The Sorting and Segregation Mechanism of the Endocytic Pathway Is Functional in a Cell-free System*

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A cell-free system which reconstitutes early stages of receptor-mediated endocytosis has been developed, based on detection of the association between avidin-β-galactosidase (AvβGal) and biotin-transferrin (B-Tf). Initially, AvβGal (a fluid-phase marker) and B-Tf (receptor-bound) are internalized and delivered to a common endosomal compartment in vivo and in vitro. Subsequently, these two probes enter divergent intracellular pathways: AvβGal is sorted from the endosome and directed for delivery to lysosomes, whereas B-Tf is segregated away from the fluid-phase marker, remaining bound to the transferrin receptor for return to the cell surface. Using the avidin-biotin association reaction to monitor the co-localization of these two probes, we have been able to reconstruct this sorting and segregation process in a cell-free system. The in vitro reaction is time-, temperature-, and ATP-dependent, and is not affected by NH4Cl; cell-free segregation of the two probes is also sensitive to N-ethylmaleimide. As these characteristics are also properties of in vitro endocytic vesicle fusion, it is likely that the latter event is a prerequisite for the sorting and segregation process. Both the in vivo and in vitro sorting of AvβGal and B-Tf to their respective and distinct destinations can be followed by subcellular fractionation on Percoll gradients. Our observations provide the first evidence that the cellular mechanism to identify, sort, and sequester endocytosed material can be reconstructed in a cell-free system.

The internalization of receptor-associated ligands from the surface of cells has been shown by a variety of physiological, morphological, and biochemical criteria to proceed via a process known as endocytosis (1–3). The cell's endocytic pathway is incorporated into a complex pattern of membrane vesicle traffic which permits the continuous flow of membrane constituents between the cell surface and various cellular compartments. A complete biochemical analysis of the protein machinery and factors governing this process requires that in vitro systems be developed which faithfully reconstitute various stages of the endocytic pathway. One of the earliest events in endocytosis is the fusion between vesicles containing the internalized ligand-receptor complex and an intracellular compartment known as the endosome or CURL (Compartment for Uncoupling of Receptor and Ligand) (4–6). This early fusion event has been reconstituted in vitro with fluid phase markers, as well as with probes internalized by receptor-mediated endocytosis (7–10). We present results here which characterize a novel cell-free system which utilizes both a fluid phase probe (avidin coupled to β-galactosidase, AvβGal) and a modified ligand of the transferrin receptor (biotin-transferrin, B-Tf) to study the fusion of vesicles derived from K562 cells. The fusion of vesicles in vitro and in vivo can be monitored through the association reaction between the internalized avidin- and biotin-linked probes and provides functional characterization of the early endocytic fusion event (8).

Subsequent events in the pathway of receptor-mediated endocytosis result, in part, from the acidic environment (pH 6) provided by the endosome, since dissociation of ligands from receptors typically occurs below neutral pH (5, 6). Endosomal sorting and separation of the dissociated ligands and receptors has been documented by morphological evidence (6, 11). The endosome, or CURL, contains both vesicular and tubular regions of membrane. The vesicular domains are eventually matured to multi-vesicular or secondary endosomes (12), containing constituents of the fluid phase with very few receptors. The contents of this compartment will eventually be found in lysosomes, resulting in the degradation of these constituents (ligands such as low density lipoprotein or macromolecules internalized by simple pinocytosis, for example) by lysosomal enzymes. Receptors, on the other hand, appear to accumulate in tubular regions of the endosome (after release of ligand) (11, 12). A unique feature of the receptor-mediated endocytosis of diferric transferrin is that, after delivery to endosomes, the acidic environment causes iron to be released from the serum protein; however, apotransferrin does not dissociate at low pH and therefore remains bound to its receptor (13–17). Thus, apotransferrin and the transferrin receptor are subsequently recycled from the tubular endosomal domains via vesicle traffic directly to the cell surface, or indirectly, by first passing through membranes of the trans-Golgi network. Exposed to the neutral pH of the cell's external milieu, apotransferrin can now dissociate from the transferrin receptor and, after re-loading, may be recycled for further rounds of iron uptake by the cell (17).

The passage of receptors and ligands through the endosomes involves the sorting and correct targeting of internal-* This work was supported in part by National Institutes of Health Grant GM34636. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1793 solely to indicate this fact.
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§ The abbreviations used are: AvβGal, avidin-β-galactosidase; B-Tf, biotin-transferrin; NEM, N-ethylmaleimide; FNS, post-nuclear supernatant; PBS, phosphate-buffered saline; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EGTA, ethylenbis(oxymethylene)tetraacetic acid; ELISA, enzyme-linked immunosorbent assay.
zized macromolecules to their appropriate destinations. Reconstitution of the endosomal sorting process in a cell-free format is a prerequisite step in dissecting the biochemical mechanism of this phenomenon. The cell-free system described (Fig. 1) here is designed to detect this sorting process, because it provides the means to detect not only circumstances when the endocytic probes reside in the same compartment (via the association between AvPGal and B-Tf), but also situations where they are not associated (via labels intrinsic to the probes themselves). Other advantages unique to this detection strategy are: 1) the association between avidin and biotin is insensitive to alterations in pH, ionic strength, and other environmental conditions which may be encountered during the course of endocytosis; and 2) transferrin has the singular property of recycling with its receptor from endosomes to the plasma membrane, while the fluid phase marker (avidin-β-galactosidase) is transported from endosomes to late endosomal/lysosomal compartments. The latter point provides the rationale through which the sorting process may be followed in a reconstituted cell-free system: segregation of either one of the probes will prevent them from associating in a common domain, as is detected subsequent to the fusion of endocytic vesicles, while the individual probes themselves should be traceable to distinct compartments after segregation has occurred. Manipulating this situation to our advantage, we are able to follow the independent endocytic pathways of avidin-β-galactosidase and biotin-transferrin both in vivo and in vitro, as documented by the results presented here. Our findings provide the first evidence of the reconstitution of the endosomal sorting mechanism in a cell-free system. This accomplishment presents the basis through which the biochemical mechanism of sorting and sequestration, as well as elements which regulate this stage of the endocytic process, may be explored.

**EXPERIMENTAL PROCEDURES**

**Materials and Cell Culture—Avidin-β-galactosidase conjugate, biotin-insulin, goat anti-rabbit IgG (IgG fraction), and apotransferrin were purchased from Sigma. Rabbit anti-human transferrin (IgG fraction) was obtained from Boehringer Mannheim. Human K562 cells were obtained from American Type Culture Collection; these cells, as well as Chinese hamster ovary cells, were maintained in a minimal essential medium containing 10 or 75% fetal calf serum (Gibco) respectively. K562 cells were propagated in suspension to 8 - 10 x 10⁷ cells/ml, whereupon fresh medium was added at 5 - 10 x 10⁴ cells/ml. CHO cells were passaged with trypsin as previously (8).

**Preparation and Characterization of Biotinylated Transferrin—** Apotransferrin was saturated with iron by incubation with a 10 mg/ml solution in PBS (0.14 M NaCl, 0.01 M sodium phosphate, pH 7.4) containing 10 mM NaHCO₃ and 0.1 mg/ml ferric ammonium citrate for 60 min at ambient temperature (18). Protein was isolated by gel filtration over Bio-Gel P6. Greater than 95% iron saturation was routinely obtained, as assessed by light absorption at 460 nm. Biotinylation of dipheric transferrin was accomplished by reacting protein with a 50-fold excess of biotinamidocaproate N-hydroxysuccinimide ester (Sigma). Biotinylated transferrin (B-Tf) was separated from unreacted reagent by gel filtration. The extent of modification was determined by the dye-binding assay described by Gerwin et al. (19) using 4-hydroxyazobenzene-2'-carboxylic acid (20). A stoichiometry of 5-10 mol of biotin/mol of Tf was routinely achieved.

The binding and internalization of ¹²⁵I-labeled B-Tf to K562 cells was measured in order to verify that the modified ligand would undergo receptor-mediated endocytosis. Both native and biotinylated dipheric Tf were iodinated by the chloramine-T method (18), using carrier-free ¹²⁵I (Du Pont-New England Nuclear); radioactivity was measured using a Beckman 4000 counter. Cells (1-5 x 10⁶ Cells) were washed three times in PBS (10 mM NaCl, 2.7 mM KCl, 10 mM sodium phosphate, pH 7.4) and resuspended in 250 µl washing buffer containing 0.5% BSA, 0.1% Triton X-100, 100 mM sodium chloride, 20 mM HEPES, pH 7.4, 20 µM EDTA. In order to disrupt cells, aliquots were rapidly frozen in liquid nitrogen and stored at -80°C until use, whereupon the aliquots were gently thawed in a 25°C water bath, followed by vigorous vortexing and rapid cooling on ice. This freeze-thaw technique results in nearly 100% breakage of K562 cells, as determined by trypsin blue exclusion. Alternatively, homogenization was performed on ice in a stainless steel homogenizer, until at least 80 - 90% of the cells were broken (8). Postnuclear supernatant preparations (PNS) were prepared by centrifugation at 800 x g for 5 min at 4°C. Aliquots of PNS stored at -80°C were found to retain fusion, sorting, and segregation activities. Cytosol fractions were prepared from PNS fractions by centrifugation at 100,000 x g for 60 min at 4°C.

**Assay Conditions to Monitor in Vitro Endocytic Vesicle Fusion—** PNS preparations containing internalized AvPGal and B-Tf were mixed on ice (final concentration of 2-4 mg/ml protein) in a mixture including 1 mM MgATP, 50 µg/ml creatine kinase, 8 mM phosphocreatine, 10 µg/ml biotin-insulin, 1 mM dithiothreitol. A third PNS fraction, containing internalized anti-transferrin antibody, was additionally present in reactions measuring multiple fusion events. When it was desirable to delete ATP, reactions were performed without the added ATP, creatine kinase, and phosphatebuffer, but with the addition of 5 mM 2-deoxyglucose and 10 units/ml hexokinase. Treatment of PNS fractions, or cytosol, with 1.5 mM N-ethylmaleimide was performed on ice for 10 min, followed by the addition of 1 mM dithiothreitol, prior to their addition to the reaction mixture. It should be noted that the addition of biotin-insulin in all of these reactions serves to scavenge any avidin-linked probe which may be present due to lysed vesicles. After incubation of the reaction mixtures at 37°C, typically for 30 min, vesicle fusion was quenched by a 1:10 dilution of lysis buffer (1% Triton X-100, 1% sodium dodecyl sulfate, 50 µg/ml biotin-insulin). Samples were brought to a final volume of 250 µl with 0.05% Triton X-100, 50 mM NaCl, 10 mM Tris, pH 7.4, 1 mg/ml heparin.

Fusion between endocytic vesicles was monitored by an ELISA procedure (8). Either goat anti-rabbit antibody (diluted 1:100) or rabbit anti-transferrin antibody (diluted 1:15,000) was added to the reaction mixture. Wells coated with the appropriate antibodies were then washed three times with PBS and incubated 30-60 min in wash buffer (1% Triton X-100, 0.1% sodium dodecyl sulfate, 50 mM NaCl, 10 mM Tris, pH 7.4, 1 mM EDTA) followed by the addition of PNS preparations in PBS (200 µl) were incubated in the prepared wells for 3 h. Wells were briefly washed three times and then incubated 15-30 min in wash buffer, followed by three washes with PBS. β-Galactosidase activity associated with the complex captured in the wells was assayed by incubation with 100 µl of reaction mixture containing 100 µl of lysis buffer (100 µM 4-methylumbelliferyl β-galactoside (0.3 mM) in 0.1 mM NaCl, 25 mM Tris, pH 7.4, 1 mM MgCl₂, 12 mM β-mercaptoethanol. Samples (200 µl) were removed and mixed with 1 ml of 133 mM glycine, 85 mM Na₂CO₃, pH 10.7. Fluorescence measurements (366 nm excitation, 450 nm emission) were performed and the results are expressed as the fraction of total counts/min resistant to enzyme digestion relative to total counts/min in the absence of enzyme.

The dissociation constant for unmodified Tf (Kn = 6.6 nM) was routinely achieved.

**Note:** The fraction of ¹²⁵I-labeled Tf and B-Tf internalized upon incubation of cells at 37°C was assessed as the fraction of counts/min resistant to extracellular digestion with trypsin (2.5 mg/ml) for 10 min on ice.
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**In Vitro Association of Endocytic Probes—**K562 cells were allowed to internalize AvpGal as described above, and then washed once with PBS containing 0.5 mg/ml biotin-insulin to block any avidin conjugate nonspecifically associated with the cell surface. This treatment was followed by two more washes in PBS, after which cells were resuspended in uptake buffer in the presence of B-Tf. B-Tf was bound to the cell surface upon incubation for 60 min at 4 °C. Cells were then warmed to 37 °C for 10 min, such that the second probe was endocytosed; internalization was quenched by the addition of 5 volumes of ice-cold PBS. Samples were washed once with PBS, suspended in breaking buffer, and lysed by the freeze-thaw method. PNS fractions were prepared and a 1:10 dilution of 10% Triton X-100, 1% sodium deoxycholate, 50 mg/ml biotin-insulin, was added to lysis vesicles containing the internalization probes. Samples were diluted to a final volume of 150 µl with 0.03% Triton X-100, 50 mM NaCl, 10 mM Tris, pH 7.4, 1 mg/ml b-merin. After clarification by microcentrifugation, b-galactosidase activity associated with the AvpGal-B-Tf complex was measured by the ELISA assay described above.

**Subcellular Fractionation on Percoll Gradients—**Subcellular fractionation was performed using self-forming Percoll gradients. PNS preparations for these gradients were isolated from cells that had internalized AvpGal and B-Tf in vivo, as described above. Alternatively, PNS fractions reacted in vitro under the conditions promoting endocytic vesicle fusion (described above) were employed. The buffer used in sample preparation for either type of experiment was triethanolamine-sucrose: 0.25 M triethanolamine, 1 mM EDTA, adjusted to pH 7.4. Post-nuclear supernatant preparations obtained in this buffer were never frozen before use for gradient experiments: the integrity of lysosomes, unlike endosomes, is not maintained during freezing and thawing. Samples were made 27% with isotonic Percoll in triethanolamine-sucrose; 9.5 ml of this mixture was underlayerd with 0.5 ml of 60% sucrose and centrifuged in a Beckman Ti-50 rotor at 17,000 rpm for 2 h (4 °C). Fractions (257 µl) of the gradients were collected from just below the interface of the sucrose cushion. Gradient fractions were assayed for counts/min of 51I-labeled B-Tf, and for b-galactosidase activity, using the assay reagent employed for the ELISA protocol. Control experiments were also performed to analyze the activity profiles of the following marker enzymes: lysosomal b-hexosaminidase, 51I-labeled placenta membrane, and galactosyl transferase (GolgI). These results were found to be consistent with data previously published by other groups using similar fractionation procedures (22-25); assays for marker enzymes were performed as described in these references.

**RESULTS AND DISCUSSION**

**Fusion Occurs in Vitro between Endocytic Vesicles Containing Fluid Phase and Receptor-associated Probes—**The endocytic vesicle fusion assay described in this paper detects the association between a fluid phase marker, avidin linked b-galactosidase (AvpGal), and a receptor-associated ligand, biotin-transferrin (B-Tf), that occurs concomitant to the fusion of vesicles containing these two probes. Previous cell-free systems for endocytic vesicle fusion (7-10) have used vesicles containing probes taken up either by fluid phase, or receptor-mediated endocytosis, although to date no clear-cut differences in the characteristics of the fusion of vesicles derived from these alternative modes of uptake have been noted (10). As a fluid phase endocytic marker, AvpGal will populate all endocytic vesicles, receptor-bearing or not, since each will contain entrapped fluid from the external milieu. Therefore, any specificity of vesicle interaction during in vitro fusion events between vesicles containing AvpGal and B-Tf should stem from the transferrin receptor-bearing vesicle component alone. PNS preparations with endocytic vesicles containing the appropriate probes are co-incubated under appropriate conditions to allow vesicle fusion (Fig. 1, top), detected via AvpGal-B-Tf complex formation by a straightforward ELISA procedure (8). The time course of the in vitro fusion reaction between vesicles harboring endocytosed AvpGal and those containing internalized B-Tf is shown in Fig. 2. The kinetics of the fusion reaction are similar to what has been reported previously (8); under these conditions of assay, fusion proceeds linearly for nearly 80 min and then plateaus, exhibiting a somewhat biphasic time course. At 4 °C, vesicle fusion is not detected (Fig. 2, open circles). Fractions prepared from cells exposed to B-Tf at 4 °C, a temperature at which endocytic uptake does not occur, produced no fusion signal in the cell-free assay at 37 °C, even though saturable binding of B-Tf to cell surface transferrin receptors does occur at 4 °C. Thus, the fusion signal was developed exclusively from B-Tf internalized by endocytic vesicles.

Vesicle fusion in this system has an absolute requirement for ATP: in the absence of ATP and in the presence of an ATP-depleting system, signals comparable to background levels are obtained. Treatment of PNS preparations (which contain endocytic vesicles) with 1.5 mM NEM prevents vesicle fusion by immediate association reaction resulting in AvpGal-B-Tf complex formation, which may be assayed on wells coated with anti-Tf antibodies by the ELISA procedure described under "Experimental Procedures." Alternatively, multiple vesicle fusion may be followed by incubation of PNS containing an additional probe, anti-Tf antibodies. The triple complex (AvpGal-B-Tf-anti-Tf) formed by inactivation of all three probes in a common compartment can be detected on wells coated with either anti-Tf antibodies or anti IgG antibodies. Sorting and segregation of the probes, which occur subsequent to the fusion of vesicles harboring them, may be followed by the reduction in fusion signal due to loss of complex formation relative to control, as well as by fractionation of reaction products on Percoll gradients.

**Fig. 1.** Cell-free assays for endocytic vesicle fusion and the sorting and segregation of ligands. PNS fractions harboring vesicles containing appropriate probes are co-incubated under conditions which support vesicle fusion. This event is detected by the avidin-biotin association reaction resulting in AvpGal-B-Tf complex formation, which may be assayed on wells coated with anti-Tf antibodies by the ELISA procedure described under "Experimental Procedures." Alternatively, multiple vesicle fusion may be followed by including PNS containing an additional probe, anti-Tf antibodies. The triple complex (AvpGal-B-Tf-anti-Tf) formed by inactivation of all three probes in a common compartment can be detected on wells coated with either anti-Tf antibodies or anti IgG antibodies. Sorting and segregation of the probes, which occur subsequent to the fusion of vesicles harboring them, may be followed by the reduction in fusion signal due to loss of complex formation relative to control, as well as by fractionation of reaction products on Percoll gradients.

**Fig. 2.** Fusion reactions are performed in vitro in the presence of ATP. Fractions of the gradient prepared from cells exposed to B-Tf at 4 °C (open circles) were assayed for fusion products in cell-free assays at 37 °C, even though saturable binding of B-Tf to cell surface transferrin receptors does occur at 4 °C. Thus, the fusion signal was developed exclusively from B-Tf internalized by endocytic vesicles.
The addition of 50 contained in cytosol which is sensitive to the alkylating agent. Thus, ‘the fusion event requires cytosol (maximum signal develops at 2 mg/ml protein) and, more specifically, a factor pH gradients and, therefore, our results support the notion that acidification is not required for vesicle fusion to occur.

Finally, the local anesthetics tetracaine and dibucaine, which disrupt a wide variety of membrane-dependent phenomena, are also found to block the fusion between endocytic vesicles at millimolar levels. All of these results for the AvPGal-B-Tf-based endocytic vesicle fusion assay are consistent with observations made for other cell free systems developed to monitor endocytic vehicle fusion (7–10).

Multiple Vesicles Can Fuse into a Common Compartment in Vitro—The in vitro fusion assay described above detects the interaction between vesicles containing only two probes, and can therefore only signal the fact that vesicle fusion of some type has occurred. To determine whether multiple vesicles can fuse together into a common compartment, a minimal requirement is that vesicles containing a third and independent probe be added to the assay system. The latter is provided by rabbit anti-Tf antibodies which recognize B-Tf. Woodward and Warren (10) have previously used endocytosed anti-Tf and Tf as probes for detecting endocytic vesicle fusion. A complex containing all three probes (AvPGal-B-Tf.anti-Tf) was formed by the fusion of multiple vesicles in vitro, are detected on anti-IgG coated wells (Fig. 3, shaded bars). Significantly, this signal is present even in the presence of exogenously added Tf. This rules out the possibility that the triple complexes are formed artifically by the association of AvPGal-B-Tf complexes with anti-Tf after detergent addition, since the exogenous Tf will scavenge any released anti-Tf. The effectiveness of adding Tf as a scavenger is clearly shown by its ability to prevent any signal at all from developing on wells coated with anti-Tf antibody (Fig. 3, non-shaded bars), moreover, omitting the Tf causes most of the complexed AvPGal to be scored on both types of wells. The ELISA fusion signal obtained from anti-IgG wells in the presence of Tf is found to be 40–50% of signal recovered on anti-Tf wells in the absence of exogenous Tf. This result implies that the fraction of fusion leading to triple complex (AvPGal-B-Tf-anti-Tf) must be quite high, compared to that resulting solely in double complex (AvPGal-B-Tf).

The fact that 40–50% of the total signal may result from fusion between multiple vesicles also implies that the cell-free fusion reaction itself is very efficient. The data shown in Fig. 3 suggest that the overall participation of endocytic vesicles in fusion events is on the order of 40–50%, assuming that the vesicle, having undergone a single fusion event, is then equally likely to undergo additional fusion events. Two requirements for this assumption to be valid are that there be no mechanistic distinction for fusion between vesicles participating in single versus multiple fusion events, and that the two types of wells provide an accurate assessment of the amount of each complex formed. In line with the first assumption, the multiple fusion reaction exhibits all of the characteristics of the single fusion process: it requires cytosol, it is sensitive to NEM, and it is dependent on the presence of ATP (data not shown).
shown). The second assumption may well be an oversimplification, since different antisera, with different titers are used to detect the double and triple complexes, and therefore the signal developed may not represent a direct proportionality between the amount of complex which bind to the different wells. However, of the two types of wells used in these assays, the anti-IgG coated wells should be more restricted in capacity, due to the lower titer of the anti-serum used to coat the wells. Thus, it is possible that the efficiency of the in vitro fusion reaction may even be greater than 40–50%. A high efficiency in co-localization of endocytosed AvpGal and B-Tf will, of course, be highly desirable in any investigation of the subsequent segregation of the co-localized probes.

In vitro, early endocytic vesicles fuse into a common compartment, the early endosome, which seems to have a composition distinct from that of the plasma membrane (26). The fact that multiple fusion events are detected in vitro implies that many endocytic vesicles can combine their contents in a common compartment during this process. Moreover, the fact that a substantial fraction (40–50%) of signal derives from multiple fusion events suggests that this common compartment may be quite large, and may possibly correspond to an endosomal body. Further work is required to define the precise composition of the in vitro compartment within which the endocytic markers are co-localized.

Co-localization and Segregation of AvpGal and B-Tf in Vivo—Although the probes initially reside in a common endosomal compartment, the fluid phase transit of AvpGal via vesicular traffic to the lysosome will direct this marker into compartments separate from those utilized by the receptor-mediated transit of B-Tf to the cell surface. Shown in Fig. 4 are results of uptake pulse-chase experiments performed in vivo, which provide experimental support for the segregation of these two probes from a common endocytic compartment in K562 cells. An initial 60-min pulse of AvpGal is internalized by K562 cells at 20 °C, after which the cells are washed to remove the probe and incubated at 37 °C for the times indicated. The cells are then allowed to take up B-Tf for a 10-min period at 37 °C, and the amount of AvpGal-B-Tf complex formed during co-localization of the two probes in intracellular compartments is subsequently determined. Initially, AvpGal is found to be accessible to internalized B-Tf (Fig. 4, top left panel). This result defines a compartment populated early in the uptake pulse-chase time period that is capable of fusing with vesicles containing the second probe, B-Tf. Within 15 min of this time point, however, AvpGal becomes almost completely inaccessible to internalized B-Tf. This is in contrast to results obtained when the first pulse of AvpGal is followed by incubation at 20 °C instead of 37 °C. The two probes are localized to a common compartment throughout the 30-min period of the uptake pulse-chase, with only a modest decline in avidin-biotin association. Other groups have previously demonstrated that at 10–20 °C, endocytosed material accumulates in endosomal compartments, failing to reach the lysosome (27, 28). The results presented in Fig. 4 are also consistent with these observations.

The decrease of avidin-biotin association signal could be due to the loss or degradation of AvpGal from the cell at 37 °C. As a fluid phase marker, AvpGal is directed to the lysosomal compartment, where, upon encountering hydrolases and proteases, the enzymatic activity would be destroyed (29). Therefore, the amount of total AvpGal-associated β-galactosidase activity in the cells was assessed for each time-point (Fig. 4, bottom right panel). The loss of internalized activity paralleled the loss of coupling with B-Tf. At 20 °C, total AvpGal β-galactosidase activity was stable throughout the time course, the expected result if the probe does not reach the lysosome at this temperature.

In order to determine whether or not the disappearance of AvpGal enzyme activity from the cells could account for the reduction in avidin-biotin association, we looked at the effects of leupeptin and NH4Cl on the uptake pulse-chase time course. As a protease inhibitor, leupeptin can slow or prevent the lysosomal degradation of proteins. NH4Cl dissipates pH gradients and, consequently, will prevent the acidification which activates lysosomal hydrolases. As demonstrated in the bottom right panel of Fig. 4, the presence of either leupeptin or NH4Cl dramatically reduces the loss of total internalized β-galactosidase activity associated with the fluid phase probe. More important, however, is the observation that the addition of leupeptin has no effect on the time course of AvpGal-B-Tf association (Fig. 4, bottom left panel). Similarly, the top right panel of Fig. 4 shows that the presence of NH4Cl does not disrupt the time course of coupling. This latter observation is consistent with results discussed earlier, which indicate that weak bases, including NH4Cl, do not interfere with the fusion of endocytic vesicles. Thus, it is unlikely that NH4Cl, in addition to inhibiting the loss of activity, is also concurrently preventing association of the probes by disrupting endocytic vesicle fusion.

The combined results presented in Fig. 4 support the conclusion that the time-dependent reduction in the ability of AvpGal to associate with the second pulse of B-Tf is due to the segregation or sequestration of the fluid phase marker in a compartment which can no longer fuse with endocytic vesicles or to a region of the endosome which is inaccessible to the receptor ligand. Qualitatively similar results are obtained if B-Tf is the first probe internalized, and followed in comparable pulse-chase experiments with a second pulse of AvpGal (data not shown). This would argue against the time-dependent inactivation or loss of the endocytic marker due to some undefined idiosyncratic effect specific to the AvpGal probe.

Endocytic Probes Are Sequestered during Incubation in Vitro—As explained above, AvpGal and B-Tf begin their endocytic journey in vivo in the same compartments, but rapidly are sorted to distinct pathways. The cell-free endocytic vesicle fusion system detects the insertion of these two probes into a common compartment, which may well be endosomal in origin. Moreover, the apparent high efficiency of this cell-free reaction argues that, at least initially, a substantial proportion of these probes can come to reside in the same compartment(s) via vesicle fusion in vitro (Fig. 3). This compartment, if it indeed represents an in vitro counterpart to the cellular endosome, might also be able to perform processes akin to endosomal segregation and sorting. Therefore, the extent of AvpGal-B-Tf association was assayed, under conditions similar to the in vivo pulse-chase experiments described above, in order to determine whether such segregation might be occurring in vitro. PNS was prepared from cells which had internalized either AvpGal or B-Tf. The PNS preparations were independently incubated at 37 °C in the presence of ATP and an ATP-regenerating system for time periods indicated. These samples were then mixed with unincubated PNS containing the complementary probe, and assayed for fusion activity. As shown in Fig. 5, both PNS containing AvpGal, and PNS containing B-Tf, exhibited a rapid, though incomplete, loss in fusion activity when treated in this manner, occurring with a time course comparable to that demonstrated for the segregation of probes in vivo (Fig. 4). The observed loss in fusion activity would be consistent with the idea that
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Fig. 4. In vivo pulse shade of AvβGal monitored by the association reaction with B-Tf. K562 cells were allowed to endocytose AvβGal for 60 min at 20 °C, with subsequent washing in PNS containing 0.5 mg/ml biotin-IgG to block any external avidin sites. Cells were then incubated at the indicated times at 37 °C in order to chase the fluid phase marker into intracellular compartments of the endocytic pathway. After washing once again with 0.5 mg/ml biotin-IgG, cells were exposed to B-Tf for 60 min at 4 °C in order to bind the ligand, after which time uptake of the second probe was allowed for 10 min at 37 °C. Co-localization of the probes may be detected by the avidin-biotin association, which is monitored by the ELISA procedure detailed under “Experimental Procedures”; shown is the averaged values of duplicate samples at each time point. Top left panel, samples were treated as described above, except that the temperature of the pulse chase incubations was either 20 °C (○) or 37 °C (●). Bottom left panel, time course of the pulse chase at 37 °C in the presence of 50 μg/ml leupeptin (▲). The protease inhibitor was also internalized for 10 min at 37 °C prior to exposure of the cells to AvβGal, and was present during the internalization of B-Tf as well. Top right panel, time course of the pulse chase at 37 °C in the presence of 20 mM NH₄Cl (■), which was included in all buffers during incubations and wash steps. Bottom right panel, control experiments performed on samples obtained under conditions represented in the other three panels in order to determine the total internal AvβGal activity during the pulse-chase time course: 37 °C (○), 20 °C (●), 50 μg/ml leupeptin (▲), and 20 mM NH₄Cl (■).

Fig. 5. In vitro pulse chase monitored by cell-free fusion. PNS fractions were prepared from cells which had internalized either AvβGal (○) or B-Tf (■) and were incubated at 37 °C for the indicated time periods in the presence of ATP and ATP-regenerating system. Samples were then assayed for fusion activity as described for Fig. 2. Data presented are averaged values from duplicate determinations at each time point. Segregation of the two probes is monitored as the loss in fusion activity under these incubation conditions.

Segregation of the two endocytic probes occurs during incubation under these conditions.

There was no further decline in fusion activity if both PNS preparations added to the assay had undergone preincubation at 37 °C prior to being mixed together (data not shown). This result, and the fact that only between 40 and 60% of the total initial fusion activity was observed to be lost in experiments of this type (Fig. 5) suggests that there is a limitation to the extent of sequestration of the endocytic probes possible under these in vitro conditions. This partial loss of activity may be a consequence of the lack of a continual flow of membrane traffic in the reconstituted system: factors which mediate and regulate the appropriate sorting and sequestration of internalized material in vivo may not be maintained or replenished in the cell-free system as efficiently as in vivo.

Treatments that prevent endocytic vesicle fusion also prevent the temporal decline in the fusion competency of these vesicles (Table I). The decline in fusion competency in vitro was found to be ATP-dependent: more than 50% of fusion activity is lost in the presence of ATP and an ATP-regenerating system, in contrast to the only 10% decline in signal when PNS fractions are incubated in the presence of the ATP-depleting system. Treatment of PNS samples with the alkylating agent NEM completely abolishes the reduction in fusion activity observed in the absence of treatment. Furthermore, segregation activity is restored by the addition of untreated cytosol to the incubation mixture. This latter result indicates that: 1) the NEM effects on sequestration are reversible; 2) a NEM-sensitive cytosolic factor is necessary for segregation or sequestration to occur. Finally, addition of weak bases such as chloroquine or NH₄Cl in the incubation medium does not inhibit the cell-free sequestration process. This observation implies that the sorting and segregation of internalized material occurs independent of pH gradient.
The sequestration of the two endocytic probes was monitored as described in Fig. 5, except that both PNS preparations were subjected to treatment at 37 °C for 20 min under the experimental conditions noted. The degree of segregation is measured as the percentage of fusion activity lost when compared to activity measured for control samples kept at 4 °C for the 20 min period of incubation prior to assessment of fusion activity.

<table>
<thead>
<tr>
<th>Experimental condition</th>
<th>Activity lost (relative to control)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Reaction requirement for ATP:</td>
<td>%</td>
</tr>
<tr>
<td>ATP-regenerating system added</td>
<td>51.4</td>
</tr>
<tr>
<td>Creatine kinase + phosphocreatine</td>
<td>10.2</td>
</tr>
<tr>
<td>ATP-depleting system added</td>
<td>60.5</td>
</tr>
<tr>
<td>Hexokinase + 2-deoxyglucose</td>
<td>62.5</td>
</tr>
<tr>
<td>Treatment of PNS by 1.5 mM NEM:</td>
<td></td>
</tr>
<tr>
<td>Untreated</td>
<td>0</td>
</tr>
<tr>
<td>NEM-treated</td>
<td>0</td>
</tr>
<tr>
<td>NEM-treated + cytosol added</td>
<td>62.6</td>
</tr>
<tr>
<td>Effects of weak bases on reaction:</td>
<td></td>
</tr>
<tr>
<td>Untreated</td>
<td>53.7</td>
</tr>
<tr>
<td>50 μM chloroquine added</td>
<td>67.4</td>
</tr>
<tr>
<td>20 mM NH₄Cl added</td>
<td>62.6</td>
</tr>
</tbody>
</table>

Fig. 6. Temperature dependencies of in vitro fusion and segregation reactions. PNS samples containing AvpGal or B-Tf were mixed and assayed for fusion or segregation activity upon incubation at different temperatures (shown as degrees centigrade). Top panel, in vitro fusion assays were performed for 30 min at indicated temperatures resulting in formation of AvpGal-B-Tf complex which was assayed as described under “Experimental Procedures.” Activity is shown as fluorescence (arbitrary units) measured by ELISA procedure. Bottom panel, cell-free segregation in the PNS fractions was accomplished by a 20-min incubation at the indicated temperatures. Activity was assessed by the loss of AvpGal-B-Tf complex formation when fusion is permitted at 37 °C for 30 min. All reactions were carried out in the presence of ATP and an ATP-regenerating system.

which would be dissipated by weak bases such as chloroquine and NH₄Cl, and that this is true, at least for K562 cells, for both in vivo and in vitro processes. These results also support the idea that the reduction of AvpGal-B-Tf complex formation is not due to nonspecific loss of factors involved in the fusion reaction, or degradation of the probes in a lysosomal compartment.

The decline in fusion competency does not occur at temperatures too low for vesicle fusion to occur. The top panel of Fig. 6 shows in vitro fusion activity plotted as a function of temperature of incubation. For comparison, in vitro decline in fusion competency versus incubation temperature is presented in the bottom panel of Fig. 6. Both activities are minimal at temperatures at or below 20 °C. However, above this limit there is a marked increase in the level of both activities. The correlation between the temperature curves for both reactions lends support to the notion that the fusion reaction is a requisite event for the decline to occur. It should also be noted that the temperature dependence observed for the cell-free system reflects in vivo observations presented in Fig. 4, and also agrees with morphological evidence previously reported by others (27, 28). The results presented in Fig. 6 and Table I are consistent with the idea that fusion of endocytic vesicles must occur prior to the sorting of internalized material. This is to be expected, if early endocytic vesicles first must fuse with an endosomal compartment which possesses the appropriate environment and apparatus for the segregation of the AvpGal and B-Tf probes to take place.

AvpGal and B-Tf become Physically Segregated during Incubation in Vivo and in Vitro—An unequivocal demonstration of the sorting and segregation of these two probes can be obtained by following the progression of endocytosed macromolecules through intracellular compartments resolved by fractionation on Percoll density gradients (22-25). Fig. 7 presents the profile of AvpGal-associated β-galactosidase activity on 27% Percoll gradients prepared with PNS isolated from K562 cells that, having internalized the AvpGal probe, were then incubated at 37 °C for the indicated times. Initially, AvpGal is contained in endocytic vesicles for low density, defined by the gradient profile observed after uptake for 60 min at 20 °C. After 15 min of incubation at the higher temperature, β-galactosidase activity has begun to shift to compartment(s) of greater density. After 60 min, activity appears predominantly in secondary lysosomes, which, as dense organelles, migrate near the bottom of the gradient. The kinetics of this maturation process are consistent with what has been observed for other endocytic marker enzymes during their progression through intracellular compartments to the lysosomes (24, 25).

Experiments similar to that presented in Fig. 7 were performed in which radiolabeled B-Tf was employed as the in vivo endocytic marker. In contrast to the results obtained with the fluid phase marker (AvpGal), internalized [125I]-B-Tf is only found in the region of low density endocytic vesicles on Percoll gradients. No change in the density of [125I]-B-Tf containing vesicles is observed upon continued incubation of cells at 37 °C, although counts/min are progressively lost with time from the low density vesicle region when PNS is fractionated on the gradient. After 60 min of incubation at 37 °C in vivo, nearly all radioactivity has disappeared from the cells (data not shown; see also Ref. 24). This is to be expected, since transferrin (bound to its receptor) is ferried back to the cell surface, and there released to the external milieu. Therefore, distinct patterns emerge between the gradient profiles of internalized AvpGal and B-Tf as a consequence of the sorting of endocytosed material for delivery to the appropriate compartment(s) in vivo.

The question may be posed: does the loss of fusion activity observed in the in vitro studies of Table I correspond to physical separation of the two endocytic probes, analogous to

**TABLE I**

Characterization of the cell-free segregation reaction.

The correlation between the temperature curves for both reactions lends support to the notion that the fusion reaction is a prerequisite event for the decline to occur. It should also be noted that the temperature dependence observed for the cell-free system reflects in vivo observations presented in Fig. 4, and also agrees with morphological evidence previously reported by others (27, 28). The results presented in Fig. 6 and Table I are consistent with the idea that fusion of endocytic vesicles must occur prior to the sorting of internalized material. This is to be expected, if early endocytic vesicles first must fuse with an endosomal compartment which possesses the appropriate environment and apparatus for the segregation of the AvpGal and B-Tf probes to take place.

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The question may be posed: does the loss of fusion activity observed in the in vitro studies of Table I correspond to physical separation of the two endocytic probes, analogous to

Control experiments revealed that the peak of β-hexosaminidase activity, which serves as a lysosomal marker, also migrated in this region of the gradient (fractions 5-11). Endosomal compartments, defined by [125I]-B-Tf uptake, as well as AvpGal gradient profiles at early uptake time points (Fig. 7, top), are located in fractions 15-21. Plasma membrane proteins (labeled with [125I] at 4 °C) migrate in fractions 18-20. The activity of galactosyl transferase (a Golgi marker enzyme) is found in fractions 16-21.
Endocytic Sorting in Vitro

FIG. 7. The internalization pathway of AvPGal followed by sub-cellular fractionation on Percoll gradients. K562 cells were allowed to endocytose the fluid phase marker for 60 min at 20 °C, following which cells were washed at 4 °C, resuspended in uptake buffer, and split into fractions which were incubated for the indicated times at 37 °C. After further washing and resuspension in tripolyphosphate buffer, cells were broken and PNS was prepared. Samples of PNS (0.5 ml) were run on 27% Percoll gradients as described under “Experimental Procedures.” Gradient fractions (275 μl) were collected and β-galactosidase activity was determined. In this latter assay, sample loadings were increased 2-fold for 15-min and 3-fold for 60-min time points in order to compensate for the reduction in enzyme activity over time (see Fig. 6, for example). Shown are the β-galactosidase activity profiles (in fluorescence units) determined for odd fractions; the left side of the figure corresponds to the bottom of the gradients.

FIG. 8. In vitro fusion, sorting and segregation of AvPGal and B-Tf. Both PNS containing AvPGal and 125I-B-Tf were mixed into a reaction medium containing ATP and an ATP-regenerating system, incubated for times indicated at 37 °C, and fractionated on 27% Percoll. The levels of AvPGal, assessed by its β-galactosidase activity with fluorescent substrate (○), and B-Tf levels, measured as 125I counts/min (×), were determined in the Percoll gradient fractions. Fusion activity was independently assayed in the reaction mixture. For details about this experiment, please refer to “Results and Discussion.”

The sorting and compartmentalization processes observed in vivo? To address this issue, PNS preparations containing AvPGal and B-Tf were co-incubated in vitro at 37 °C in the presence of ATP and ATP-regenerating system, and then fractionated on Percoll density gradients. As depicted in Fig. 1, portions of these reactions were removed and assayed by the ELISA technique to confirm the endocytic fusion had indeed occurred during incubation. The top panel of Fig. 8 shows the Percoll gradient profiles of the activities for both probes in the initial incubation mixture. After 60 min of incubation, the two endocytic markers have separated: AvPGal now migrates with vesicles of a greater density than those containing radiolabeled B-Tf. Due to the fusion between endocytic vesicles, a portion of B-Tf will be coupled to AvPGal: this may account for a minor shift in the gradient profile of the radiolabeled ligand. The AvPGal that remains with the light membranes could conceivably be as much as 40% of the total activity, and may well remain unsorted by virtue of its association with B-Tf and the transferrin receptor. However, as demonstrated by the data presented in the bottom panel of Fig. 8, significant fractions of the probes do not associate under these conditions of incubation, since they can be separated on the gradient after the in vitro reaction. In all probability, this material represents that fraction of probe which escaped association with its complementary probe during its progression through the fusion-competent compartment, and has been subsequently sorted and segregated to a denser vesicular compartment.

The profile of AvPGal-related β-galactosidase activity in Fig. 8 clearly shows a shift to a denser region of the gradient after incubation at 37 °C, comparable to results obtained in vivo (Fig. 7). The total β-galactosidase activity summed across the gradient fractions for the 60-min incubation is within 15% of the activity on the gradient containing unincubated material, thus ruling out an artifactual change in the density gradient profile due to degradative loss of β-galactosidase activity in the lighter fractions. It should be noted that the β-galactosidase activity peak observed in vitro does not correspond to the density of secondary lysosomes, even after 60 min of incubation. This is in contrast to the gradient profile from in vivo experiments, in which, after a 60-min incubation at 37 °C, the AvPGal activity is almost exclusively found at the density of secondary lysosomes, with little or no activity observed in the region of endocytic vesicle migration (Fig. 7, bottom panel). The in vitro gradient profile of Fig. 8 is consistent, however, with the results of Fig. 5, wherein only about 50% of the total initial activity is lost during the in vitro sequestration reaction. This is approximately the portion of β-galactosidase activity which has shifted to the denser region of the gradient after the 60-min incubation (Fig. 8, bottom panel, closed circles). It seems probable that the density shift observed in the cell-free system after 60 min corresponds to a maturation of roughly half the AvPGal probe to a dense prelysosomal or early lysosomal vesicle fraction. Further maturation to secondary lysosomes may well be prevented simply by the current limitations imposed by our in vitro conditions.

Unlike the shift in AvPGal activity, the gradient profile of 125I-B-Tf after the 60-min incubation period remains almost identical to that observed under initial conditions (Fig. 8,
In vitro segregation of AvpGal to dense vesicles

PNS fractions containing the two endocytosed probes were incubated at 37°C in vitro under the experimental conditions indicated, followed by subcellular fractionation as shown in Fig. 8. Endocytic marker activity was measured in terms of β-galactosidase activity for AvpGal, and 125I counts/min for B-TF, as described under "Experimental Procedures." The activity ratio is defined as the total endocytic marker activity found in dense vesicle fractions (corresponding to fractions 7-13 in Fig. 8) relative to the total endocytic marker activity found in light vesicle fractions (corresponding to fractions 10-21 in Fig. 8).

<table>
<thead>
<tr>
<th>Experimental condition</th>
<th>Time of incubation</th>
<th>Activity ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>min</td>
<td>βGal activity</td>
</tr>
<tr>
<td></td>
<td></td>
<td>dense fractions</td>
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<tr>
<td>ATP-regenerating system added</td>
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<td>0.66</td>
</tr>
<tr>
<td></td>
<td>15</td>
<td>0.90</td>
</tr>
<tr>
<td></td>
<td>90</td>
<td>0.90</td>
</tr>
<tr>
<td>ATP-depleting system added</td>
<td>90</td>
<td>0.71</td>
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<table>
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<th>Experimental condition</th>
<th>Time of incubation</th>
<th>Activity ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>min</td>
<td>cpm dense fractions</td>
</tr>
<tr>
<td></td>
<td></td>
<td>cpm light fractions</td>
</tr>
<tr>
<td>ATP-regenerating system added</td>
<td>0</td>
<td>0.69</td>
</tr>
<tr>
<td></td>
<td>60</td>
<td>0.72</td>
</tr>
</tbody>
</table>

open circles); no density shift is observable. This result indicates that the segregation mechanism appropriately sorts the two internalized probes in vitro, directing the fluid phase marker to dense prelysosomal or lysosomal vesicles, while excluding the receptor-associated ligand from the latter compartments.

Fractionation of the in vitro reaction products on Percoll gradients was explored under a variety of experimental conditions, summarized in Table II. For convenience, the appearance of endocytic marker activity is presented as the ratio of activity found in "dense" vesicle gradient fractions (corresponding to fractions 7-13 in Fig. 8, for example), relative to activity found in "light" vesicle gradient fractions (corresponding to fractions 15-21 in Fig. 8). The density shift in AvpGal-associated β-galactosidase is observed after only 15 min of incubation at 37°C, and remains unchanged after 90 min of incubation. The timing of this shift corresponds to the timing of the loss of fusion activity observed both in vitro and in vivo (Figs. 4 and 5), as well as to the density shift observed for AvpGal enzyme activity in vivo (Fig. 7). With reactions performed in the presence of an ATP-depleting system, no alteration in gradient profile is observed for the AvpGal β-galactosidase activity (Table II). This observation indicates that in vitro segregation is an ATP-dependent process, in parallel with the ATP dependence of the decline in fusion competence seen in Table I.

The density profile of radiolabeled B-TF on Percoll gradients is identical to that found initially for the fluid phase AvpGal marker, and is not altered by incubation in vitro at 37°C; the dense/light ratio for B-TF remains essentially unaltered after an hour of incubation at 37°C (Table II). During this time, B-TF remains intra-vesicular, as the radiolabeled probe is only susceptible to digestion by protease K in the presence of added detergent (data not shown). Has the B-TF remained in a fusion-competent compartment throughout the incubation period, or has it progressed beyond this compartment? The biochemical evidence presented earlier (Fig. 5 and Table I) does strongly imply that B-TF has been moved to some later compartment which is not capable of fusing with endocytic vesicles (or the endosomal apparatus), by comparison to the activity assessed for the initial compartment containing the probe. Normally, B-TF would be expected to enter vesicles destined to recycle to the plasma membrane. The precise pathway of recycling of the Tf receptor in K562 cells is unclear: two separate routes have been defined, based on monensin sensitivity (30). One of these pathways seems to involve transit through the trans-Golgi network, based on the observed resialylation of desialated Tf receptors (31) and the localization of Tf receptors in this region by immuno-electron microscopy (32). Alternatively, direct transfer to exocytic vesicles may occur. However, any such exocytic movement will involve transport via light membrane fractions that would not be resolved from the initial endocytic compartment on these gradients. Hence, no alteration in the density profile of B-TF during the incubation period is to be expected in such a case. It is thus possible that B-TF has segregated to an exocytic compartment, and perhaps has even passed on into plasma membrane vesicles, in the in vitro system.

In conclusion, we have demonstrated both the in vivo and in vitro sorting and segregation of the endocytic markers, AvpGal and B-TF, to independent and distinct compartments. These two probes are extremely useful for the study of intracellular vesicle traffic: since AvpGal serves as a fluid phase marker and B-TF is recycled with the transferrin receptor, different and independent intracellular pathways can be analyzed, both in vivo and in vitro. The significant observation that segregation and sorting of these ligands can occur in a cell-free system provides a format by which those biochemical factors responsible for directing this process can now be studied. Key questions which can now be addressed in this manner are: what cell factors recognize and direct the recycling receptors into the exocytic pathway and through what mechanism are fluid phase probes shuttled into prelysosomal and lysosomal compartments? The observation that AvpGal shifts to vesicles of greater density upon incubation in vitro suggests there is reconstitution of either the vesicular transfer of AvpGal, or the remodeling of an endosomal compartment containing this probe to a pre-lysosomal or lysosomal compartment. Morphological data will be required to distinguish between these possibilities. Thus, further understanding of the complex process of lysosome biogenesis or vesicular traffic to this cellular organelle may also be gained through future studies in this system.
Endocytic Sorting in Vitro

REFERENCES


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The sorting and segregation mechanism of the endocytic pathway is functional in a cell-free system.
M Wessling-Resnick and W A Braell


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