Real-Time Fluorescence Measurement of Cell-Free Endosome Fusion: Regulation by Second Messengers

Neil Emans and A. S. Verkman
Departments of Medicine and Physiology, Cardiovascular Research Institute, University of California, San Francisco, California 94143-0521 USA

ABSTRACT A quantitative real-time assay of cell-free endosomal vesicle fusion was developed and applied to study fusion mechanisms in endosomes from baby hamster kidney (BHK-21) cells. The assay is based on an irreversible ~10-fold increase in BODIPY-avidin fluorescence on binding of biotinylated conjugates. BODIPY-avidin and biotin-dextran were internalized for 10 min at 37°C into separate populations of BHK-21 cells, and endosome fractions were prepared. Postnuclear supernatant fractions underwent ATP- and temperature-dependent fusion, as measured in a sensitive custom-built microfluorimeter by the continuous increase in BODIPY-avidin fluorescence. Fusion processes of efficiency >2.5% could be detected with 200-ms time resolution in sample volumes of 50 μL containing endosomes derived from ~4×10^4 cells. The fusion time course consisted of a distinct lag phase (up to 10 min) in which little fusion occurred, followed by an approximately exponential rise ($t_{1/2}$ 10–30 min; fusion efficiency ~15%). The lag phase was reduced by preincubation of separate endosome fractions with ATP at 37°C and by coincubation of endosomes at 22°C before the assay, suggesting a rate-limiting step involving binding of a soluble protein to the endosome membrane. Endosome fusion was strongly inhibited by GTPγS, *N*-ethylmaleimide, and AlF_4^-. Endosome fusion was not affected by phorbol myristate acetate but was significantly inhibited by cAMP and bovine brain calmodulin. The results establish a sensitive real-time fluorescence assay to quantify the kinetics and extent of endosome fusion in a cell-free system and demonstrate regulation of early endosome fusion by cytosolic second messengers.

INTRODUCTION

Fusion between endocytic compartments underlies the processes of receptor-mediated and fluid-phase endocytosis in mammalian cells (Kornfeld and Mellman, 1989; Gruenberg and Howell, 1989; Braell, 1992; Goda and Pfeffer, 1989, 1991; Gruenberg et al., 1989; Aniento et al., 1993; Emans et al., 1993, 1995). Clathrin-coated vesicles derived from the cell plasma membrane fuse with a peripheral tubulovesicular compartment, defined as the early endosome, delivering receptor-bound ligands and fluid-phase markers from the extracellular medium. Most internalized markers subsequently appear in perinuclear late endosomes after passage through an intermediate endosomal carrier vesicle. Several steps of the endocytic pathway have been reconstituted in cell-free assays (for review: Goda and Pfeffer, 1989). Early endosomes undergo homotypic fusion in vitro (Gruenberg et al., 1989; Braell, 1987; Diaz et al., 1988) but do not fuse with late endosomes or endosomal carrier vesicles (Gruenberg et al., 1989; Aniento et al., 1993; Emans et al., 1993; Goda and Pfeffer, 1988; Woodman et al., 1992; Braell, 1987; Davey et al., 1985; Clague et al., 1994). Fusion of endosomal carrier vesicles to late endosomes has been shown to require intact microtubules and cytoplasmic dynein (Aniento et al., 1993). Early endosome fusion is regulated by rab5, a member of the sec4/ypt1/rab family of small ras-like monomeric GTPases (Gorvel et al., 1991) and by protein phosphorylation (Woodman et al., 1992) and to utilize factors that are also involved in intra-Golgi transport (Diaz et al., 1989a). Many of these factors may constitute regulatory components of the ubiquitous membrane fusion/recognition apparatus proposed in the SNARE hypothesis (Sollner et al., 1993).

Classical endosome fusion assays have exploited high affinity avidin-biotin or antibody-antigen interactions to measure the extent of fusion between vesicle preparations labeled independently by internalization (Colombo et al., 1992a; Braell, 1987; Gruenberg and Gorvel, 1992). Various detection methods have been used to quantify the amount of a complex formed from fusion-dependent intermixing of endosomal contents. For example, avidin or biotinylated horseradish peroxidase is internalized into cells. Endosomes are prepared separately and mixed under in vitro conditions similar to those used for cell-free reconstitution of intra-Golgi transport (Braell, 1987; Gruenberg and Gorvel, 1992; Balch et al., 1984; Braell et al., 1984). After specified incubation time(s) at 37°C, the endosome mixture is solubilized, immunoprecipitated with an antiavidin antibody, and the coimmunoprecipitated peroxidase activity taken as a measure of the intermixing of endosomal contents (Gruenberg et al., 1989; Gruenberg and Gorvel, 1992).

Cell-free assays provide a general method to identify the factors that are involved in the regulation of endosome fusion (Goda and Pfeffer, 1989; Gorvel et al., 1991). In addition, the analysis of fusion kinetics permits the identi-

Received for publication 10 January 1996 and in final form 31 March 1996.

Address reprint requests to Dr. Neil Emans, 1246 Health Sciences East Tower, Cardiovascular Research Institute, University of California, San Francisco, San Francisco, CA 94143-0521. Tel.: 415-476-8530; Fax: 415-665-3847; E-mail: emans@itsa.ucsf.edu.

0006-3495/96/07/487/08 $2.00

fication of rate-limiting steps in the fusion process and the site of action of regulatory factors. A limitation of available assays is that data can be obtained only at single time points because the fusion reaction must be stopped and the extent of intermixing of endosome contents measured. Because of technical concerns in the accurate measurement of immunoprecipitated substrates at multiple time points, the assays are not easily applicable to quantify the detailed kinetics of fusion and to define the mechanisms by which regulatory factors modulate fusion.

We report here a sensitive fluorescence assay that monitors endosome fusion quantitatively as it proceeds in real time. Compared with conventional biochemical assays referred to above, the fluorescence assay permits the accurate analysis of detailed fusion kinetics in very small samples. Our approach was based on the finding that the fluorescence of BODIPY-avidin (B-av) increases strongly (by ~10 fold) on binding of a biotinylated substrate and that this avidin-biotin interaction could be exploited to follow endosome fusion in living cells by ratio imaging of individually labeled endosomes (Emans et al., 1995). The B-av/biotin system satisfies the major requirements of a cell-free endosome fusion assay: the increase in BODIPY fluorescence signal on biotin binding is easily measured, very rapid ($t_{1/2}$ < 25 ms), pH insensitive, and essentially irreversible (dissociation $t_{1/2}$ > 24 h). In addition, the validity of various avidin and biotin conjugates as markers of fluid-phase or receptor-mediated endocytosis has already been established in cell-free endosome assays and in our recent study of endosome fusion in living cells. The experiments reported here utilized established in vitro fusion conditions in which endosome fractions were prepared from cells after internalization of B-av or biotin-dextran. After mixing of the labeled vesicles, endosome fusion was detected from the time course of increase in BODIPY fluorescence. A sensitive microfluorimeter was constructed to optimize the fluorescence signal from a small sample volume and to perform multiple fusion assays in parallel. The cell-free assay was first validated by use of known modulators of endosome fusion and then applied to characterize an interesting lag phase in fusion kinetics and the influence of cytosol second messengers.

MATERIALS AND METHODS

Materials

BODIPY-avidin (B-av), amino dextran (MW 10,000), and biotinamido caproate-*N*-hydroxysuccinimidyl ester (biotin-X-NHS) were purchased from Molecular Probes (Eugene, OR). Creatine kinase, ATP, GTPγS, and creatine phosphate were purchased from Boehringer Mannheim (Mannheim, Germany). Purified bovine brain calmodulin and the sodium salts of cAMP, phorbol 12-myristate 13-acetate, H89, Go6976, and KN-62 were from Calbiochem (San Diego, CA). All other chemicals were purchased from Sigma (St. Louis, MO). Tissue culture reagents were obtained from the University of California, San Francisco, tissue culture facility. Transparent plastic vials were from Continental Labsystems-Denley (Needem Heights, MA). Amino dextran was biotinylated by reaction at 10 mg/mL in 0.1 M $NaHCO_3$ for 2 h at 20°C with a fivefold excess of biotin-X-NHS. Products were dialyzed against 10 mM Na_2CO_3 for 36–72 h and lyophilized.

Cell culture

Baby hamster kidney cells (BHK-21 [C-13] ATCC CCL 10, passages 1–15 after cloning) were grown at 37°C in 95% air/5% CO_2 in DME-21 medium supplemented with 10% heat-inactivated (20 min at 56°C) fetal bovine serum and 1% penicillin-streptomycin. Cells were plated at a density of 10^5/mL on 10-cm-diameter tissue culture-treated plastic dishes (Falcon Labware) and used when confluent.

Endosome labeling procedures

Cells were released into suspension by a 20–30-min incubation in Ca^{2+}- and Mg^{2+}-free phosphate buffered saline containing 10 mM D-glucose, 10 mM Hepes-NaOH (pH 7.4), and 4 mM EGTA at 37°C in a 95% air/5% CO_2 atmosphere. The suspended cells were washed twice with Hepes buffered saline (20 mM Hepes-NaOH pH 7.4, 5 mM KCl, 5 mM D-glucose, and 0.15 M NaCl) by centrifugation (800 × g) for 10 min at 4°C. Cells were labeled in suspension by a 10-min incubation at 37°C with 0.5 mg/mL B-av in 50 mM Na_2CO_3, 5 mM KCl, 10 mM D-glucose, 12 mM Hepes-NaOH (pH 7.4), 75 mM NaCl, or 4 mg/mL biotin-dextran (in phenol red-free DME-H21 containing 10 mM Hepes-NaOH and 5 mM D-glucose). Labeled cells were then washed three times with Hepes buffered saline by centrifugation (800 × g, 5 min, 4°C) and then pelleted in homogenization buffer (250 mM D-glucose, 3 mM imidazole pH 7.4, 2 μg/mL aprotinin, 2 μM pepstatin) at 3000 × g for 15 min at 4°C.

Preparation of endosome fractions and cytosol

Cells were homogenized by passage through a 22-gauge steel needle until cell breakage was estimated to be 80% by phase contrast microscopy (Emans et al., 1993; Gruenberg and Gorvel, 1992). Nuclei and cell debris were removed by centrifugation (3000 × g) for 10 min at 4°C. The resultant postnuclear supernatants (8–13 mg protein/mL) were pooled and snap frozen in liquid nitrogen before storage at −80°C. Rat liver cytosol was prepared as described (Bomsel and Mostov, 1993), snap frozen, and stored at −80°C. Protein concentration was determined by the Bradford microassay (Bradford, 1976).

Cell-free fusion reactions

Postnuclear supernatants or cytosol fractions were rapid thawed at 40°C for 30 s and placed on ice. Solution concentrations were raised to 50 mM KOAc, 400 μg/mL unlabeled avidin, 12.5 mM Hepes-NaOH (pH 7.4), 1 mM DTT, and 2.5 mM MgOAc as described (Gruenberg and Gorvel, 1992) and incubated for 1 min at 4°C. Complete assay mixtures (generally 25 μL [acceptor] avidin-labeled fraction: 25 μL [donor] biotin-labeled fraction) were deposited in plastic vials precoated with rat liver cytosol (2 mg protein/mL in homogenization buffer, 12 h at 4°C). An ATP-regenerating mixture (Colombo et al., 1992a) (freshly dissolved 6400 units/mL creatine kinase in 10 mM Na_2HPO_4) was mixed with an equal quantity of homogenization buffer and then mixed 1:1 with 100 mM ATP (pH 7.2–7.6, adjusted with NaOH) and 800 mM creatine phosphate. 1.5 μL of the ATP regenerating mixture was added to the assay mixture in the vials (final 1 mM ATP, 8 mM creatine phosphate, 32 units/mL creatine kinase). In some experiments, ATP was depleted by addition to the assay mixture of 1.5 μL of 1500 units/mL yeast hexokinase lyophilizate in 250 mM D-glucose (30 units/mL final) (Balch et al., 1984). Assays were generally begun by rapid warming of the mixture to 37°C (<2 min) in a custom-designed microscope stage (see below) and continued for >1 h. At the completion of the assay, biotin methyl ester (2 μL of 50-mM stock in DMSO) was added to the reaction mixture in situ to give maximum B-av fluorescence for determination of the absolute fusion efficiencies (see below).

Fluorescence instrumentation

A computer-controlled multisample microfluorimeter was constructed on the chassis of an upright Nikon Labophot (Melville, NY) microscope. A 14.0 cm × 17.5 cm × 3.0 cm aluminum block was machined to give a centered insert, which accommodated six plastic vials. A 36-inch-long, three-sixteenth-inch-diameter heating wire (Cole Palmer, Niles, IL) and a PVC-coated, type J thermocouple were embedded in the block for continuous temperature control. Temperature control was accomplished with a closed-loop feedback controller (Yokogawa model 89700, Cole Palmer) coupled to the thermocouple and the heating wire. Temperature was maintained constant to within 0.1°C. The block was mounted on the microscope stage. One-dimensional stage translation was accomplished by mechanical coupling of the stage translator to a five-phase microstepper motor (UPS502–535MA, Nyden Corp., Santa Clara, CA) driven by a MAC 300 (Nyden) motor controller. Stage x-position was computer controlled with a precision of better than 0.05 mm and a maximum velocity of 50 mm/s.

The light source was a 100-W tungsten-halogen lamp powered by a stabilized power supply (model 68735, Oriel, Stratford, CT). The lamp was housed and light collimated by a Nikon focusing accessory designed as a light source for epi-illumination. Excitation light was filtered by two 485 ± 22 nm six-cavity interference filters (blocked from UV to IR) in series (Omega Optical, Brattleboro, VT). The monochromatic light was reflected by a 45° concave mirror and focused onto the sample by a plano-convex glass lens (focal length 5 cm) fixed beneath the stage. Emitted fluorescence was collected by a custom-built objective (N.A. 0.16, working distance 5 cm) and filtered by three 535 ± 35 nm interference filters (Omega Optical) in series mounted directly above the objective. The signal was detected by an 11-dynode photomultiplier (R928S, Hamamatsu, Middlesex, NJ) powered by a dc high-voltage supply (Ealing model 110, Natick, MA). The signal was amplified and digitized at 30 Hz by a 12-bit analog-to-digital converter (ADALAB, Interactive Microware, State College, PA). Motor movement and signal acquisition were controlled by a 486 PC. Software was written in Quick Basic 4.5 to move the stage from well to well with specified dwell times (generally 1 s, average of 25 points at 40 ms/point, 24 wells measured/min) and to record averaged photomultiplier signals together with corresponding times. The microscope optics were encased in a light-tight box and mounted upon a self-leveling floating optical table (Newport NS 1000, Irvine, CA). The light source and the detection electronics were stable over 1 h to better than 0.2% as judged from the signal from fluorophore standards.

Data analysis

Data arrays were stored as space delimited ASCII files for analysis by EXCEL software and custom-written macros. Fluorescence intensity (F) was converted to the percentage fusion of B-av labeled endosomes by correction for signal background and normalization to the maximal intravesicular B-av signal. Maximal total B-av fluorescence was measured at the completion of each assay by addition of biotin methyl ester. The fraction of total B-av that was intravesicular was determined from the fluorescence enhancement of an acceptor endosome fraction on addition of biotin dextran (membrane impermeant) to enhance the fluorescence of external B-av, followed by addition of biotin methyl ester (membrane permeant) to enhance the fluorescence of all B-av. Generally, 20–50% of B-av was intravesicular.

RESULTS

Endosome–endosome fusion resolved in real time

Separate populations of endosomes from BHK-21 cells were labeled by internalization for 10 min at 37°C in intact cells with either B-av ("acceptor" endosomes) or biotinylated-dextran ("donor" endosomes). Previous research demonstrated that B-av is a fluid phase marker of endocytosis and is delivered to fusogenic early endosomes under these conditions in vivo (Emans et al., 1995). Postnuclear supernatant fractions containing the acceptor and donor endosomes were prepared as described in Materials and Methods and mixed in a buffer containing an ATP-regenerating or -depleting system. Excess unlabeled nonfluorescent avidin was added to scavenge extravesicular biotin dextran and thus prevent any fluorescence enhancement of extravesicular B-av. Assay mixtures were placed in the microfluorimeter at 37°C for continuous measurement of B-av fluorescence.

Endosome–endosome fusion results in an enhancement of B-av fluorescence as donor endosomes containing an excess of biotin dextran fuse with acceptor endosomes containing B-av. Fig. 1A shows the time course of fluorescence enhancement of B-av on incubation of the donor and acceptor fractions at 37°C in the presence of an ATP-regenerating (+ATP) or -depleting (-ATP) system. In the presence of an ATP-regenerating system, the "fusion signal" consisted of a lag phase (10–15 min) in which little rise

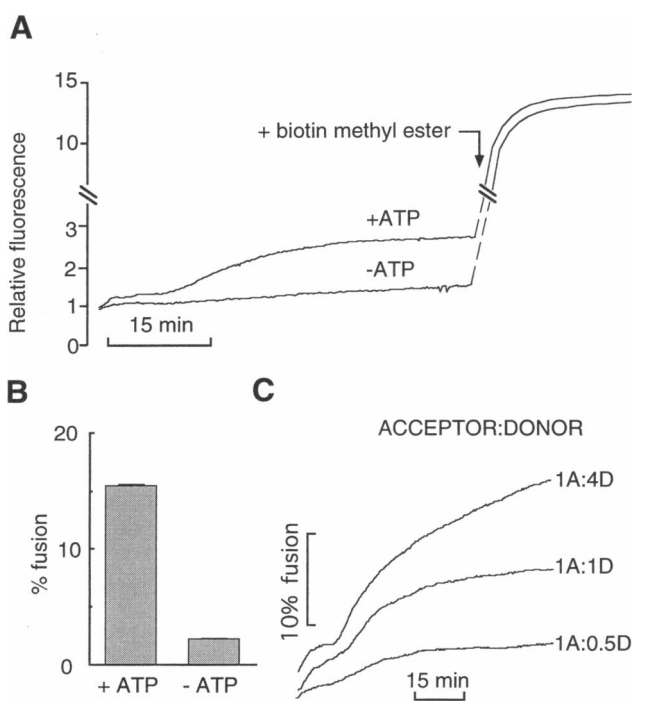

FIGURE 1 Real-time assay of endosome–endosome fusion. (A) Postnuclear supernatants were prepared from cells that internalized B-av or biotin-dextran for 10 min at 37°C. Endosome fractions (8 mg total protein/mL) were mixed in fusion buffer containing an ATP-regenerating system (+ATP) or an ATP-depleting system (−ATP). Assays were performed at 37°C. Maximum BODIPY fluorescence was obtained by addition of membrane-permeable biotin methyl ester (+biotin methyl ester). (B) Mean ± SE for percentage fusion at 1 h: n = 27 (+ATP), n = 12 (−ATP). (C) A constant volume of acceptor endosomes was diluted with increasing volumes of donor, supplemented with an ATP-regenerating system (representative of four sets of experiments). Data are expressed as % fusion as described in Materials and Methods.

in fluorescence occurred, followed by a monotonic rise to a stable level after ~45 min. A lag phase has been reported in comparable classical assays of endosome fusion (Diaz et al., 1989a). In addition, a small increase in fluorescence (<5% fusion) was often observed in the first 2 min of the assay (see below). At the end of the assay, a cell-permeable biotin derivative (biotin methyl ester) was added to give the maximal B-av fluorescence enhancement for calculation of fusion efficiency (see Materials and Methods). The ATP-dependent fluorescence signal was 15 ± 0.1% (SE, n = 27) of the latent avidin signal after addition of biotin methyl ester, compared with 2.2 ± 0.1% (n = 12) in the presence of the ATP-depleting system (Fig. 1 *B*).

The fusion signal was sensitive to temperature. Fusion was undetectable when the assay was carried out at 22°C and 26°C; at higher temperatures, where fusion could be detected, the percentage fusion at 1 h relative to that at 37°C was 58 ± 8% (32°C, n = 3) and 46 ± 7 (42°C, n = 3). These data are in qualitative agreement with previous temperature-dependence studies (Braell, 1987, 1992; Colombo et al., 1992a).

In control studies (in the presence of an ATP-regenerating system), addition of detergent (0.1% Triton X-100) at 0 or 60 min did not produce an enhancement in fluorescence signal, indicating that sufficient excess unlabeled avidin was present to block any signal increase from extravesicular B-av. The fluorescence of BODIPY-fluorescence from a "blank" assay containing acceptor endosomes alone changed by <1% over 60 min, indicating absence of photobleaching under the experimental conditions. It is noted that, for experiments performed on six parallel samples, each vial was illuminated for only ~7% of the time; furthermore, the large (4-mm-diameter) spot size of the incident beam produced a relatively low illumination intensity in the aqueous sample.

The dependence of the fusion signal on the relative proportion of acceptor and donor endosomes was investigated. Fig. 1 *C* shows that an increasing proportion of donor (biotin-containing) endosomes produced a larger fusion signal with little effect on the lag phase. This result is in agreement with the prediction that the probability of an avidin-containing endosome successfully fusing with a biotin-containing endosome should increase with the number of available biotin-containing endosomes. It is also noted that the relative proportion of donor and acceptor endosomes (at constant total protein) had relatively little influence on the duration of the lag phase (see below).

Sensitivity of the assay to known modulators of endosome fusion

There is a considerable body of evidence indicating that endosome fusion is sensitive to modulators of monomeric and trimeric GTPase activity (Colombo et al., 1992b; Gorvel et al., 1991; Lenhard et al., 1994; Mayorga et al., 1989; Colombo et al., 1994). Assays were performed as in Fig. 1 *A* with addition of the nonhydrolyzable GTP analog GTPγS just before the assay (Fig. 2A). Fusion was effectively blocked at 50 μM GTPγS, in agreement with previous observations (Gorvel et al., 1991). Interestingly, the small increase in fluorescence over the first few minutes was not blocked by GTPγS. The compound AlF_4^- activates trimeric G proteins but does not affect ras-like small GTPases (Kahn, 1991; Stryer and Bourne, 1986; Gilman, 1987). Assays were carried out with $AlCl_3$ alone, KF alone, or $AlCl_3$ and KF in combination to give the final specified concentrations of AlF_4^- (Fig. 2 *B*). $AlCl_3$ and KF had little effect individually but inhibited fusion when added together, in agreement with previous cell-free-fusion studies (Mayorga et al., 1989). The *N*-ethylmaleimide (NEM) sensitivity of endosome fusion has implicated NEM-sensitive factor in endosome fusion; effects of NEM can be reversed by addition of purified NEM-sensitive factor (Diaz et al., 1989b). To determine whether an NEM-sensitive factor was required here, we performed experiments with endosomes pretreated with 1 mM NEM for 30 min at 4°C. NEM pretreatment completely abolished the fusion signal (Fig. 2 *B*). Taken together, the ATP and temperature dependencies of the fusion signal, and its sensitivity to GTPγS, AlF_4^-,

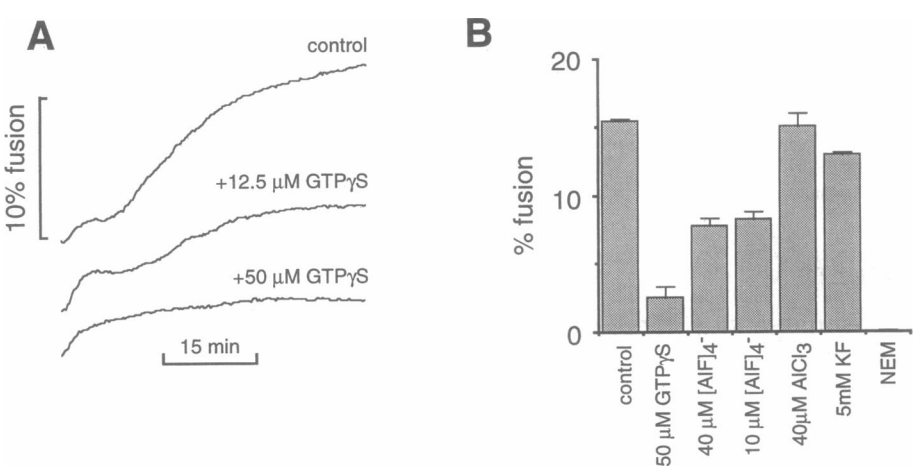

FIGURE 2 Sensitivity of endosome–endosome fusion to known modulators of fusion, nucleotide analogs, and NEM. Acceptor and donor fractions were mixed, and the assays were carried out at 37°C in the presence of an ATP-regenerating system. (*A*) GTPγS was added before the temperature was raised. Plots are representative of six sets of experiments. (*B*) Summary (mean ± SE, n = 3) of endosome fusion inhibition studies. Where indicated, acceptor and donor fractions were separately pretreated with 1 mM NEM for 30 min at 4°C. Excess NEM was quenched by 2 mM DTT for 15 min at 4°C before mixing endosome fractions, adding the ATP-regenerating system, and raising the temperature (n = 6).

and NEM, support the conclusion that the fluorescence assay is detecting endosome–endosome fusion.

Endosome–endosome fusion is preceded by a lag phase

Fig. 1 *A* showed that the increase in endosome fusion was preceded by a lag phase of 10–15 min during which little fusion was detected, similar to previous observations for cell-free endosome fusion (Diaz et al., 1989a) and intra-Golgi transport (Balch et al., 1984). The characteristics of the lag phase were investigated under conditions in which fusion was reversibly inhibited (low temperature) or would not be detected (separate incubation of acceptor and donor membranes at 37°C).

The lag time was significantly reduced by separate preincubation of acceptor and donor fractions at 37°C (in the presence of an ATP-regenerating system) for a time comparable with the lag time (Fig. 3*A*; the dashed lines indicate preincubation time period). Preincubation of the donor and acceptor fractions together at 4°C had little effect on the lag (Fig. 3 *A*, 4°C coincubation), yet coincubation at 22°C, a temperature that blocks fusion (see above), markedly reduced the lag phase. The lag time was progressively shortened with increasing coincubation time at 22°C. Also, the lag phase was nearly eliminated when the assay was performed at higher concentrations of endosomes and cytosol (13 mg protein/mL; Fig. 3 *B*). At this higher protein concentration, coincubation of endosome fractions at 22°C resulted in an increased rate of fusion after the temperature was increased to 37°C (Fig. 3 *C*).

As explained below, the preincubations and increased protein concentration could reduce the lag time by permitting one or more of the following rate-limiting steps to occur: 1) binding of a soluble protein to the endosome surface (priming), 2) binding of endosomes to form a prefusion intermediate (binding/recognition), and/or 3) creation of a transport intermediate that accumulates because it is not consumed by fusion (vesicle budding). It was reasoned that the results support possibility 1 in which there is priming of endosomes, likely corresponding to the rate-limiting binding of a soluble protein required for endosome fusion (see Discussion).

Endosome fusion is sensitive to cytosolic second messengers

The fluorescence assay was next utilized to test whether in vitro fusion of early endosomes is regulated by activation of protein kinases involved in signal transduction: protein kinase A, protein kinase C, and Ca^{2+}-calmodulin-dependent protein kinase. Representative fusion data are shown in Fig. 4, and the results from a series of studies are summarized in Fig. 5. Addition of the protein kinase A activator cAMP remarkably reduced the extent of fusion without blocking the initial early fusion signal (Fig. 4 *A*). Addition of the kinase inhibitor H89 had a slight stimulatory effect on fusion but at concentrations of up to 2.5 μM did not reverse the cAMP effect (data not shown). Activation of protein kinase C by PMA or inhibition by Go6976 had no significant effect on fusion. Inhibition of the Ca^{2+}-calmodulin kinase with KN-62 did not affect fusion; however, addition of purified bovine brain calmodulin (single band by SDS-PAGE, not shown) at concentrations lower than those used in permeabilized cell assays of exocytosis (Chamberlain et al., 1995) inhibited fusion (Fig. 4 *B*).

DISCUSSION

The initial aim of this study was to develop a sensitive fluorescence assay to monitor cell-free endosome fusion continuously in real time. The development of a real time assay of endosome fusion was inspired by the real-time assay of influenza virus: cell fusion and the subsequent

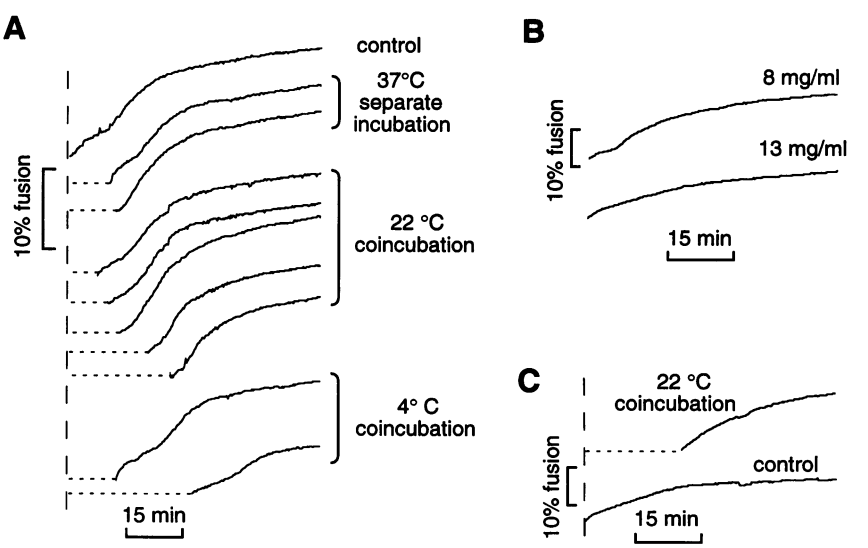

FIGURE 3 The lag phase is sensitive to preincubation, temperature, and protein concentration. (*A*) The control assay was performed as in Fig. 1 *A*. Acceptor and donor fractions (8 mg protein/mL) were separately preincubated at 37°C (37°C separate incubation) or together at 22°C (22°C coincubation) or 4°C (4°C coincubation). An ATP-regenerating system was present during the preincubations. The dashed lines indicate the time of preincubation. Plots are representative of four sets of experiments. (*B*) The lag phase was abolished by carrying out the fusion assay at a higher protein concentration (13 mg/mL). (*C*) At the higher concentration (13 mg protein/mL), incubation of endosome fractions at 22°C (22°C coincubation) resulted in an increased initial rate of fusion. Representative of three sets of experiments.

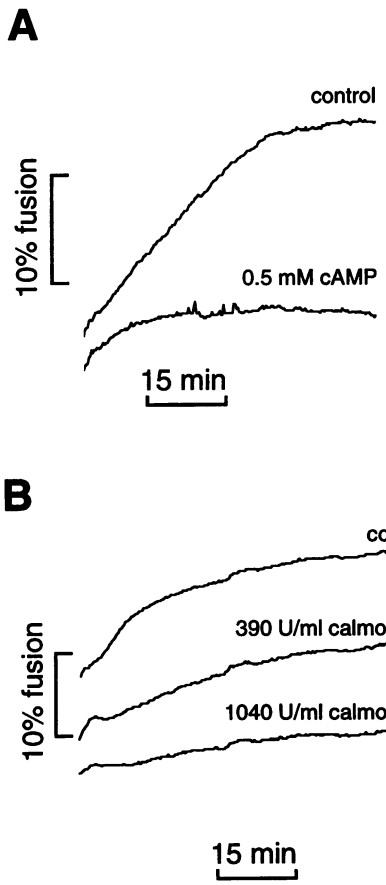

FIGURE 4 Endosome fusion is sensitive to cAMP and calmodulin. (*A*) Inhibition of endosome fusion by cAMP. Curves shown are representative of 10 experiments in which cAMP was added just before the assay. (*B*) Purified bovine brain calmodulin was added before the assay was supplemented with an ATP-regenerating system and the temperature was raised. Plots are representative of eight sets of experiments.

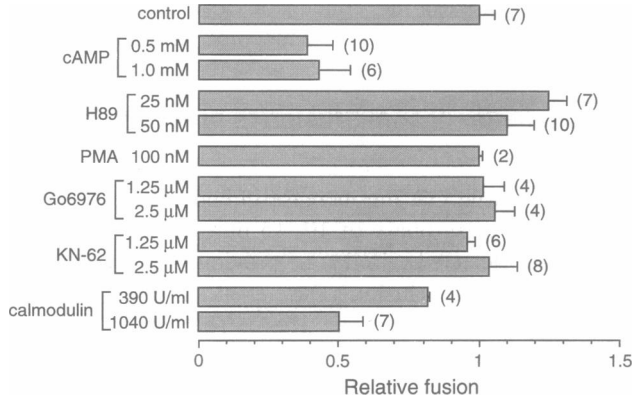

FIGURE 5 Summary of effects of cytosolic second messengers and inhibitory factors on fusion of early endosomes. Data indicate percentage fusion at 60 min (mean ± SE), with numbers of experiments shown in parentheses.

elegant elucidation of viral fusion mechanisms (Zimmerberg et al., 1995; Hoekstra and Klappe, 1993; Puri et al., 1993). Fusion of early endosomes in BHK cells was chosen as the model system because of the relative ease of endosome loading with fluorescent markers and the substantial body of experimental data from classical assays on the factors regulating fusion (Gruenberg and Howell, 1989). Our assay exploited the discovery that B-av undergoes a large, rapid, irreversible, and pH-independent fluorescence enhancement on biotin binding and that this enhancement could be used to quantify endosome fusion in vivo (Emans et al., 1995). Endosome fusion could be reproducibly measured in a relatively crude postnuclear supernatant fraction with high sensitivity from endosomes derived from as few as 4×10^4 cells.

As mentioned in the Introduction, the fluorescence assay has several advantages over conventional biochemical assays of the intermixing of endosomal contents. The fluorescence assay permits continuous quantitative resolution of vesicle fusion over time in a single experiment, whereas conventional assays are limited to single-time-point resolution per assay. Generally, conventional assays have been applied to measure the extent of fusion at a predetermined end point and give no information on the kinetics of fusion during the experiment; in some studies, limited time resolution of the order of tens of minutes was obtained from measurements performed at multiple time points (Aniento et al., 1993; Diaz et al., 1989a). The real-time assay here provides a complete profile of fusion kinetics in a single assay, permitting the resolution of rate-limiting processes (e.g., lag phases) and the determination of the effects of putative regulatory factors on the rate *and* extent of endosome fusion. We have estimated that the present-day cost of the classical avidin assay is >12 U.S. dollars per single time point (estimated from Gruenberg and Gorvel, 1992), compared with a total cost of <1 dollar for a complete time course with the fluorescence assay.

In addition to the kinetic information about fusion rates, the assay provides accurate and reproducible information on the extent of fusion at specific end points (see Fig. 1 *A*). In agreement with previous results from research that utilized biochemical assays, the fluorescence assay indicated that fusion of early endosomes was sensitive to ATP, temperature, a nonhydrolyzable guanine nucleotide analog, activation of trimeric G proteins, and the sulphydryl modifier *N*-ethylmaleimide. The fluorescence assay thus displays qualitatively similar characteristics to established assays of cell-free endosome fusion, and the findings here are in agreement with a large body of evidence that implicates a key role in endosome fusion of monomeric GTPases, trimeric G proteins, and NSF (Colombo et al., 1992b; Diaz et al., 1989b, Gorvel et al., 1991; Lenhard et al., 1994; Colombo et al., 1994).

There were several important technical considerations in the design of the microfluorimeter for measurement of endosome fusion. Microscope optics were chosen to quantify fluorescence in a small volume of a turbid endosome suspension. A movable microscope stage was

constructed to make parallel measurements in multiple samples over an extended assay period (generally >1 h) with a time resolution of seconds. 180° illumination and detection optics were chosen over epi-illumination based on initial results showing significant autofluorescence (compared to a sample signal) from dichroic mirrors. In addition, because of the small BODIPY fluorescence enhancement signal, the illumination and detection electronics were selected for high stability and low drift; it was found that commercial multiplate fluorescence readers did not have adequate sensitivity or stability to detect endosome fusion in our assay.

The time course of endosome fusion demonstrated a distinct lag phase that was dependent on protein concentration and various incubation conditions (Fig. 3). The lag was remarkably diminished by preincubation of endosome fractions separately with ATP at 37°C and by coincubation of endosomes at 22°C, a temperature at which fusion did not occur. Several rate-limiting processes could account for these observations; they include 1) binding of a soluble protein to the endosome membrane (priming), 2) binding of endosomes to form a prefusion intermediate (binding/recognition), and 3) creation of a transport intermediate that accumulates because it is not consumed by fusion (vesicle budding). It is unlikely than an endosome–endosome interaction (binding/recognition) is responsible for the lag because preincubation of endosomes separately at 37°C abolished the lag. The observations that coincubation at 22°C diminished the lag but that coincubation at 4°C had no effect suggest that vesicle budding is not the rate-limiting process accounting for the lag. Vesicle budding is temperature sensitive in vitro (Tooze and Huttner, 1990), and there is biochemical evidence that endosomes fuse directly with endosomes (Emans et al., 1993).

Our working hypothesis is that a "priming" factor must be recruited to the endosome before fusion and that this process is inhibited at 4°C but can proceed at 22°C. A potential candidate for such a priming factor is ARF6, a member of the ARF-like multigene family defined by the ability to activate cholera toxin (Moss and Vaughan, 1995). ARFs have been demonstrated to regulate the priming of endosome membranes before fusion and to stimulate the rate of fusion in vitro (Lenhard et al., 1992, 1994). Overexpressed ARF6 localizes to endosomes and to the plasma membrane (D'Souza-Schorey et al., 1995; Peters et al., 1995) and slows the rate of transferrin uptake without effect on the secretory pathway (D'Souza-Schorey et al., 1995). Association of the AP-1 adaptor complex with Golgi membranes is mediated by ARF1, and a lag phase that precedes binding has been proposed to represent the time required to recruit ARF1 to the membrane (Traub et al., 1993). The association of ARF with Golgi has also been demonstrated to be temperature sensitive and blocked at 0°C (Traub et al., 1993). Further studies are indicated to test the hypothesis that the lag phase is dependent on ARF6 recruitment.

Cyclic AMP activates a diverse set of cellular responses generally involving protein phosphorylation by the protein kinase A pathway. The rate of fluid-phase endocytosis is slowed by cAMP agonists in a number of cell types (Zen et al., 1992; Bradbury and Bridges, 1992). In vitro endosome fusion was found here to be remarkably inhibited by addition of cAMP. Further research will be required to determine whether this inhibition is a primary interaction of cAMP with the fusion machinery or a secondary effect on other regulators of endosomal fusion such as Gs (Colombo et al., 1994). In any case, the cAMP effect provides evidence that cytosolic second messengers modulate endosome fusion in vitro and may provide an explanation for the inhibition of endocytosis in intact cells by cAMP agonists.

Calmodulin has been demonstrated to be involved in exocytosis in chromaffin cells (Chamberlain et al., 1995) and endocytosis in yeast (Mu et al., 1995). Calmodulin kinase antagonists perturb apical and basolateral endocytic pathways in polarized cells, suggesting a role in maintaining separate endocytic pathways (Hunziker, 1994; Apodaca et al., 1994). The addition of purified bovine brain calmodulin significantly inhibited endosome fusion (Fig. 5). A calmodulin-binding protein present in early endosomes was cloned recently (EEA-1; Mu et al., 1995) and shares homology with yeast proteins involved in endocytosis [FAB1] and vacuolar protein sorting [VPS11, VPS18p, VAC1] (Robinson et al., 1991; Weisman and Wickner, 1992). EEA-1 may thus be the calmodulin target protein responsible for the inhibition of fusion found here.

The fluorescence-based cell-free assay established in this study should have a number of applications in the identification and mechanistic analysis of factors affecting the fusion of various intracellular vesicles. The possibility of comparing cell-free and intact-cell data directly, utilizing essentially the same assay conditions, should be useful in testing in vivo the implications of observations made in cell-free assays.

We thank Drs. S. Bicknese and N. Periasamy for help in software development, Dr. Keith Mostov for helpful discussions, and Laszlo Bocskai of the University of California, San Francisco, physiology machine shop for construction of the microfluorimeter. This research was supported by grants HL42368 and DK39354 from the National Institutes of Health grant R613 from the National Cystic Fibrosis Foundation and U.C.TRDRP grant 4RT-0072. Dr. Emans was supported by a 2-year postdoctoral fellowship from the Boehringer Ingelheim Fonds.

REFERENCES

Aniento, F., N. Emans, G. Griffiths, and J. Gruenberg. 1993. Cytoplasmic dynein-dependent vesicular transport from early to late endosomes. *J. Cell Biol.* 123:1373–1387.

Apodaca, G., C. Enrich, and K. E. Mostov. 1994. The calmodulin antagonist, W-13, alters transcytosis, recycling and the morphology of the endocytic pathway in Madin-Darby canine kidney cells. *J. Biol. Chem.* 269:19,005–19,013.

Balch, W. E., B. S. Glick, and J. E. Rothman. 1984. Sequential intermediates in the pathway of intercompartmental transport in a cell-free system. *Cell.* 39:525–536.

Bomsel, M., and K. E. Mostov. 1993. Possible role of both the alpha and beta gamma subunits of the heterotrimeric G protein, Gs, in transcytosis of the polymeric immunoglobulin receptor *J. Biol. Chem.* 268: 25,824–25,835.

Bradbury, N., and R. J. Bridges. 1992. Endocytosis is regulated by protein kinase A, but not protein kinase C in a secretory epithelial cell line. *Biochem. Biophys. Res. Comm.* 184:1173–1180.

Bradford, M. M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding *Anal. Biochem.* 72:248–254.

Braell, W. A. 1987. Fusion between endocytic vesicles in a cell-free system. *Proc. Natl. Acad. Sci. USA.* 84:1137–1141.

Braell, W. A. 1992. Detection of endocytic vesicle fusion in vitro, using assay based on avidin-biotin association reaction. *Methods Enzymol.* 219:12–20.

Braell, W. A., W. E. Balch, D. C. Dobbertin, and J. E. Rothman. 1984. The glycoprotein that is transported between successive compartments of the Golgi in a cell-free system resides in stacks of cisternae. *Cell.* 39:511–524.

Chamberlain, L. H., D. Roth, A. Morgan, and R. D. Burgoyne. 1995. Distinct effects of α-SNAP, 14–3-3 proteins and calmodulin on the priming and triggering of regulated exocytosis. *J. Cell Biol.* 130: 1063–1070.

Clague, M. J., S. Urbe, F. Aniento, and J. Gruenberg. 1994. Vacuolar ATPase activity is required for endosomal carrier vesicle formation. *J. Biol. Chem.* 269:21–24.

Colombo, M. I., J. M. Lenhard, L. S. Mayorga, and P. D. Stahl. 1992a. Reconstitution of endosome fusion: identification of factors necessary for fusion competency. *Methods Enzymol.* 219:32–44.

Colombo, M. I., L. S. Mayorga, P. J. Casey, and P. D. Stahl. 1992b. Evidence of a role for heterotrimeric GTP-binding proteins in endosome fusion. *Science.* 255:1695–1697.

Colombo, M. I., L. S. Mayorga, I. Nishimoto, E. M. Ross, and P. D. Stahl. 1994. Gs regulation of endosome fusion suggests a role for signal transduction pathways in endocytosis. *J. Biol. Chem.* 269: 14,919–14,923.

Davey, J. S., S. M. Hurtley, and G. Warren. 1985. Reconstitution of an endocytic fusion event in a cell free system. *Cell.* 43:643–652.

Diaz, R., L. S. Mayorga, L. E. Mayorga, and P. Stahl. 1989a. In vitro clustering and multiple fusion among macrophage endosomes. *J. Biol. Chem.* 264:13,171–13,180.

Diaz, R., L. Mayorga, and P. Stahl. 1988. In vitro fusion of endosomes following receptor mediated endocytosis *J. Biol. Chem.* 263: 6093–6100.

Diaz, R., L. Mayorga, P. Weidman, J. Rothman, and P. D. Stahl. 1989b. Vesicle fusion following receptor mediated endocytosis requires a factor active in Golgi transport *Nature (London)* 339:398–400.

D'Souza-Schorey, C., G. Li, M. I. Colombo, and P. D. Stahl. 1995. A regulatory role for ARF6 in receptor-mediated endocytosis *Science.* 267:1175–1178.

Emans, N., J. Biwersi, and A. S. Verkman. 1995. Imaging of endosome fusion in BHK fibroblasts based on a novel fluorimetric avidin-biotin binding assay. *Biophys. J.* 69:716–728.

Emans, N., J.-P. Gorvel, C. Walter, V. Gerke, G. Griffiths, and J. Gruenberg. 1993. Annexin II is a major component of fusogenic endosomal vesicles. *J. Cell Biol.* 120:1357–1370.

Gilman, A. G. 1987. G proteins: transducers of receptor-generated signals. *Annu. Rev. Biochem.* 56:615–649.

Goda, Y., and S. R. Pfeffer. 1988. Selective recycling of the mannose-6-phosphate/IGF-11 receptor to the trans Golgi network in vitro. *Cell.* 55:309–320.

Goda, Y., and S. R. Pfeffer. 1989. Cell-free systems to study vesicular transport along the secretory and endocytic pathways. *FASEB J.* 3:2488–95.

Goda, Y., and S. R. Pfeffer. 1991. Identification of a novel, *N*-ethylmaleimide-sensitive cytosolic factor required for vesicular transport from endosomes to the trans-Golgi network in vitro. *J. Cell Biol.* 112:823–831.

Gorvel, J.-P., P. Chavrier, M. Zerial, and J. Gruenberg. 1991. Rab5 controls early endosome fusion in vitro. *Cell.* 64:915–925.

Gruenberg, J. E., and J.-P. Gorvel. 1992. In vitro reconstitution of endocytic vesicle function. *In*: Protein Targeting: A Practical Approach. A. L. Magee and T. Wileman, editors. Oxford University Press, New York. 187–215.

Gruenberg, J. E., G. Griffiths, and K. E. Howell. 1989. Characterization of the early endosome and putative endocytic carrier vesicles in vivo and with an assay of vesicle fusion in vitro. *J. Cell Biol.* 108:1301–1316.

Gruenberg, J. E., and K. E. Howell. 1989. Membrane traffic in endocytosis: insights from cell-free assays. *Annu. Rev. Cell Biol.* 5:453–481.

Hoekstra, D., and K. Klappe. 1993. Fluorescence assays to monitor fusion of enveloped viruses. *Methods Enzymol.* 220:261–276.

Hunziker, W. 1994. The calmodulin antagonist W-7 affects transcytosis, lysosomal transport and recycling but not endocytosis. *J. Biol. Chem.* 269:29,003–29,009.

Kahn, R. A. 1991. Fluoride is not an activator of the smaller (20–25 kDa) GTP-binding proteins. *J. Biol. Chem.* 266:15,595–15,597.

Kornfeld, S., and I. Mellman. 1989. The biogenesis of lysosomes. *Annu. Rev. Cell Biol.* 5:483–525.

Lenhard, J. M., M. I. Colombo, and P. D. Stahl. 1994. Heterotrimeric GTP-binding proteins (G proteins) and ADP-ribosylation factor (ARF) regulate priming of endosomal membranes for fusion. *Arch. Biochem. Biophys.* 312:474–479.

Lenhard, J. M., R. A. Kahn, and P. D. Stahl. 1992. Evidence for ADP-ribosylation factor (ARF) as a regulator of in vitro endosome–endosome fusion. *J. Biol. Chem.* 267:13,047–13,052.

Mayorga, L. S., R. Diaz, and P. D. Stahl. 1989. A regulatory role for GTP binding proteins in endocytosis. *Science.* 244:1475–1477.

Moss, J., and M. Vaughan. 1995. Structure and function of ARF proteins: activators of cholera toxin and critical components of intracellular vesicular transport activities. *J. Biol. Chem.* 270:12,327–12,330.

Mu, F. T., M. J. Callaghan, O. Steele-Mortimer, H. Stenmark, R. Parton, P. L. Campbell, J. McClusky, J. P. Yeo, E. P. Tock, and B. H. Toh. 1995. EEA1, an early endosome-associated protein. EEA1 is a conserved alpha-helical peripheral membrane protein flanked by cysteine "fingers" and contains a calmodulin-binding IQ motif. *J. Biol. Chem.* 270: 13,503–13,511.

Peters, P. J., V. W. Hsu, C. E. Ooi, D. Finazzi, S. B. Teal, V. Oorschot, J. G. Donaldson, and R. D. Klausner. 1995. Overexpression of wild-type and mutant ARF1 and ARF6: distinct perturbations of non-overlapping membrane compartments. *J. Cell Biol.* 128:1003–1017.

Puri, A., M. J. Clague, C. Schoch, and R. Blumenthal. 1993. Kinetics of fusion of enveloped viruses with cells. *Methods Enzymol.* 220:277–287.

Robinson, J. S., T. R. Graham, and S. D. Emr. 1991. A putative zinc finger protein, saccharomyces cerevisiae Vps18p, affects late Golgi functions required for vacuolar protein sorting and efficient alpha-factor prohormone maturation. *Mol. Cell Biol.* 12:5813–5824.

Sollner, T., S. W. Whiteheart, M. Brunner, H. Erdjument-Bromage, S. Geomanos, P. Tempst, and J. E. Rothman. 1993. SNAP receptors implicated in vesicle targeting and fusion. *Nature (London)* 362:318–324.

Stryer, L., and H. R. Bourne. 1986. G proteins: a family of signal transducers. *Annu. Rev. Cell Biol.* 2:391–419.

Tooze, S. A., and W. B. Huttner. 1990. Cell-free protein sorting to the regulated and constitutive secretory pathways. *Cell.* 60:837–847.

Traub, L. M., J. A. Ostrom, and S. Kornfeld. 1993. Biochemical dissection of AP-1 recruitment onto Golgi membranes. *J. Cell Biol.* 123:561–573.

Weisman, L. S., and W. Wickner. 1992. Molecular characterization of VAC1, a gene required for vacuole inheritance and vacuole protein sorting. *J. Biol. Chem.* 267:618–623.

Woodman, P. G., D. I. Mundy, P. Cohen, and G. Warren. 1992. Cell-free fusion of endocytic vesicles is regulated by phosphorylation. *J. Cell Biol.* 116:331–338.

Zen, K., J. Biwersi, N. Periasamy, and A. S. Verkman. 1992. Second messengers regulate endosomal acidification in Swiss 3T3 fibroblasts. *J. Cell Biol.* 119:99–110.

Zimmerberg, J., R. Blumenthal, D. P. Sarkar, M. Curran, and S. J. Morris. 1995. Restricted movement of lipid and aqueous dyes through pores formed by influenza hemagglutinin during cell fusion. *J. Cell Biol.* 127:1885–1894.