successive waves fits well with the interpretation that they represent the successive transit steps between reticulum and Golgi and Golgi and plasma membrane, respectively. Specifically, half of the pulse-labeled G in total cell homogenates is Endo H-resistant (and therefore has reached the Golgi)[1] after 7 min chase (Fig. 1), at which point about half of the early Endo H-sensitive G_1 wave is complete (Fig. 5) but virtually all of the [^{35}S]G in coated vesicles is still Endo H-sensitive (Fig. 6). Furthermore, about 55% of G has appeared on the cell surface by 65 min (Fig. 1), a time at which the specific radioactivity of G_2 in coated vesicles has dropped to about half of its peak value (Fig. 5).

Other VSV-specific Proteins are also Found in Coated Vesicle Preparations

As can be seen from Figs. 4 and 7, a considerable amount of the other viral encoded proteins, M, N, and L, (but not NS) are found in the coated vesicle containing fractions from ^{35}S-labeled infected cells: Table II quantitates the ratio of G to these other proteins and the percentage of the total radioactivity as G in the preparation. A progressive enrichments in G protein is observed throughout purification, and after anticlathrin precipitation following the second sucrose gradient. It thus seems likely that the N, L, and M proteins originate as contaminants of coated vesicles, and that their presence in coated vesicle preparation does not reflect a specific association with coated vesicles per se. N and L may be complexed as nucleocapsids, whose sedimentation properties would be expected to overlap with those of coated vesicles. Consistent with this, marked random variations in the extent of contamination by N and L proteins occur from sample to sample. The origin of M protein is unclear. M protein is not found in steady-state amounts in coated vesicles prepared from tsG31-infected CHO cells (30); the finding of only pulse-labeled M in coated vesicle fractions may be related to the rapid turnover of M protein in ts31-infected cells at the restrictive temperatures (33). When nor-

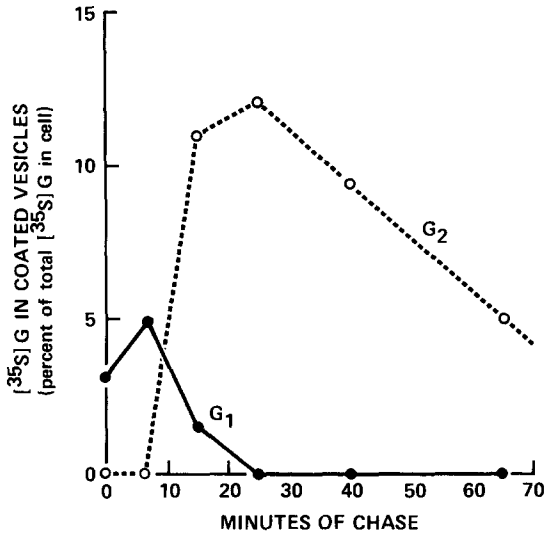


FIGURE 5 Kinetics of passage of newly synthesized G protein through the coated vesicle fraction obtained after the first two sucrose gradients. The autoradiograph of an experiment similar to that of Fig. 4 was quantitated. The recoveries of G_1 and G_2 were determined separately, normalized according to the internal standard of [^{3}H]leucine so as to account for sample-to-sample variation in recovery, and then adjusted for the yield of the purification at the two gradient stage (Materials and Methods). Data shown are the percentage of the total (G_1 plus G_2) in [^{35}S]G protein in the cell which can be ascribed to coated vesicles, for both G_1 (●) and G_2 (○).

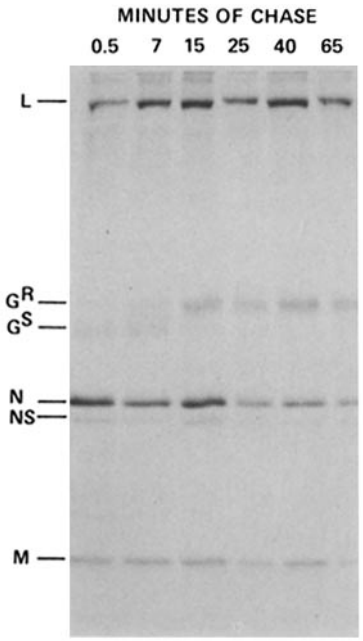


FIGURE 6 Treatment of coated vesicle with endoglycosidase H. Samples of the coated vesicle fractions obtained from the second sucrose gradient (15 μg protein) were heated at 105°C for 3 min in 20 μl total volume containing 50 mM Tris HCl pH 6.8, 1% SDS, 15 mM dithiothreitol. Then, 20 μl of 0.3 M citrate buffer (pH 5.5) was added, followed by 1.5 ng protein of pure endoglycosidase H (36). After 20 h at 37°C, the 10% trichloroacetic acid precipitate was subject to SDS-polyacrylamide gel electrophoresis and autoradiography; the autoradiograph is shown. The symbols G^S and G^R refer to forms of G whose Asn-linked oligosaccharides have been cleaved (sensitive) or not cleaved (resistant) by the treatment with Endo H (27). The times of chase are also indicated.

malized for differential recovery among samples by the internal standard ([^{3}H]Leu or vesicle protein), the amount of [^{35}S]M protein is independent of time of chase.

Immunoprecipitation with Anticoated Vesicle Serum Distinguishes Coated Vesicles Containing G_1 from Those Containing G_2

It was a matter of some concern that purification through the third gradient resulted in an enrichment of G_2 but not G_1 (Figs. 5 and 8), raising the possibility that G_2 but not G_1 was actually in coated vesicles. To check this, [^{35}S]Met-labeled G_1- and G_2-containing coated vesicles (obtained from the second sucrose gradient) were separately immunoprecipitated with anticoated vesicle serum (whose properties have been described earlier [6]), as shown in Fig. 9. When the degree of precipitation of G_1- and G_2-containing coated vesicle fractions by increasing amounts of anticoated vesicle serum were quantitated, a rather surprising result (Fig. 10) was obtained. An even higher percentage of G_1-containing coated vesicles than of G_2-containing coated vesicles was precipitated, for each dilution of serum employed. These results suggest that the two functionally distinct classes of coated vesicles are also structurally distinguishable, and can be differentially purified. G_2-containing coated vesicles are enriched relative to those containing G_1 by the third sucrose gradient; enrichment in the opposite direction is achieved by immune precipitation with anticoated vesicle antibodies.

Further increases in the amount of antibody used did not result in further precipitation of either clathrin, G_1, or G_2. A number of attempts to increase the yield of immunoprecipitation using two stage indirect immunoprecipitation techniques were hampered by extensive nonspecific binding of coated vesicles to immunosorbents. The addition of detergents to such systems is usually used to minimize such difficulties, but was not possible in the present case because G is extracted under these circumstances from coated vesicles.

DISCUSSION

Coated Vesicles Transport G Protein to the Cell Surface

It is clear (Figs. 5 and 8) that we have identified a transit form of the VSV glycoprotein in highly enriched coated vesicle fractions whose kinetics show that it is in passage from the endoplasmic reticulum towards the plasma membrane, via the Golgi apparatus.[1] This transit form of G is contained in clathrin-coated (Fig. 10) particles whose sedimentation properties are indistinguishable from those of coated vesicles (Table I). Furthermore, we have previously shown (30) that G protein is found in highly purified preparations of coated vesicles obtained from infected cells in steady-state amounts nearly stoichiometric with clathrin.

From this data we conclude that coated vesicles transport G in two successive stages during the passage of this membrane protein from its site of synthesis in the rough endoplasmic reticulum to the plasma membrane. The earlier stage is characterized by a high mannose-containing and Endo H-sensitive form of G (G_1), whereas the later stage involves a mature G protein (G_2) containing sialic acid and resistant to Endo H. Therefore, we believe that the early stage corresponds to coated vesicles that bud off from the endoplasmic reticulum and fuse with the Golgi apparatus,[1] thereby delivering G to this organ-

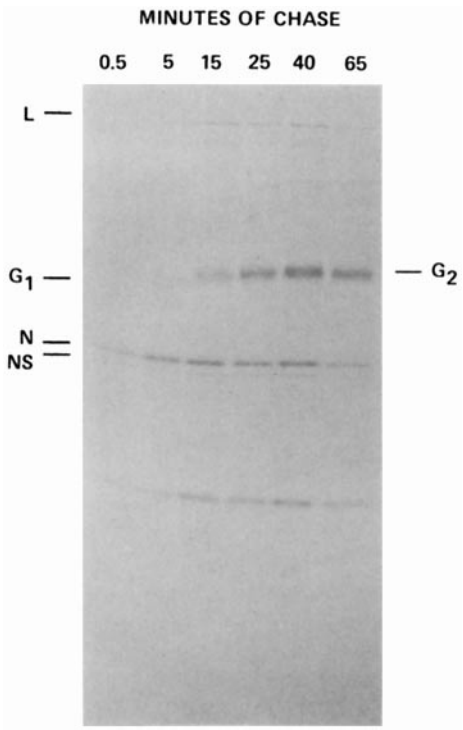


FIGURE 7 Coated vesicle fractions obtained by purification through all three sucrose gradients. Times of chase are indicated on the autoradiograph.

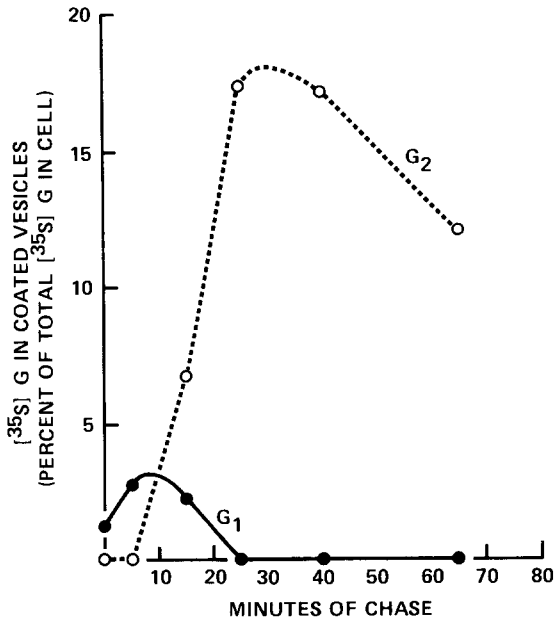


FIGURE 8 Kinetics of passage of newly synthesized G protein through the coated vesicle fraction obtained by purification through three gradients. Same experiment as Fig. 7, quantitated as described in Materials and Methods. G_1 (●), G_2 (○).

elle, within which Endo H-resistance is conferred and the terminal sugars including sialic acid are added. The late stage would then correspond to coated vesicles that bud from the Golgi and fuse with the plasma membrane.

Since our results derive exclusively from cell fractionation, the evidence that coated vesicles transport G necessarily is of a correlative rather than a functional nature. It is an inherent limitation of all cell fractionation studies, no matter how pure the fraction in question, that the source of any individual protein could be a trace impurity of unknown origin. We believe, however, that the biochemical and immunochemical evidence demonstrating the purity of coated vesicle fractions presented here, their strikingly high content of G protein (30), and the quantitative arguments presented in Table I make this possibility remote.

From our results we fully anticipate that membrane glycoproteins of plasma membranes are, quite generally, transported intracellularly within coated vesicles in a manner similar to that of the VSV G protein. Additionally, because coated vesicles are well-defined entities in intact cells, our observations, coupled with arguments given earlier (14, 15), would appear to rule out those models in which intracellular transport is proposed to be accomplished by lateral diffusion between the occasionally reported zones of membrane continuity between organelles such as the endoplasmic reticulum and Golgi apparatus.

Coated Vesicles Can Account for the Transport of All of the G Protein to the Cell Surface

Fig. 5 shows that the first and second waves of transport contain about 5 and 15% of the total pulse-label, respectively. This modest amount of labeled G found in coated vesicles at any one time is a necessary consequence of the rapid turnover of these vesicles, and, indeed, would be expected for any transient species.

Coated vesicles form and fuse extremely rapidly. A number of estimates of the rate of this process have been made for coated endocytic vesicles (1, 11). The time required for a complete cycle of transport in coated vesicles (i.e., formation of the vesicle, fusion with target organelle, and recycling of receptor and possibly coat to the plasma membrane) varies from only 1 to 5 min.

Times much longer than this are required to complete the two waves of transport (about 20 min for the early wave, over 60 min for the late wave). Therefore, if these waves were the result of the activity of coated vesicles, then each wave of transport would result from multiple, successive cycles of transport, each cycle requiring only a very short time. Thus, only a small fraction of the total pool of G would be found in coated vesicles at any one time, corresponding to the amount being transported in coated vesicles at that one instant. But the cumulative effect would be to transport much larger amounts

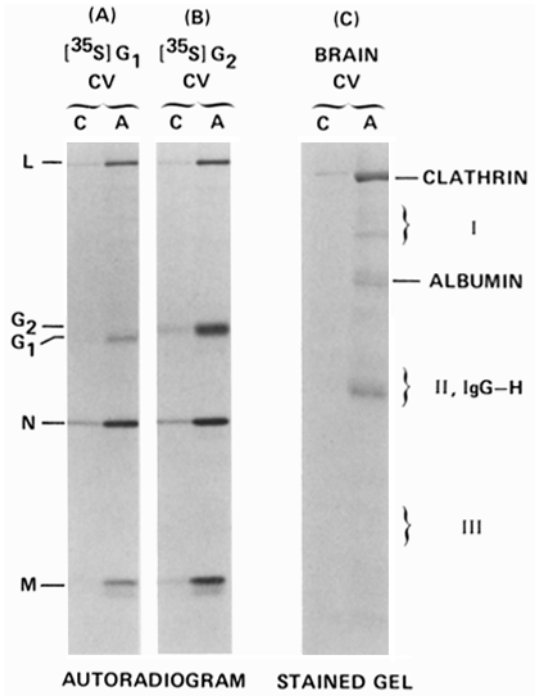


FIGURE 9 Anticoated vesicle precipitation of coated vesicles. G protein in coated vesicle fractions is precipitated by anticoated vesicle serum. Coated vesicles were copurified from [^{35}S]Met pulse-labeled, VSV-infected CHO cells after 5 min (*A*, [^{35}S]G_1 CV) and 30 min (*B*, [^{35}S]G_2 CV) of chase. Only the first two sucrose gradients were employed. For immunoprecipitation, ^{35}S-labeled coated vesicles (60 μg protein) were disaggregated by brief sonication (two bursts in 5 s) on ice in 1.5 ml of Tris-buffered saline. Portions (0.5 ml) of these coated vesicles were incubated with 0.1 ml of either pre-immune or anticoated vesicle serum (6). Following incubation for 2 d at 4°C, precipitates were collected by centrifugation at 5,000 rpm for 10 min in the Sorvall HS-4 rotor, were washed twice with Tris-buffered saline, and then subjected to SDS-polyacrylamide gel electrophoresis. The gel was stained (*C*) and then autoradiographed (*A* and *B*). The letters *C* and *A* underneath the brackets indicate whether control (*C*) or anticoated vesicle (*A*) sera were utilized. (*A*) [^{35}S]G_1-containing coated vesicles from 5 min chase were precipitated. (*B*) [^{35}S]G_2-containing coated vesicles from 30 min chase were used. (*C*) Stained polypeptide pattern of a similar precipitation in which brain coated vesicles were employed. Albumin and IgG bands originate from the serum. Coated vesicle specific polypeptides (clathrin, I, II, III) are indicated. This same immunoprecipitation procedure enriches for G protein (Table II) and specifically precipitates coated vesicles from crude cellular extracts (6).

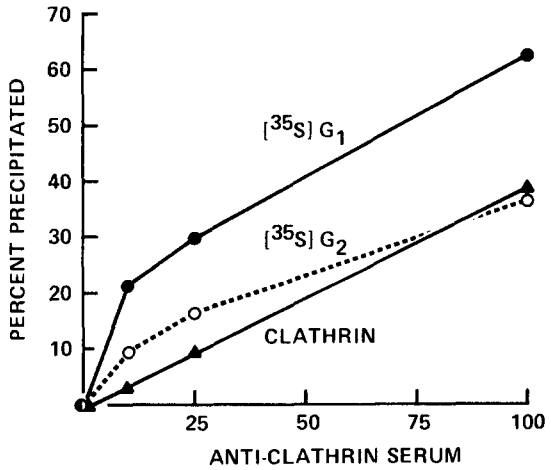


FIGURE 10 Differential precipitation of G_1 and G_2-containing coated vesicles by anticoated vesicle serum. The percent of the initially added [^{35}S]G_1 (●), [^{35}S]G_2 (○), and of clathrin (△) that had been precipitated by anti-clathrin (Fig. 9) was determined from densitometry tracings of autoradiographs (for G_1 and G_2) and of stained gels (for clathrin, derived principally from the brain carrier used in the copurification). Samples of the same incubations were analyzed to determine precipitation of clathrin and G protein.

of G through multiple cycles. For example, the early phase of transport requires about 20 min for completion (Figs. 1 and 5), during which time virtually all of the G is transported. If we assume that 1 min is required for a transport cycle, then during these 20 min 20 successive individual cycles would have taken place, each having had to carry 5% of the total pool of G. So, the peak of pulse-label in coated vesicles would have been around 5% of the total, similar to that observed. An analogous rough calculation can be made for the late phase to show that the level of G in coated vesicles is consistent with a reasonable turnover time for these vesicles.

Therefore, on the basis of the rapid turnover of coated vesicles in other systems, the amount of pulse-label recovered in coated vesicles appears to be sufficient to account for most, if not all, of the intracellular transport of G. However, any pulse-chase experiment involving transient species is inherently incapable of rigorously proving that all of the flux is through the pathway under study.

Coated Vesicles Mediating the Two Transit Steps Appear to Constitute Distinct Subpopulations

Figs. 5, 8, and 10 indicates that G_1- and G_2-containing coated vesicles can be distinguished on the basis of their differential immunoprecipitation by an anticoated vesicle serum and their differential purification by the third sucrose gradient. One attractive explanation would be that the clathrins that coat these two classes of vesicles are similar but distinct polypeptides, and this difference is recognized by the antibodies used. Consistent with this explanation is the previously demonstrated immunochemical differences between coated vesicles from different tissues in the same species (6). Indeed, the study of Friend and Farquhar (9) made clear that multiple, functionally distinct classes of coated vesicles do exist, differing in both enzyme content and size. The latter difference would be best explained if different classes of vesicles were to have different coats with different lattice sizes.

Whatever the correct explanation(s) for the difference between G_1- and G_2-containing coated vesicles, the existence of such a difference would appear crucial in considering how coated vesicles could function to transport proteins and lipids through several distinct routes within the same cell. Perhaps this would be best accomplished by distinct classes of coated vesicles, each class specialized to a particular transport step.

A simple but reasonable point of view would be that coated vesicles are a family of specific intracellular carriers of lipids and proteins which are the key intermediates in most sorting and transport events in higher cells. Indeed, current evidence points to an involvement of coated vesicles in each of the major intracellular transport routes (29). A particularly attractive aspect of viewing the sorting problem from this unitary standpoint is that the biochemical mechanisms underlying the sorting process can be expected to be homologous among the various transport routes, since homologous subpopulations of coated vesicles would mediate each; only the specificities involved in each route would differ.

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