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An internalized transmembrane protein resides in a fusion-competent endosome for less than 5 minutes

(endosome subpopulations/cell-free system/membrane protein recycling/"immuno-isolation"/G protein of vesicular stomatitis virus)

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ABSTRACT **We have used our assay for the cell-free reconstitution of vesicle fusion occurring in endocytosis to investigate the fusion competence of defined endosomal fractions containing the G protein of vesicular stomatitis virus. G protein was first implanted into the plasma membrane, and endocytosis was then allowed to proceed for defined periods of time. Endosomal fractions were prepared by "immuno-isolation" on a solid support with a monoclonal antibody against the cytoplasmic domain of the G protein. Maximal internalization of the G protein occurred within 5 min at 37°C. From this early endosome the G molecules follow a branched pathway: 50% recycles to the cell surface, while 50% is transported along the endocytic route to the lysosomal compartment. The proportion of G protein following each pathway can be modulated. When the amount of implanted G protein was increased, the fraction of G molecules recycling to the cell surface was reduced. When the G molecules were cross-linked with an antibody prior to the internalization step, recycling to the cell surface was abolished. The cell-free analysis of vesicle fusion was carried out with endosomal fractions immuno-isolated after 5, 10, 15, and 30 min of G-protein internalization at 37°C. Fusion competence was at a maximum with the fraction isolated 5 min after internalization and then decreased with a half-life of ≈3 min with fractions isolated at later time points. The fusion-competent compartment is the early endosome where sorting of the transmembrane G protein to recycling or degradation occurs.**

Endosomes consist of a series of morphologically distinct vesicular, tubular, and multivesicular elements where internalized molecules and membrane proteins, including receptors and their ligands, are sorted. In nonpolarized cells, the destinations are essentially recycling to the cell surface for reutilization and routing to the lysosomes for degradation (for reviews see refs. 1 and 2). Additional routes leading to the Golgi complex also have been reported (for review see ref. 3). Morphological studies have shown that endocytosed membrane proteins destined to be recycled or degraded share common endosomal elements before they are sorted (4). Although biochemical evidence is still lacking, transport from the plasma membrane to these common elements is believed to occur by way of coated vesicles that deliver their cargo by membrane fusion. It remains unclear at what stage of endocytosis the recycling proteins leave the common endosomal elements to return to the plasma membrane or at what stage the molecules selected for degradation are collected. It is not known whether sorting and export of proteins to be recycled or degraded occur from this common compartment with the same kinetics. Nor is it known whether this common compartment and the subsequent compartments involved in transport to the lysosomes are distinct biochemical entities connected by vesicular traffic.

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We have described (5) an experimental system established for the cell-free reconstitution of the vesicle fusions occurring in endocytosis. This system uses defined endosomal fractions "immuno-isolated" on a solid support. The membrane-spanning glycoprotein G of vesicular stomatitis virus (VSV) was used to provide the antigen. The G protein was first implanted in the plasma membrane in its membrane-spanning conformation by low pH-mediated fusion of the viral envelope with the plasma membrane (6, 7) and then was internalized by incubating the cells at 37°C. Endosomal fractions were immuno-isolated using a monoclonal antibody raised against the cytoplasmic domain of G protein. An endosomal fraction isolated 15 min after G-protein internalization differed in its protein composition from a plasma membrane fraction and retained its acidic luminal pH. More importantly, both a biochemical and a morphological detection system showed that these endosomal vesicles immuno-isolated on a solid support were capable of undergoing fusion with other vesicles of the endocytic pathway and that this process required ATP. This experimental reconstitution of vesicle fusion was specific, since fusion was not detected with a plasma membrane fraction and since fusion competence was lost with a fraction prepared 30 min after G-protein internalization.

In the present paper we have used this cell-free system to identify the fusion-competent subcompartment of the endocytic pathway. To address this question it was important to first characterize the routes as well as the kinetics of G-protein endocytosis after implantation and internalization from 0 to 30 min at 37°C. Then, we immuno-isolated the subpopulations of endosomes as the G protein traveled through the endocytic pathway (time-defined fractions). Finally, the fusion competence of these time-defined fractions was determined in the cell-free assay. Functional differences are expected between the compartments through which endocytosed molecules travel from the plasma membrane to the lysosomes.

MATERIALS AND METHODS

Cells, Viruses, and Immunological Reagents. Monolayers of BHK-21 cells, VSV, and [^{35}S]methionine-labeled VSV were prepared as described (5, 7). All manipulations of the cells and cell fractions were at ice temperature, except when indicated. The solid supports were either polyacrylamide beads (Bio-Rad) or monodisperse magnetic beads (8) with a covalently coupled linker antibody against the Fc portion of mouse IgG. The immunoadsorbent was prepared by binding the monoclonal antibody against a cytoplasmic epitope of the G protein (clone P5D4; ref. 9) to the solid support by the linker antibody as described (7). The polyclonal antibody was raised against a micellar preparation of the G protein.

Abbreviations: VSV, vesicular stomatitis virus; PNS, post-nuclear supernatant; LPO, lactoperoxidase; HRP, horseradish peroxidase.

Immuno-Isolation of Time-Defined Endosomal Fractions. The protocol used for VSV binding to the cell surface, the implantation of the G protein in the plasma membrane, and the subsequent endocytosis of the G protein in the presence or absence of horseradish peroxidase (HRP) at 5 mg/ml was as described (5). In all experiments (except when indicated), 41 μg of VSV was added to 1.3×10^7 cells (in a 75-cm^2 Petri dish, 2.5 mg of total cellular protein); 8 μg of VSV was fused with the plasma membrane, resulting in a density of 70 G molecules implanted per μm^2 of membrane surface area (for calculations, see ref. 7; the BHK plasma membrane surface area is 3400 μm^2, ref. 10).

The cells with G protein implanted and internalized for 5–30 min at 37°C were homogenized as described (5) in 250 mM sucrose buffered to pH 7.4 with 10 mM Tris·HCl, and a post-nuclear supernatant (PNS) was prepared. The immunoisolation was initiated by mixing 500 μg of PNS protein with 1 mg of immunoadsorbent. The mixture was rotated at 2 rpm for 2 hr at 4°C. Then, the fraction bound to the immunoadsorbent was retrieved and washed to remove the unbound cellular material (5).

Immunoradiometric Quantitation of the G Protein on the Cell Surface. Cells with G protein implanted and internalized for 5–30 min at 37°C were incubated at ice-temperature for 30 min with the antiserum against the G protein diluted 1:250 in 3 ml of 7.4/MEM/BSA [minimal essential medium (MEM), 10 mM *N*-tris(hydroxymethyl)methyl-2-aminoethane sulfonic acid (Tes), 10 mM Mops, 15 mM Hepes, 2 mM NaH_2PO_4, $NaHCO_3$ at 350 mg/liter, pH 7.4] containing 20 mM *N*-acetylglucosamine and 0.2% IgG-free bovine serum albumin. IgG-free bovine serum albumin was used to reduce the background. The excess antibody was removed, and the cells were washed three times for 5 min with PBS^{++} [137 mM NaCl, 2.7 mM KCl, 1.5 mM KH_2PO_4, 6.5 mM Na_2HPO_4, 1 mM $CaCl_2$, and 1 mM $MgCl_2$ (pH 7.4)] conatining 0.5% bovine serum albumin. Then the cells were incubated for 1 hr with 3 ml of 7.4/MEM/BSA containing 10^6 cpm/0.5 μg of ^{125}I-labeled protein A (Amersham). The excess label was removed, and the washing sequence was repeated. The cells were then scrapped from the dishes with a rubber policeman, collected by centrifugation ($100 \times g$, 5 min), and counted.

Cell-Free Reconstitution. The cell-free fusion assay was carried out as described (5) using the time-defined fractions immuno-isolated on the solid support (acceptors). The donor fraction was always a PNS prepared from cells without G protein that had internalized lactoperoxidase (LPO) at 2 mg/ml continuously for 30 min at 37°C. The reconstitution was carried out by mixing each acceptor with the donor for 15 min at 37°C in a final volume of 0.3 ml adjusted to 25 mM Hepes·KOH, pH 7.0/25 mM KCl/1.5 mM $Mg(OAc)_2$/1 mM dithiothreitol in the presence of either an ATP-regenerating system (1 mM ATP, 8 mM creatine phosphate, and creatine phosphokinase at 0.043 mg/ml) or an ATP-depleting system (5 mM D-glucose and hexokinase at 0.2 mg/ml). The amount of PNS protein (0.5 mg) from the donor fraction in the cell-free assay was the same as used for the immuno-isolation of the acceptor fraction.

After reconstitution, the acceptor fraction bound to the solid support was retrieved, washed to remove the unreacted donor, and resuspended in 0.5 ml of PBS^{++} containing 20 mM D-glucose and 0.05 ml of 0.5 M acetic acid, pH 5.7 (final pH 6.6). The iodination reaction was initiated by the addition of 55 μl of D-glucose oxidase at 4 μg/ml as a H_2O_2-generating system and 1 mCi (1 Ci = 37 GBq) carrier-free ^{125}I (Amersham). The reaction was allowed to proceed on ice for 15 min and stopped by the addition of 0.1 ml of 50 mM KI in PBS. The acceptor fraction was retrieved, washed to remove the excess ^{125}I, and then solubilized in 0.1% $NaDodSO_4$ and 2% (vol/vol) Triton X-100. For immunoprecipitation (11), the polyclonal antibody against the G protein and, after 12 hr, protein A-Sepharose were added without removing the immunoadsorbent. The G protein immunoprecipitated on the immunoadsorbent and on protein A-Sepharose was analyzed by $NaDodSO_4$/PAGE using the system of Maizel (12), and the ^{125}I-labeled G protein was detected by autoradiography. The regions of the gels corresponding to the immunoprecipitated ^{125}I-labeled G protein were excised, and the ^{125}I was measured.

RESULTS

Kinetics of G-Protein Endocytosis. A single and synchronous wave of G-protein internalization can be studied, because the G protein is restricted to the plasma membrane after implantation. Endocytosis is initiated by raising the temperature to 37°C and is stopped at defined times by returning the cells to ice temperature.

The amount of G protein at the cell surface was quantitated after 0, 5, 15, and 30 min of internalization at 37°C using an immunoradiometric assay (Fig. 1*A*). The G protein was rapidly internalized; over 60% of the G protein was endocytosed within 5 min. From this early endocytic compartment, ≈60% of the G protein recycled back to the cell surface within 15 min and 40% of the G molecules were transported through later endosomal elements (see below) to the lysosomal compartment where degradation of the G protein occurred. The synchrony and efficiency of this wave of implanted G-protein internalization are due to the transient lateral association of the cytoplasmic domains of G protein with the viral M protein–nucleocapsid complex (see *Discussion*). Our studies had shown (5) that after implantation this complex remained associated with the plasma membrane at 4°C and was rapidly released upon incubation at 37°C. Fig. 1*A* shows that after this first round of endocytosis the G protein that had recycled to the cell surface remained on the plasma mem-

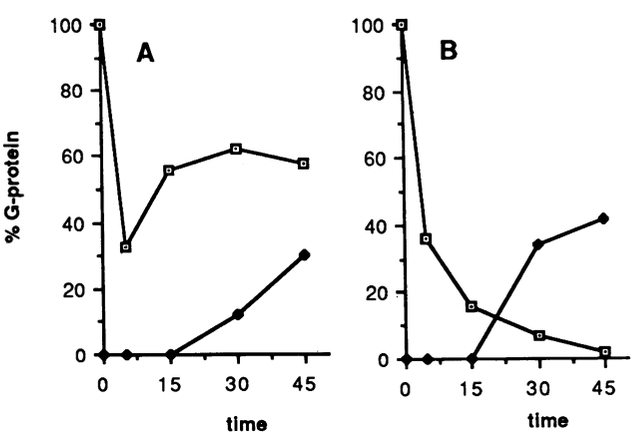

FIG. 1. Implanted G-protein recycling and degradation. (*A*) The amount of G protein on the cell surface (open symbols) was quantitated with an immunoradiometric assay using a polyclonal antibody against the G protein exoplasmic domain and is expressed as percent of the amount originally implanted G protein (70 G molecules per μm^2 of membrane surface area). To quantitate the amount of G-protein degraded in the lysosomal compartment, ^{35}S-labeled VSV was used for G-protein implantation. After ^{35}S-labeled G-protein internalization for the indicated time, the cells were extracted in Triton X-114 (13). All viral proteins were labeled but only the intact ^{35}S-labeled G protein partitioned into the detergent phase. The amount of G protein degraded was calculated by subtracting the G protein still intact at each time point from the G protein originally implanted and is expressed as percent of the total (solid symbols). (*B*) Effect of cross-linking with antibody on G-protein recycling and degradation. The experiments were as in *A* except that a polyclonal antibody against the G protein was bound to the cell surface prior to the internalization step.

brane; a steady state was reached and only a minor portion of the G protein was endocytosed at any time.

Lysosomal degradation of the ^{35}S-labeled G protein was quantitated by determining the amount of ^{35}S-labeled G protein still intact at the various time points (Fig. 1*A*). Degradation was not detected during the first 15 min of internalization. After 30 min at 37°C, 12% of the total G protein was degraded. All the G molecules transported along the endocytic pathway (40% of total G protein) were degraded by 60 min (data not shown).

Modulation of G-Protein Endocytosis. Additional evidence that the G protein follows a branched pathway in endocytosis was obtained by cross-linking the implanted G molecules with a polyclonal antibody against G protein prior to the internalization step (Fig. 1*B*). Cross-linking abolished the recycling of the G molecule to the cell surface. Internalization occurred at the same rate as the noncrossed-linked G protein (60% in 5 min at 37°C), and the time course of transport to the lysosomes was also similar.

The pathway that the G protein follows in endocytosis could also be modulated by varying the amount of G protein originally implanted into the plasma membrane (Fig. 2). At low densities of implanted G protein (7 G molecules per μm^2), essentially all the G molecules were internalized and subsequently recycled back to the cell surface. When the G-protein density was increased, the fraction recycling back to the cell surface decreased. At high G-protein density (270 G molecules per μm^2), only 45% of the G molecules was on the cell surface after a 30-min incubation. At high densities, G-protein internalization may have lost its synchrony or potential transport sites may have become saturated, resulting in a less-apparent profile of internalization and subsequent recycling. However, recycling is likely to have occurred since the G protein did not clear from the plasma membrane.

Immuno-Isolation of Time-Defined Endosomal Fractions. Time-defined endosomal fractions were prepared by immuno-isolating the successive compartments of the pathway where the G protein distributed after increasing times of incubation at 37°C. This was carried out under the conditions shown in Fig. 1*A*, where ≈40% of the G molecules was routed to degradation. These conditions guaranteed a high enough density of G molecules for efficient immuno-isolation (see Fig. 3) and provided approximately equal amounts of G protein in each time-defined fraction (see Fig. 4).

As an independent marker of the endosomes, HRP was cointernalized with the G protein. The amount of HRP internalized into the cells increased with the time of incubation (Fig. 3). A parallel increase in the amount of HRP recovered in the immuno-isolated fractions was observed. At 5 min the cells and the immuno-isolated fractions contained 20% of the HRP found at 30 min. Morphological studies show that the endocytic vesicles immuno-isolated at 5 and 15 min correspond to the morphologically distinct elements observed in the cell (14). The amount of G protein in these endosomal fractions was quantitated using ^{35}S-labeled VSV

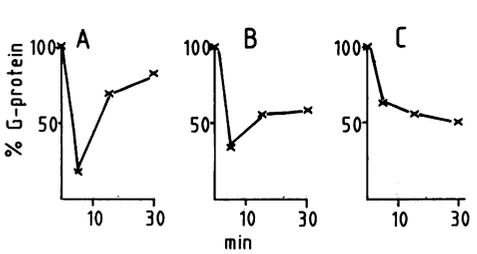

FIG. 2. Modulation by increasing amounts of implanted G protein. The amount of G protein on the cell surface was quantitated after internalization at 37°C for the indicated times as in Fig. 1. The input VSV originally added to 1.3×10^7 cells was 8 μg (*A*), 41 μg (*B*), and 200 μg (*C*).

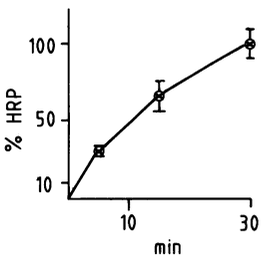

FIG. 3. HRP quantitation in time-defined endosomal fractions. HRP internalized in endosomal elements was quantitated as described (5) either in the cells (crosses) or in isolated endosomal fractions (open circles). To allow comparison between the cells and the fractions, the amount at each time point is expressed in percent of the total amount present, respectively, in the cells and in the fractions after 30 min. After 30 min of incubation in the presence of HRP at 5 mg/ml, 1.12 μg of HRP was internalized by 1.3×10^7 cells. The PNS used as input for isolation contained 0.67 μg of HRP; the remainder, 0.45 μg, was lost in the nuclear pellet. The amount isolated from this PNS was 0.28 μg or 70% of the membrane-enclosed HRP.

(Fig. 4). In each fraction the amount of ^{35}S-labeled G protein isolated represented ≥80% of the G molecules internalized and present in the PNS.

Fusion Competence of Time-Defined Endosomal Fractions. We have used these time-defined fractions (acceptors) in the cell-free assay to test whether each had the same competence for fusion with other endocytic vesicles.

Since the acceptor was immuno-isolated on the solid support, it could be introduced into and retrieved from the reaction mixtures at each step of the reconstitution. The donor fraction was a PNS prepared from cells that had internalized LPO for 30 min at 37°C. This allowed the detection of fusion events with any acceptor, since the LPO used in the fusion detection reaction was present in the lumen of all endocytic vesicles along the entire pathway.

The reconstitution was carried out by mixing acceptor and donor at 37°C for 15 min in the presence of an ATP-regenerating system or an ATP-depleting system. It has been shown by us (5) and others (15) that the fusion of endocytic vesicles requires ATP *in vitro*. After reconstitution, the acceptor bound to the solid support was retrieved, and the occurrence of fusion was monitored by an iodination reaction. Because only fusion can deliver LPO to the lumen of the acceptor vesicles, iodination of the G protein by LPO was used as the specific criterion of fusion. The G protein was then immunoprecipitated, and the extent of iodination was analyzed by NaDodSO$_4$/PAGE and autoradiography.

The cell-free reconstitution of ATP-dependent fusions with the time-defined endosomal fractions is shown in Fig. 5*A*. The maximal amount of ^{125}I-labeled G protein was detected in the early fraction prepared 5 min after G-protein internalization. We had estimated (5) using a morphological detection

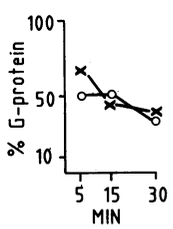

FIG. 4. G-protein quantitation in time-defined endosomal fractions. The ^{35}S-labeled G protein isolated at each time point (open circles) is compared to the G protein internalized at each time point (crosses, obtained from Fig. 1 by subtracting the G protein at the cell surface from the total amount implanted). Both values are expressed in percent of the total G protein present.

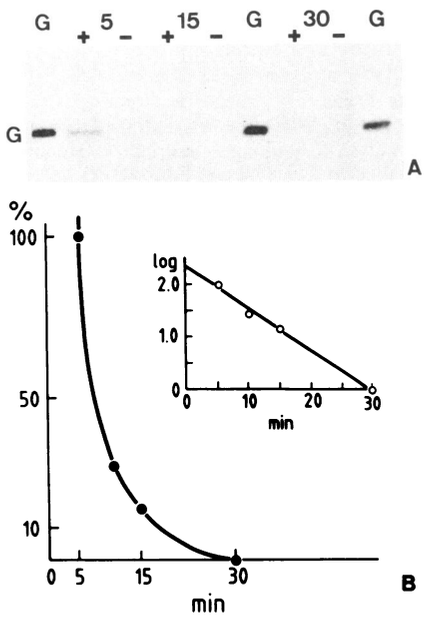

FIG. 5. Time-course of fusion in endocytosis. (*A*) A representative cell-free fusion experiment shows an autoradiogram of immunoprecipitates of the G protein using acceptors isolated after 5, 15, and 30 min of internalization. The experiments were carried out in the presence (+) or in the absence (−) of ATP. The mobility of the G protein itself is indicated using a VSV G-protein standard. (*B*) Fusion competence of the time-defined endosomal fractions was quantitated from four experiments with acceptors prepared after 5, 10, 15, and 30 min of internalization. The amount of G protein iodinated with each acceptor fraction was counted and expressed as percent of the amount of ^{125}I-labeled G present after 5 min of internalization for each experiment. (*Inset*) The exponential decrease of the fusion competence is shown on a semi-logarithmic scale (regression coefficient, 0.9966). This permits the calculation of a half-life for this process of 3.1 ± 0.6 min.

system that 50% of the vesicles in a 5-min acceptor preparation fused in the assay. Since the present study compares the fusion competence of successive time-defined fractions of the endosomal compartment, we calculated the relative efficiencies from the amount of ^{125}I-labeled G protein present in each fraction normalized to the amount present in the 5-min fraction (Fig. 5*B*). G-protein iodination decreased exponentially to 28% at 10 min and 15% at 15 min. By 30-min of internalization no fusion was detected, confirming our earlier findings (5). The fusion-competent compartment (5-min internalization) is likely to be the compartment where membrane proteins are sorted because transport of the G protein from this compartment either through the endocytic route (fusion competence) or back to the plasma membrane (recycling) occurred with similar kinetics.

The cell-free assay was designed to detect the major profile of vesicle fusion in endocytosis. These results do not preclude that other fusions that occurred at a very slow rate or that depended on different functional requirements remained undetected. Our experimental approach was not optimized to detect the occurrence of endosome–lysosome fusion, if it exists, nor would it detect any fusion with vesicles coming from the exocytic pathway.

These results demonstrate that an early compartment where the G protein is present after 5 min of internalization is fusion competent. The residual fusions detected with the later fractions may reflect some overlapping between the fractions, due to a few G molecules still present in the early fusion competent elements. The linear character of the semi-logarithmic plot calculated from the data presented in Fig. 5*B* shows that the fusion-competent compartment has a half-life of 3.1 min (Fig. 5*B*, *Inset*). This value may overestimate the half-life of an individual endosomal element, since complete synchrony of G-protein internalization is not expected.

DISCUSSION

We have made use of a viral glycoprotein to identify the fusion-competent subcompartment in endocytosis. Our experimental approach was first to study G-protein endocytosis, following its implantation into the plasma membrane. Endocytosis of the G protein had been reported in polarized cells after transfection (16) as well as transcytosis after implantation (17). Next, we immuno-isolated endosomal fractions using the G protein as antigen at different stages of the pathway and determined the fusion competence of each fraction in a cell-free assay. Because we had characterized the stages of G-protein endocytosis, it was possible to identify the fusion-competent elements as the early subcompartment where sorting of plasma membrane proteins occurs (Fig. 6).

The implanted G protein was quickly, within 5 min, internalized into an early endosomal compartment. From this early compartment, the G protein followed the two major pathways reported for membrane proteins. The G protein was either recycled to the plasma membrane with kinetics similar to the transferrin receptor (half-life, 7.5 min; ref. 18) or transported along the endocytic pathway to the lysosomes with kinetics also reported in other systems (≥30 min; refs. 19–21). An explanation for this apparently unique feature of G-protein endocytosis may be derived from our observation that the proportion of G molecules routed to the lysosomes increases with the amount of G-protein present (see Fig. 2). The G molecules that would otherwise recycle may be directed to the lysosomes because of interactions between the luminal portions of the G molecules in acidic endosomes. The degree of these lateral interactions is likely to increase as the density of the G molecules increases in the endosomal

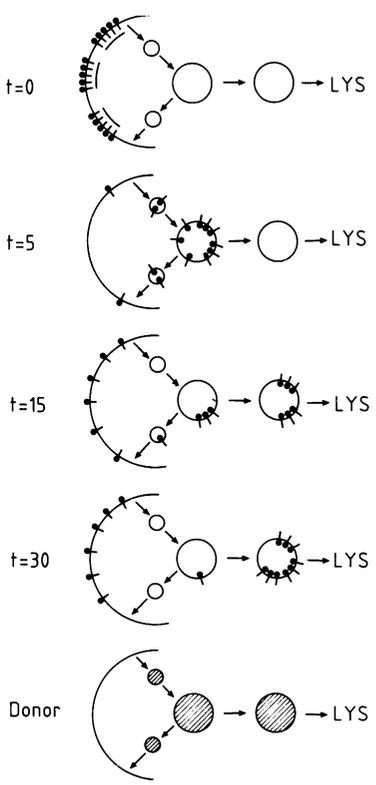

FIG. 6. Outline of G-protein endocytosis and recycling. LYS, lysosome.

membrane. Cross-linking of the G molecules with antibodies at the cell surface may mimic this process, as this abolishes recycling and directs the G protein to the lysosomes for degradation (see Fig. 1*B*). A similar role can be ascribed to the transient association of G molecules by way of the M protein–nucleocapsid complex following implantation. These findings are consistent with the observation that routing of the Fc receptor to degradation in macrophages depends on its interaction with polyvalent complexes (20).

Essentially all the G molecules that do not recycle to the plasma membrane represent an initial, single wave transported along the pathway since they are quantitatively immunoisolated in the time-defined fractions and eventually degraded (40% of the total G protein in these experiments). The G molecules that do recycle to the cell surface remain on the plasma membrane (60% of the total G protein after 30 min). Continuous recycling probably still occurs but accounts for a minor portion of the amount on the surface at any one time.

We have used this wave of G-protein transport along the endocytic pathway to immuno-isolate time-defined fractions (acceptors) and assay their capacity to undergo fusion with other components of the endocytic pathway (donor) in the cell-free system. Fusion competence was maximal when the acceptor was prepared 5 min after G protein internalization, corresponding to the endosomal compartment shared by both the recycling and the degradation pathway. A high level of fusion activity of these endosomal elements is expected. A continuous flow of membrane vesicles between the plasma membrane and the endosomal compartment is required to account for the rapid turnover of the plasma membrane (1). It has been estimated that 1100–2300 coated vesicles leave the plasma membrane each minute in a BHK cell (22). These as well as the endocytic vesicles from the 5-min subcompartment provide likely candidates for the donor in the cell-free assay. Identification of the donor will require a modification of the detection system, so that the amount of donor fused can be quantitated. An alternative is to combine the use of time-defined acceptors with a detection system using the high affinity of avidin for biotin similar to that reported by Braell (23).

After 15 min of incubation at 37°C, the wave of internalized G protein was transported to later elements of the pathway. As a result the early endosomal elements (the 5-min compartment) were depleted of G molecules (Fig. 6). This process was completed by 30 min, when the wave of internalized G protein reached the lysosomal compartment. The kinetics of loss of fusion competence (half-life of <5 min) agrees well with the kinetics of endocytosis and recycling measured with fluid-phase markers (24, 25). Besterman *et al.* (24) have estimated to 5 min the half-life of recycling of [^{14}C]sucrose from a first compartment of endocytosis. Our results strongly suggest that the fusion competent endosomal elements are a subclass of endosomes where membrane proteins are sorted to be recycled or transported to the lysosomes.

The lack of vesicle fusions observed at later stages of the pathway may reflect a maturation process required for the transport and delivery of these elements to the lysosomes (see ref. 26). However, functional differences between the endosomal subcompartments lead us to believe that distinct proteins exist to carry out these functions. This implies that vesicular traffic must occur albeit at a low rate at later stages (not detected here). Therefore, it becomes more informative to consider the mechanisms of endocytosis as a combination of vesicular traffic and maturation.

Note Added in Proof. To provide a direct measurement of the efficiency of acceptor vesicle fusion we adapted the detection system reported by Braell (23). Avidin was internalized simultaneously with the G protein in acceptor cells, and biotin-HRP was internalized in donor cells. The fusion was carried out as described in the presence of biotin-insulin to quench any free avidin. After detergent solubilization, the amount of avidin–biotin-HRP complex formed during the fusion was quantitated with an ELISA assay using an antibody against avidin. The total amount of immuno-isolated avidin was assayed in parallel by adding exogenous biotin-HRP and omitting the donor fraction. With this protocol, 63% of the acceptor vesicles prepared after 5 min of internalization were fusion competent, confirming the data reported in this paper.

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