

Isolation of Functional, Coated, Endocytic Vesicles

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Abstract. Brief internalization of [125 I]transferrin was used to label coated endocytic vesicles, which were then purified using a combination of $^2\text{H}_2\text{O}$ and $^3\text{H}_2\text{O}$ /Ficoll density gradients. Purification was monitored using an assay measuring fusion of endocytic organelles, so as to isolate functional vesicles. Isolated vesicles had all the properties of clathrin-coated vesicles, being

enriched for the major components of clathrin coats and uncoated by either 1 M Tris-HCl or an uncoating ATPase. Nearly half of the labeled vesicles were able to participate in subsequent fusion events, as measured by the cell-free assay. Fusion was specific, requiring energy and cytosol, and being sensitive to N-ethyl maleimide.

INTERNALIZATION of receptors is mediated by clathrin-coated pits (Goldstein et al., 1985). These specialized areas of plasma membrane contain clustered receptors, which are concentrated by interaction between adaptor complexes and a motif within the cytoplasmic domain of the receptors (Vigers et al., 1986; Pearse, 1988; Davis et al., 1986; Lazarovits and Roth, 1988). The adaptor complexes also bind clathrin triskelions (Zaremba and Keen, 1983; Pearse and Robinson, 1984), which form a lattice on the membrane. Rearrangement of this lattice (Heuser, 1980) accompanies the invagination of membrane, and a scission event (Kosaka and Ikeda, 1983; Smythe et al., 1989) releases a free, clathrin-coated vesicle.

Consumption of this vesicle and delivery of receptors to the endosome is thought to occur by the following sequence of events. First, the clathrin lattice is removed, at least in part, from the vesicle. It is known that clathrin triskelions are present in a soluble pool, which is presumably employed to support further rounds of receptor internalization (Goud et al., 1985; Rothman and Schmid, 1986). The most likely agent for release is uncoating ATPase, an enzyme that can disassemble clathrin cages and release clathrin triskelions from bovine brain coated vesicles (Schlossman et al., 1984; Greene and Eisenberg, 1990). Removal of the clathrin coat enables the vesicle to deliver its contents to the endosome. The nature of this transfer is not certain (Helenius et al., 1983; Schmid et al., 1988; Griffiths et al., 1989). According to one model, endocytic vesicles combine with each other, and with vesicles derived from the Golgi complex, to form an endosome *de novo*. Alternatively, stable endosomes might accept membrane directly from endocytic vesicles. Whichever model is correct, endocytic vesicles must recognize and fuse with a target organelle.

The molecular basis for fusion between endocytic compartments remains poorly understood. Our approach, and that of other groups, has been to reconstitute the event as a biochemical reaction (for review see Warren et al., 1989; Gruenberg and Howell, 1989). Together, these studies have

established certain broad requirements for the reaction. Fusion is energy dependent and requires proteins present on the vesicle membranes, as well as components within the cytosol. Fusion is sensitive to the action of the alkylating agent, N-ethyl maleimide (NEM).¹ Furthermore, fusion is inhibited when nonhydrolyzable analogues of GTP, such as GTP γ S, are included in the incubation, consistent with a role for GTP-binding proteins (Mayorga et al., 1989).

Studies on intra-Golgi transport (Balch et al., 1984) indicate that fusion of transport vesicles with the acceptor cisternum is dependent upon the recruitment of components from the cytosol to assemble a fusion complex at the site of membrane contact (Malhotra et al., 1988; Orci et al., 1989; Clary and Rothman, 1990; Clary et al., 1990). Since endocytic fusion shares several properties with fusion of Golgi transport vesicles, it is thought that endocytic fusion might require an analogous complex (Schatz, 1989). Indeed, there is some evidence that one of these components, the NEM-sensitive factor, is also involved in endocytic fusion (Diaz et al., 1989). It should, therefore, be possible to isolate such a complex using the present cell-free systems.

However, analysis of the interaction of a putative fusion complex with the membrane of endocytic compartments requires the isolation of active endocytic organelles. Isolation will also permit the study of components on the vesicle membrane that are required for recognition and fusion with target organelles. With this in mind, we have isolated an endocytic intermediate labeled with a marker that can be employed in a fusion assay. We have chosen the clathrin-coated vesicle because, firstly, its generation and consumption are well characterized, compared with later endocytic compartments. Secondly, it is the earliest endocytic compartment, so it is easy to label specifically by employing a short internalization time. Thirdly, the unique composition of the coated vesicle makes it relatively easy both to isolate and to identify, and there are several well-established purification procedures.

1. *Abbreviation used in this paper:* NEM, N-ethyl maleimide.

We have therefore used the framework provided by these procedures to purify coated vesicles, but have followed their isolation using a cell-free assay for endocytic vesicle fusion (Woodman and Warren, 1988). This has allowed us to isolate functional, coated endocytic vesicles.

Materials and Methods

Materials

All reagents, unless otherwise specified, were obtained from Sigma Chemical Co. or BDH Chemicals Ltd. (Poole, Dorset, UK). Ficoll (type 400 DL) was dialyzed against water and freeze dried before use.

Cells

A431 cells were maintained in DME supplemented with 10% (vol/vol) FCS and 100 U/ml of both penicillin and streptomycin. All media and supplements were obtained from Northumbria Biologicals Ltd. (Cramlington, Northumberland, UK).

Antibodies

Sheep antihuman transferrin serum was obtained from the Scottish Antibody Production Unit (Carluke, Scotland). An antiserum to human clathrin light chain was prepared by immunizing a rabbit with light chains purified from placental-coated vesicles according to the method of Lisanti et al. (1982). Rabbit antiserum to human transferrin was prepared as described (Woodman and Warren, 1989).

Radiolabeling

Human transferrin was radioiodinated by the Iodogen method (Fraker and Speck, 1978), as described previously (Woodman and Warren, 1989). Normally, 100 μ g transferrin was labeled with 2.5 mCi Na¹²⁵I (16 mCi/ μ g; Amersham International, Bucks, UK), to achieve an activity of $\sim 10^7$ cpm/ μ g.

A431 cells were metabolically labeled with [³⁵S]methionine (800 Ci/mmol; Amersham International) according to the following procedure. Cells ($\sim 5 \times 10^7$ on a 24 \times 24 cm dish) were washed three times in Dulbecco's PBS, then incubated for 16 h with 80 ml labeling medium (DME with 0.75 mg/l methionine [MEM Select-Amine kit; Gibco Laboratories, Grand Island, NY] and supplemented with dialyzed FCS) containing 60 μ Ci/ml [³⁵S]methionine.

Coated Vesicle Preparations

Isolation of donor coated vesicles was based on the method used by Pearse (1982) to isolate coated vesicles from placenta. A431 cells (1–4, 24 \times 24 cm dishes, each containing 10^8 cells) were washed four times in PBS and incubated for 2 h at 4°C with 1.5 μ g/ml [¹²⁵I]transferrin in binding medium (DME containing 20 mM Hepes, pH 7.4, and 0.2% [wt/vol] BSA). After washing in PBS, the cells were warmed for 2 min at 31°C in binding medium, and then washed four times in vesicle buffer (140 mM sucrose, 75 mM potassium acetate, 10 mM MES, pH 6.6, 1 mM EGTA, 0.5 mM magnesium acetate). After draining the dishes, the cells were scraped with a rubber policeman, and DTT (1 mM) and protease inhibitors (1 μ g/ml chymostatin, 1 μ g/ml pepstatin, 40 μ g/ml PMSF, 2 μ g/ml E64, 1 μ g/ml antipain, all from a 1,000 \times concentrate in DMSO) were added. Cells were homogenized on ice by passing the suspension 10 times through a 0.2540-inch bore containing a 0.2530-inch diameter ball (Balch et al., 1984), and a postnuclear supernatant prepared by centrifugation at 500 g_{av} for 5 min. Ribonuclease type A (50 μ g/ml; Worthington Enzymes Ltd., Freehold, NJ) was added to disassemble polyribosomes and, after 30 min at 4°C, the preparation was centrifuged at 7,000 g_{av} for 30 min to obtain a postmitochondrial supernatant. This supernatant (~ 3 ml) was applied to a 10-ml continuous gradient of 10–90% (wt/vol) ²H₂O in vesicle buffer, containing 1 mM DTT, and centrifuged in an SW 40 rotor (Beckman Instruments, Inc., Palo Alto, CA) at 45,000 g_{av} for 30 min. 1-ml fractions were collected and those of peak fusion activity (fractions 5–9) were pooled, diluted in vesicle buffer to 18 ml, and applied to a 20-ml continuous gradient of 2% (wt/vol) Ficoll/9% (wt/vol) ²H₂O–20% Ficoll/90% ²H₂O in vesicle buffer containing 1 mM DTT throughout. This gradient was centrifuged to equilibrium

in an SW 28 rotor at 80,000 g_{av} for 16 h and 20, 1-ml fractions were taken from the bottom for analysis. Where indicated, untreated A431 cells were added as a carrier to preparations prior to homogenization. To obtain preparations labeled with both [¹²⁵I]transferrin and [³⁵S]methionine, metabolically labeled cells were combined with cells containing internalized [¹²⁵I]transferrin. The above protocol was followed, except that colchicine (10 μ g/ml) was added before the final density gradient, in order to reduce contamination by microtubules. This treatment did not affect the sedimentation of the coated vesicles. Coated vesicles were isolated from human placenta on ²H₂O/Ficoll gradients exactly as described by Pearse (1982). All coated vesicle preparations were frozen and stored in liquid nitrogen.

Desferroxamine Treatment

Desferroxamine (Ceiba-Geigy, Cambridge, UK) was used to remove [¹²⁵I]transferrin associated with the cell surface (Klausner et al., 1983) as follows. Cells were washed twice in ice-cold citrate saline (130 mM NaCl, 25 mM NaCitrate, pH 5.0) containing 50 μ M desferroxamine and incubated in the same buffer for 5 min at 4°C. After washing twice in PBS containing 50 μ M desferroxamine, cells were incubated for a further 5 min at 4°C in PBS/desferroxamine. This treatment removes 85–90% of transferrin bound to cells at 4°C.

Acceptor Preparations

Preparations were based on those described previously (Woodman and Warren, 1989), with the following modifications. Cells (10^8) were washed four times with PBS and incubated with 10 μ g/ml transferrin in BM for 1 h at 4°C, washed again four times, and incubated with BM containing 1 ml sheep antitransferrin antiserum. Cells were warmed for 5 min at 31°C to internalize antibody, then washed four times in ice-cold homogenization buffer (HB; 140 mM sucrose, 70 mM potassium acetate, 20 mM Hepes, pH 7.2). Protease inhibitors and DTT were added as for coated vesicle preparations, cells were homogenized and a postnuclear supernatant was prepared. To obtain crude acceptor membrane fractions, the postnuclear supernatant was applied to a discontinuous sucrose gradient of 2.5 ml HB containing 40% (wt/vol) sucrose overlaid with 8 ml of the same buffer containing 20% (wt/vol) sucrose, with 1 mM DTT throughout. The gradient was centrifuged at 155,000 g_{av} for 2 h and the crude membrane fraction was recovered from the 20–40% interface by tube puncture. All preparations were frozen and stored in liquid nitrogen.

Crude Donor Preparations

A431 cells (2×10^8) were incubated with 1.5 μ g/ml [¹²⁵I]transferrin in binding medium at 4°C for 2 h, warmed at 31°C for 5 min, then washed four times in HB. Homogenization and preparation of crude membranes was performed exactly as for acceptor membrane preparations. Samples were frozen and stored in liquid nitrogen.

Cytosol Preparations

Confluent monolayers of A431 cells were washed four times with HB and scraped. DTT and protease inhibitors were added as above and the cells were homogenized. Postnuclear supernatants were centrifuged at 400,000 g_{av} for 30 min in a TL100 bench-top ultracentrifuge (Beckman Instruments, Inc.), and supernatants desalted on Biogel P6 columns (Bio-Rad Laboratories, Cambridge, MA) into HB containing 1 mM DTT. Aliquots were frozen and stored in liquid nitrogen.

Fusion Assays

Fusion assays using coated vesicle preparations as donors were carried out as described before (Woodman and Warren, 1988, 1989), with the following modifications. To effectively dilute the vesicle isolation buffer and Ficoll, coated vesicle preparations were diluted by a factor of five into the final assay mix. A typical incubation contained donor coated vesicles (50 μ l), ATP-depleting or -regenerating cocktails (25 μ l), 1 mg/ml transferrin in HB (25 μ l), acceptor postnuclear supernatant (50 μ l), or acceptor membranes (50 μ l) with cytosol (final concentration 2 mg/ml). The volume was made up to 250 μ l with HB. Samples were incubated for 2 h at 37°C, transferred to 4°C, and diluted to 1 ml with immunoprecipitation buffer (IB; 0.1 M Tris-HCl, pH 8.0, 0.1 M NaCl, 5 mM MgCl₂, 1% [wt/vol] Triton X-100, 0.5% [wt/vol] SDS, 1% [wt/vol] sodium deoxycholate, 0.1% BSA). *Staphylococcus aureus* cells (20 μ l of a 10% suspension, washed three times in IB) were

added to each sample and, after 1 h at 4°C, the precipitates were washed twice in IB by centrifugation and counted for radioactivity.

Latency Assays

The proportion of [¹²⁵I]transferrin within sealed vesicles was determined by measuring inaccessibility to antitransferrin antibody. Donor vesicles (10 μl) were incubated with antitransferrin antiserum (0.5 μl) in a total volume of 200 μl, made up either with IB, or with IB lacking detergent. To control for nonspecific binding, parallel incubations included 100 μg unlabeled transferrin. After 1 h at 37°C, unlabeled transferrin was added to each sample, followed by IB (0.8 ml) and *Staphylococcus aureus* cells (50 μl). After a 1-h incubation at 4°C, precipitates were washed twice with IB by centrifugation and counted for radioactivity.

Uncoating ATPase

Preparation of uncoating ATPase from human placenta or bovine brain was adapted from the method of Schlossman et al. (1984), except that the hydroxylapatite stage was omitted. Preparations were stored in ATPase buffer (25 mM KCl, 20 mM Hepes, pH 7.0, 2 mM magnesium acetate, 1 mM DTT) at -80°C. Preparations from bovine brain were homogeneous, while preparations from placenta were contaminated with the related grp78, which has no uncoating activity (Chappell et al., 1986). Contamination never exceeded 20–30% of total protein.

Uncoating Assays

[¹²⁵I]Transferrin-labeled vesicles were diluted tenfold into HB containing protease inhibitors and 1 mM DTT. ATP-regenerating or -depleting cocktails were used as for fusion assays. In a standard incubation 10 μg placental uncoating ATPase was used. Bovine brain uncoating ATPase could be used to equal effect. Samples were incubated for 15 min at 37°C, diluted to 2 ml with ice-cold vesicle buffer, and centrifuged on 10 ml 2H₂O/Ficoll gradients at 80,000 g_{av} for 16 h. The gradient was sampled from the bottom and the first 15 fractions (0.5 ml) were counted for radioactivity. [³⁵S]Methionine-labeled vesicles were treated as above, except that dilution into vesicle buffer was followed by centrifugation at 200,000 g_{av} for 15 min. Pellets were resuspended in sample buffer. Protein from supernatants was precipitated with 70% (vol/vol) ethanol and resuspended in sample buffer.

EM

Donor vesicles were diluted five times in vesicle buffer and centrifuged at 100,000 g_{av} for 2 h. The pellet was washed in vesicle buffer and fixed in 3% (wt/vol) glutaraldehyde, in the same buffer. Pellets were rinsed three times in 0.1 M sodium cacodylate, pH 7.4, and postfixed in 1% osmium tetroxide and 1.5% potassium ferrocyanide for 30 min at 4°C. Pellets were rinsed as above, dehydrated in graded ethanols, and embedded in Epon. Sections (70 nm) were cut and stained with alcoholic uranyl acetate and Reynold's lead citrate.

Protein Assays

Protein concentrations were determined by the method of Bradford (1976).

SDS-PAGE

Samples were run on 10% acrylamide gels according to the method of Laemmli (1970). Standard molecular mass markers for Coomassie-stained gels were (from Bio-Rad Laboratories): 200 kD (myosin); 116 kD (β-galactosidase); 97-kD (phosphorylase b); 66 kD (BSA); 45 kD (ovalbumin); 31 kD (carbonic anhydrase), and for fluorographed gels ([¹⁴C]; from Amersham International): 200 kD (myosin); 97 kD (phosphorylase b); 69 kD (BSA); 46 kD (ovalbumin); 30 kD (carbonic anhydrase). Western blotting was performed according to the method of Towbin et al. (1979). Gradient fractions were diluted in vesicle buffer, and centrifuged at 100,000 g_{av} for 2 h. The pellets were resuspended in sample buffer and applied to a 10% SDS gel. Proteins were transferred to nitrocellulose and probed with anti-light chain serum (diluted 1:200), followed by [¹²⁵I]protein A (0.1 μCi/ml, 30 μCi/μg; Amersham International). For fluorography, fixed [³⁵S]-labeled gels were equilibrated with Amplify (Amersham International). Dried gels were exposed to X-OMAT XAR-5 film (Eastman Kodak Co., Rochester, NY).

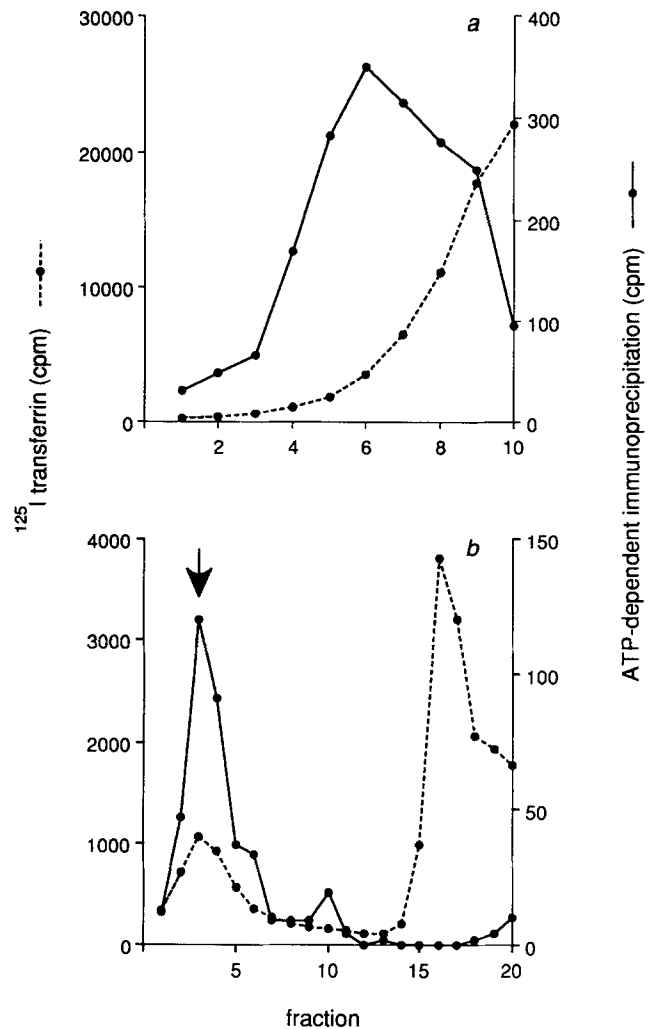


Figure 1. Isolation of endocytic vesicles labeled with [¹²⁵I]transferrin. [¹²⁵I]transferrin was bound to A431 cells and internalized. Cells were homogenized and a postmitochondrial supernatant prepared before density gradient centrifugation. (a) ²H₂O gradient. Fractions (1 ml, fraction 1 = bottom) were sampled for radioactivity and assayed for fusion activity using acceptor endocytic vesicles present in a postnuclear supernatant. Activity is expressed as the ATP-dependent immunoprecipitation of [¹²⁵I]transferrin. (b) ²H₂O/Ficoll gradient. Fractions from the ²H₂O gradient with most fusion activity (fractions 5–9) were applied to a continuous ²H₂O/Ficoll gradient. Fractions (1 ml, fraction 1 = bottom) were counted for radioactivity and assayed for fusion activity against an acceptor postnuclear supernatant. The arrow indicates the peak position of placental coated vesicles applied to an equivalent gradient.

Results

Isolation of Functional Endocytic Vesicles

Endocytic vesicles were labeled with [¹²⁵I]transferrin, and their isolation was monitored by the use of a functional assay that measures endocytic vesicle fusion. The assay measures fusion between vesicles, containing internalized [¹²⁵I]transferrin, which donate their contents to acceptor vesicles, containing polyclonal antitransferrin antibody (Woodman and Warren, 1988, 1989). The resulting radiolabeled immunocomplex is recovered on *Staphylococcus aureus* cells after solubilizing the vesicles in detergent, and the activity of the

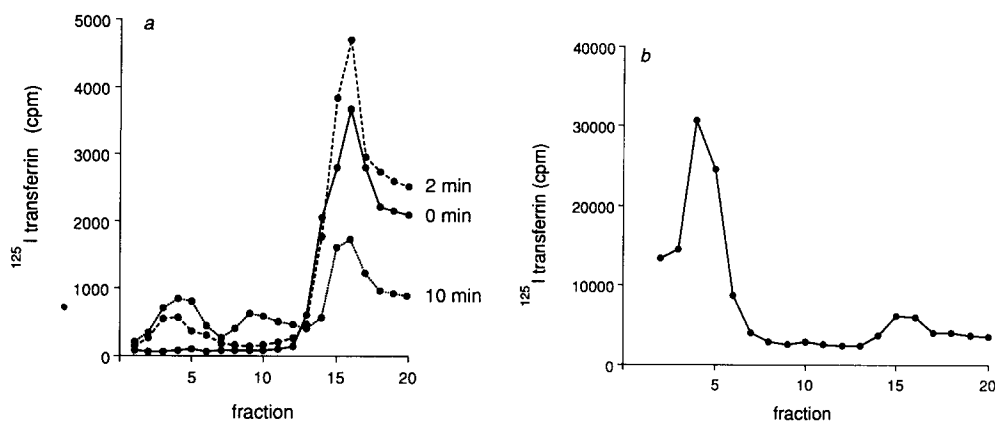


Figure 2. Isolated vesicles are early endocytic intermediates. (a) A431 cells were labeled with [¹²⁵I]transferrin for 2 h at 4°C, and kept at 4°C (solid line), or warmed for 2 min at 31°C (broken line), or 10 min at 31°C (dotted line). Coated vesicle isolation was carried out and fractions (1 ml) were removed from the final ²H₂O/Ficoll gradient and counted. (b) Coated vesicles were prepared as normal, except that [¹²⁵I]transferrin remaining on the cell surface was removed by desferroxamine treatment before homogenizing the cells.

isolate gives an indirect measure of the extent of vesicle fusion.

Transferrin is internalized rapidly by A431 cells, a human cell line rich in transferrin receptors (Hopkins and Trowbridge, 1983). After binding [¹²⁵I]transferrin to receptors on the plasma membrane at 4°C, cells were warmed for 2 min at 31°C, before homogenization. This brief incubation was chosen in order to maximize the proportion of internalized transferrin that labeled coated vesicles, given their short half-life (Anderson et al., 1977). The purification of coated vesicles was based on the use of a ²H₂O rate sedimentation gradient, followed by a ²H₂O/Ficoll isopycnic gradient (Pearse, 1982). Representative purification data are shown in Fig. 1. Fig. 1 *a* demonstrates a typical profile after a post-mitochondrial supernatant was applied to the first gradient. Fractions were sampled for [¹²⁵I]transferrin and for fusion activity against crude acceptor membranes, supplied as a postnuclear supernatant. Activity was expressed as the ATP-dependent immunoprecipitation of [¹²⁵I]transferrin. When each fraction was assayed in the presence of an ATP-depleting cocktail, immunoprecipitation of [¹²⁵I]transferrin could not be detected above background levels (data not shown; see also Fig. 8). Clearly, the fusion activity is separated from the bulk of [¹²⁵I]transferrin, much of which will have been released from plasma membrane receptors before homogenization. Activity is found in a broad peak at a similar position to the protein profile obtained when placental coated vesicles were applied to equivalent gradients (Pearse, 1982; our observations, not shown).

The fractions of peak fusion activity were applied to the ²H₂O/Ficoll density gradient and centrifuged to equilibrium. Upon fractionation, two major peaks of [¹²⁵I]transferrin were identified; one near the top of the gradient and one near the bottom (Fig. 1 *b*). The position of each peak on the gradient is reproducible between preparations. The bottom peak migrated to the same position as coated vesicles isolated from placenta (Fig. 1 *b*, arrow). In a typical preparation ~1.0% of the cell-associated transferrin was associated with this peak.

The upper peak of [¹²⁵I]transferrin was devoid of fusion activity. This suggests that it represented cell-surface label, since we and others (Davey et al., 1985; Braell, 1987) have shown that fusion occurs specifically between endocytic vesicles and does not involve plasma membrane-derived vesicles.

Only the lower peak of [¹²⁵I]transferrin had any significant fusion activity, suggesting that this was an endocytic compartment.

Isolated Vesicles Are Early Endocytic Intermediates

When [¹²⁵I]transferrin was bound at 4°C, but not internalized, no peak of [¹²⁵I]transferrin was observed at the bottom of the ²H₂O/Ficoll gradient (Fig. 2 *a*). The presence of [¹²⁵I]transferrin near the top of the gradient from such preparations confirms it as plasma membrane labeling. Increasing the incubation time at 31°C to 10 min resulted in the appearance of an intermediate peak on the ²H₂O/Ficoll

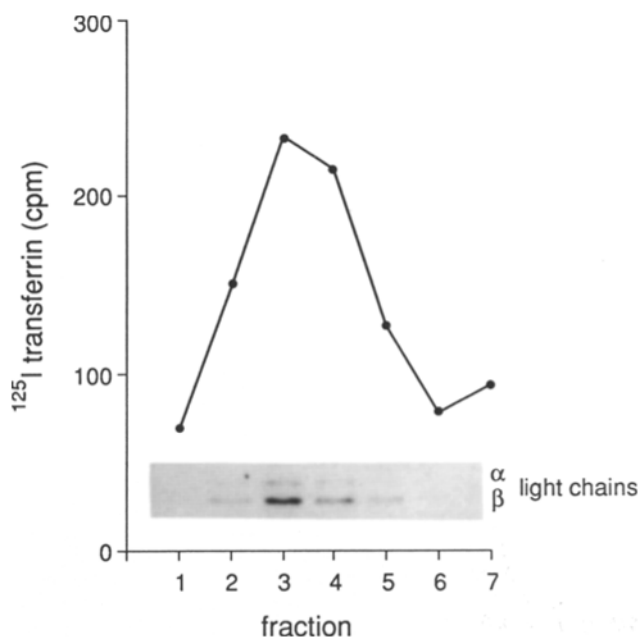


Figure 3. [¹²⁵I]Transferrin-labeled vesicles sediment with clathrin light chains on ²H₂O gradients. A431 cells (10⁸) were labeled with [¹²⁵I]transferrin and coated vesicles isolated. A tenfold excess of untreated cells was added as carrier. 1-ml fractions from the ²H₂O/Ficoll gradient were counted for radioactivity. Fractions were centrifuged and pellets resuspended in sample buffer, applied to a 10% SDS gel, and blotted with anti-light chain serum. The molecular mass of α and β light chains was ~36 and 34 kD, respectively.

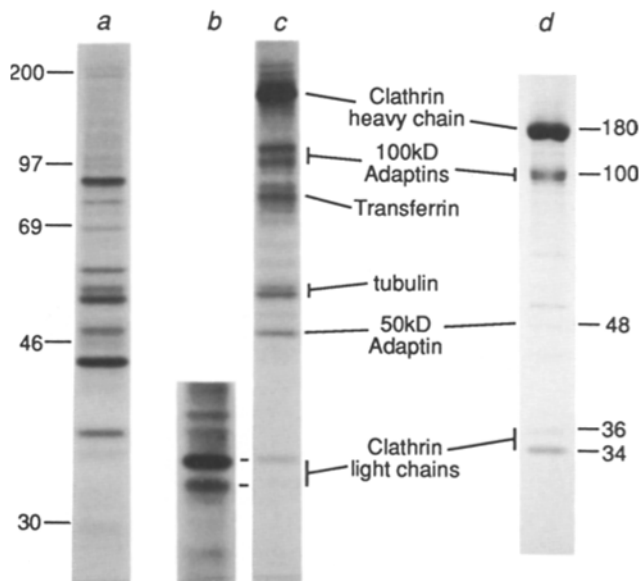


Figure 4. Isolation of metabolically labeled coated vesicles. (a) Cells labeled with [³⁵S]methionine were mixed 1:4 with those containing [¹²⁵I]transferrin and coated vesicles prepared and analyzed by SDS-PAGE and fluorography. (a) Postnuclear supernatant. (b and c) Coated vesicles. (d) The profile of placental coated vesicles, isolated in a similar fashion and revealed by Coomassie stain is shown for comparison.

gradient, with a consequent diminution of the top peak. Given the kinetics of transferrin internalization, and the activity of this peak in endocytic fusion assays (data not shown), it most likely represents labeled endosomes. The increase in the lower peak may be due to labeling of coated vesicles in-

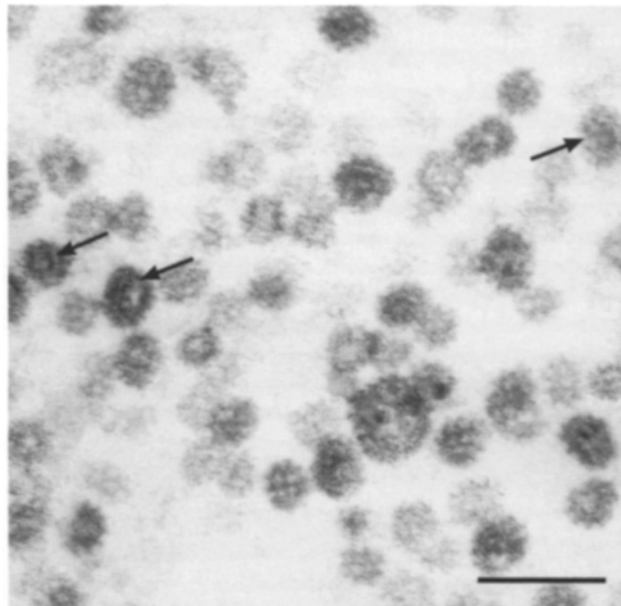


Figure 5. Electron micrograph of coated vesicle preparation. A431 cells were labeled with [¹²⁵I]transferrin (specific activity 10⁶ cpm/ μ g) and coated vesicles prepared. Unlabeled cells were included as carrier. Peak fractions from the ²H₂O/Ficoll gradient were combined and processed for EM. Arrows point to examples of vesicle membranes. Bar, 0.2 μ m.

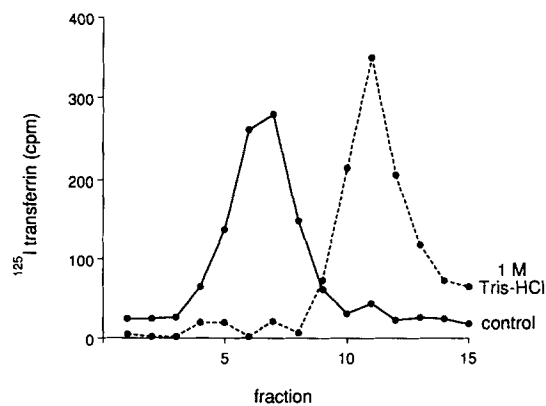


Figure 6. Tris-HCl removes vesicle coats. [¹²⁵I]transferrin-labeled vesicles were diluted in HB (solid line) or 1 M Tris-HCl, pH 7.5 (broken line). Samples were applied to ²H₂O/Ficoll gradients and fractions (0.5 ml, fraction 1 = bottom) were counted for radioactivity.

involved in transferrin receptor recycling, in addition to endocytic vesicles, although further work is required to substantiate this argument. In all, the data in Fig. 2 a provide evidence that the active vesicles in the bottom peak are the earliest labeled endocytic compartment. Further evidence that the compartment is inside the cell was provided by treating cells after internalization of [¹²⁵I]transferrin with the iron chelator desferroxamine which, under appropriate conditions, releases cell-surface transferrin (Klausner et al., 1983). In preparations treated thus, the lower peak was still observed while the upper peak was almost completely lost (Fig. 2 b).

Isolated Vesicles Possess Clathrin Coats

Fractions from the ²H₂O/Ficoll gradient were analyzed by Western blot using an antibody raised against clathrin light chains isolated from human placental coated vesicles. This demonstrated that the peak of [¹²⁵I]transferrin sedimented to the same position as the vesicle coat proteins; the peak coincides with the distribution on the gradient of α and β clathrin light chains (Fig. 3). Clathrin light chains were not detected in any other part of the gradient. In addition, preparations were made from a combination of untreated, [³⁵S]methionine-labeled cells and cells containing internalized [¹²⁵I]transferrin. SDS-PAGE analysis of the lower peak from the ²H₂O/Ficoll density gradient revealed a profile from isolated vesicles (Fig. 4 c) that was very similar to that obtained from placental coated vesicles (Fig. 4 d). The dominant labeled protein was the 180-kD clathrin heavy chain (Pearse, 1975). Also present were proteins migrating at \sim 100 kD and 45–50 kD, consistent with the presence of the adaptor complexes. The doublet migrating at \sim 55 kD is probably tubulin, since it was more heavily labeled in preparations not treated with colchicine. [¹²⁵I]Transferrin was also detected. Clathrin light chains appeared faint, due to their low methionine content (Jackson and Parham, 1988), so the appropriate region of a longer exposure is shown (Fig. 4 b). The degree of purification can be judged by comparison of the profile of isolated vesicles with that of a postnuclear supernatant from [³⁵S]methionine-labeled cells (Fig. 4 a).

Electron micrographs (Fig. 5) of the preparation revealed structures with polygonal coats characteristic of clathrin-

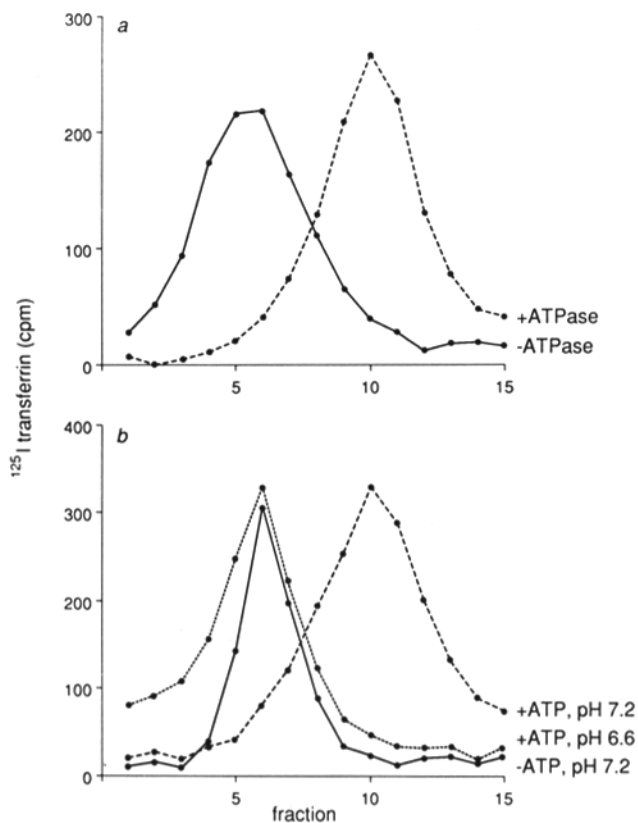


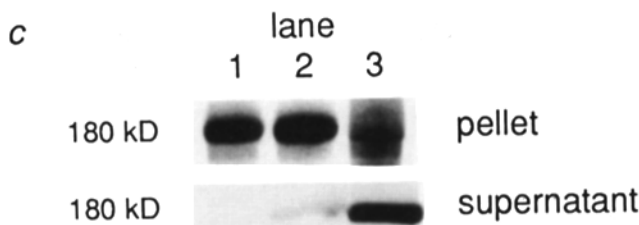
Figure 7. Vesicles are substrates for uncoating ATPase. (a) Vesicles were incubated with ATP-regenerating cocktails, with (broken line) or without (solid line) placental uncoating ATPase (10 μ g). (b) Vesicles and placental uncoating ATPase (10 μ g) were incubated with ATP-regenerating, or -depleting (dotted line) cocktails in HB or vesicle buffer, pH 6.6 (broken line). Samples were processed as in Fig. 6. (c) [35 S]methionine-labeled coated vesicles were incubated with the following. (lane 1) An ATP-regenerating cocktail. (lane 2) An ATP-depleting cocktail and placental uncoating ATPase (10 μ g). (lane 3) An ATP-regenerating cocktail and placental uncoating ATPase (10 μ g). Samples were centrifuged, and then pellets and supernatants were analyzed by SDS-PAGE. The 180-kD region is shown.

coated vesicles. In many cases a membrane could be discerned beneath the coat (Fig. 5, arrows), demonstrating that at least some of the structures were coated vesicles, rather than empty clathrin cages. Virtually all identifiable membrane vesicles were coated.

Table I. Latency Assay

Excess transferrin	Detergent	Immunoprecipitation	Immunoprecipitation (relative)
		% of total	
Donor coated vesicles			
-	+	76	100
-	-	11	14
+	+	1	1
+	-	1	1
Free [125I]transferrin			
-	+	80	100
-	-	79	99
+	+	1	1
+	-	1	1

Donor vesicles were assayed for latent [125 I]transferrin as described in Materials and Methods. Total radioactivity in each sample was 4,280 counts. Free [125 I]transferrin was treated in the same fashion. Total radioactivity in each sample was 18,286 counts. Results are the mean of triplicate determinations, each of which differed by no more than 6% from the mean.



Vesicles Containing [125 I]Transferrin Are Coated

To confirm that isolated vesicles labeled with [125 I]transferrin were coated, preparations were treated with 1 M Tris-HCl, an agent that removes the coat proteins (Keen et al., 1979). Since the position of coated vesicles on $^2\text{H}_2\text{O}$ /Ficoll gradients was due to the high density conferred on the vesicle by the clathrin coat, removal of the coat should have displaced the vesicles to a higher position on a similar gradient. Fig. 6 shows that, when isolated vesicles were treated with 1 M Tris-HCl at 37°C, the entire population of [125 I]transferrin-labeled vesicles were reduced in density compared with control vesicles, incubated in HB at 37°C. These vesicles migrated to the same position as vesicles maintained at 4°C in vesicle buffer before reisolation (not shown).

Vesicles Containing [125 I]Transferrin Are Clathrin Coated

The entire population of [125 I]transferrin-labeled endocytic vesicles were substrates for uncoating ATPase (Fig. 7 a), proving that these vesicles were clathrin coated. The product of the incubation migrated to a higher position, just below that of Tris-HCl-treated vesicles. This intermediate position is consistent with the action of uncoating ATPase in removing only clathrin, whereas Tris-HCl removes both clathrin and the adaptor complexes. The density shift did not

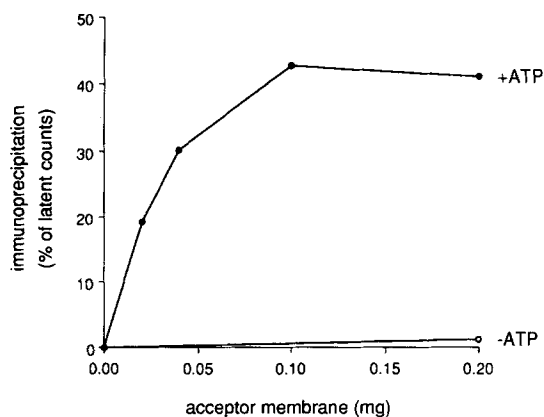


Figure 8. Fusion efficiency depends on acceptor membrane concentration. [125 I]Transferrin-labeled coated vesicles were incubated, as described, with cytosol, ATP-regenerating or -depleting cocktails, and different amounts of acceptor membrane. Values are expressed as the percentage of the total input radioactivity (4,010 counts) that were latent (see Table I), and are means of triplicate determinations, differing by no more than 12% from the mean.

occur if vesicles were incubated with uncoating ATPase in the presence of an ATP-depleting cocktail, or if the incubation was performed at pH 6.6, a condition that stabilizes the clathrin coat (Fig. 7 b). These observations are consistent with the properties of the uncoating reaction described by Schlossman et al. (1984). Treatment with cytosol from A431 cells in the presence of ATP effected a similar decrease in the density of [125 I]transferrin-labeled vesicles (data not shown).

Confirmation that the ATPase removes the clathrin coat was provided by analysis of preparations from metabolically labeled cells. When these vesicles were treated with uncoating ATPase and then centrifuged, there was a clear redistribution of clathrin heavy chain from the pellet to the supernatant (Fig. 7 c, lane 3). No uncoating was observed when the reaction was performed at 4°C (Fig. 7 c, lane 1), or at 37°C in the presence of an ATP-depleting cocktail (Fig. 7 c, lane 2).

Coated Vesicle Fusion Is Efficient

To estimate the efficiency of fusion, it was first necessary to quantitate the proportion of latent [125 I]transferrin present within sealed vesicles, and not exposed when vesicles became damaged upon isolation or during storage. This value was determined as the relative amount of [125 I]transferrin that was protected from immunoprecipitation by antitransferrin antibody in the absence of detergent. The result of a typical latency assay is shown in Table I. When fractions were incubated with antiserum in the presence of detergent, 76% of the radioactivity could be isolated on *Staphylococcus aureus* cells. In contrast, when the incubation was performed without detergent, only 11% of the radioactivity was subsequently bound to *Staphylococcus aureus* cells. This data gave a latency value of 86%. Inclusion of an excess of unlabeled transferrin prevented immunoprecipitation with or without detergent. As a control, free transferrin gave a latency value of 1% (Table I). Hence, the majority of the [125 I]transferrin in the coated vesicle preparation was contained within sealed vesicles.

The observed efficiency of fusion was dependent upon the amount of acceptor membranes in the incubation. As shown in Fig. 8, at a constant level of cytosol, the percentage of ATP-dependent immunoprecipitation of latent [125 I]transferrin rose with increasing concentration of acceptor membranes, the peak of activity being 43%. Virtually no precipitation of [125 I]transferrin was observed when an ATP-depleting cocktail was included, even when the highest level of acceptor membranes was used.

Coated Vesicle Fusion Is Specific

Fusion of coated vesicles showed similar requirements as fusion between other endocytic vesicles, demonstrating that it was not a nonspecific consequence of vesicle damage arising from the isolation. We have previously shown that fusion between endocytic vesicles requires cytosol and is sensitive to the alkylating agent, NEM (Woodman and Warren, 1988). We examined the characteristics of coated vesicle fusion with crude acceptor membranes (Table II). Fusion was absolutely dependent upon a cytosol fraction. In addition, fusion was sensitive to NEM. Prior incubation of cytosol and acceptor membranes with NEM, followed by quenching with DTT, reduced the efficiency of fusion by 89%. The effect of NEM was substantially reduced by including DTT in the incubation. Similar results were obtained when crude donor and acceptor membranes were mixed (Table II), showing that purification had not altered the properties of fusion. Cytosol-dependent fusion was almost totally abolished by pretreatment of cytosol and acceptor membranes with NEM. Again, NEM was ineffective when incubated together with DTT.

Discussion

We have described the isolation of coated endocytic vesicles

Table II. Properties of Endocytic Fusion

Treatment		Immunoprecipitation % of control
Cytosol	Acceptor membranes	
Donor coated vesicles		
No treatment	No treatment	100
Cytosol absent	No treatment	2
NEM	NEM	11
NEM/DTT	NEM/DTT	79
Donor crude membranes		
No treatment	No treatment	100
Cytosol absent	No treatment	5
NEM	NEM	7
NEM/DTT	NEM/DTT	111

[125 I]Transferrin-labeled coated vesicles were incubated with an ATP-regenerating cocktail, cytosol (final concentration 2 mg/ml when present), and acceptor membranes (50 μ g). Cytosol and acceptor membranes were pretreated as indicated. (NEM) 3 mM NEM at 4°C for 30 min, then quenched with 3 mM DTT; (NEM/DTT) 3 mM NEM, together with 3 mM DTT, at 4°C for 30 min. Results are expressed as immunoprecipitation relative to the untreated control (7,500 counts). Values are averages of duplicate determinations. Individual samples differed by no more than 7% from the average. Crude donor membranes (1 μ g) were incubated with acceptor membranes (50 μ g), ATP, and cytosol as above. Results are expressed as immunoprecipitation relative to the untreated control (2,740 counts). Values are the mean of triplicate determinations. Individual samples differed from the mean by no more than 13%.

from A431 cells containing internalized [¹²⁵I]transferrin, by centrifugation on 2H₂O and 2H₂O/Ficoll density gradients. [¹²⁵I]transferrin isolated from the final density gradient is contained within clathrin-coated vesicles, confirmed by the following criteria: (a) the peak of [¹²⁵I]transferrin at high density is present only in preparations from cells containing internalized transferrin, and represents the first compartment labeled upon internalization; (b) virtually all the [¹²⁵I]transferrin within this peak is inside sealed vesicles; (c) [¹²⁵I]transferrin sediments to the same position on the gradient as clathrin-coated vesicles, determined both by SDS-PAGE analysis of preparations from metabolically labeled cells and by Western blotting using antiserum against clathrin light chains; (d) electron micrographs of peak fractions reveal structures typical of clathrin-coated vesicles; and (e) the density of [¹²⁵I]transferrin-labeled vesicles is altered by Tris-HCl and uncoating ATPase in a manner consistent with the actions of these reagents on clathrin coats.

Furthermore, we have demonstrated that the isolated vesicles are active transport intermediates, on the basis of their ability to fuse with other endocytic compartments. Fusion of labeled vesicles is efficient, almost 50% of latent [¹²⁵I]transferrin being immunoprecipitated in an ATP-dependent fashion. At the moment we cannot be sure whether this value represents the proportion of coated vesicles that are active, or is the maximum signal obtainable in the fusion assay due to a limit of antibody in the acceptor preparation. The reaction also retains the features of fusion between unpurified endocytic membranes, namely the dependence upon added cytosol and energy, and sensitivity to NEM. This rules out the possibility that vesicles are damaged during the isolation in such a way as to allow nonspecific fusion with other membranes. Further evidence that these vesicles are bona fide transport intermediates has been provided by examining their ability to prepare for fusion in the manner expected. Coated endocytic vesicles must have clathrin removed, at least in part, to allow the apposition of membranes participating in the fusion event. The most likely agent for this is an uncoating ATPase (Schlossman et al., 1984), which has been shown to release clathrin from brain coated vesicles *in vitro*. We have shown that the [¹²⁵I]transferrin-labeled vesicles are uncoated by this enzyme. Indeed, preliminary results indicate that the uncoated intermediate can be reisolated using density gradients and retains the ability to fuse (Woodman, P. G., and G. Warren, unpublished observations). Not only does enzymic uncoating emphasize the functional integrity of the vesicles, it also demonstrates that all of the [¹²⁵I]transferrin-labeled vesicles are clathrin-coated.

This is the first time an active transport intermediate has been isolated. Other systems for studying fusion between endocytic vesicles rely on the use of postnuclear supernatants (Braell, 1987) or relatively crude membrane preparations (Mayorga et al., 1989). Use has been made of endocytosed markers to immunoisolate fusion competent endocytic compartments (Gruenberg and Howell, 1986). However, the success of this approach in isolating a single, defined population of endocytic vesicles is limited by the kinetics of internalization, since at a given time the endocytosed marker will not be confined to any one compartment. Instead, we have elected to isolate a transport intermediate according to its physical properties. A similar approach was taken by Malhotra et al. (1989) to isolate a novel class of vesicles medi-

ating the transfer of newly synthesized proteins through the Golgi stack. However, because they contained no labeled marker protein, and since isolation was dependent upon inhibiting the fusion of these vesicles with their target membranes, these preparations could not be used as active transport intermediates. Similarly, exocytic transport vesicles derived from the *trans*-Golgi network have been isolated (Bennett et al., 1988; Wandinger-Ness et al., 1990), but these have not so far been demonstrated to be functional intermediates. Functional vesicular intermediates that mediate the transport of proteins from the ER to the Golgi complex have been isolated from permeabilized yeast cells (Groesch et al., 1990), but they have still to be characterized biochemically.

The successful isolation of an active transport intermediate should enable us to understand in greater detail the molecular basis for vesicle recognition and fusion. Although coated vesicle preparations are likely to contain Golgi-derived clathrin-coated vesicles and recycling vesicles in addition to endocytic vesicles, the use of a functional assay will select for those proteins involved in endocytic fusion. For example, it should be possible to look for the association of cytosolic proteins with coated vesicles under conditions that block fusion, and thereby isolate components of a putative endocytic fusion intermediate. Confirmation that these proteins are required for fusion would follow from experiments to reconstitute activity.

In addition, we hope to extend our studies and isolate acceptor coated vesicles so as to test the two models of endocytosis reviewed in the introduction. If coated vesicles fuse with each other to form endosomes, then fusion would be expected to occur between donor and acceptor coated vesicles. If, however, coated vesicles deliver their contents to a stable endosome, then one would not expect coated vesicles to be able to fuse directly with each other. Reaction product would be observed only in the presence of unlabeled endosomes, which would act as a "sink" for the coated vesicle contents.

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