Sequential Actions of Rab5 and Rab7 Regulate Endocytosis in the *Xenopus* Oocyte

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Abstract. To explore the role of GTPases in endocytosis, we developed an assay using *Xenopus* oocytes injected with recombinant proteins to follow the uptake of the fluid phase marker HRP. HRP uptake was inhibited in cells injected with GTP\textsubscript{ε}S or incubated with aluminimum fluoride, suggesting a general role for GTPases in endocytosis. Injection of Rab5 into oocytes, as well as Rab5:Q79L, a mutant with decreased GTPase activity, increased HRP uptake. Injection of Rab5:S34N, the dominant-negative mutant, inhibited HRP uptake. Injection of N-ethylmaleimide–sensitive factor (NSF) stimulated HRP uptake, and ATPase-defective NSF mutants inhibited HRP uptake when co-injected with Rab5:Q79L, confirming a requirement for NSF in endocytosis. Surprisingly, injection of Rab7:WT stimulated both uptake and degradation/activation of HRP. The latter appears to be due to enhanced transport to a late endosomal/prelysosomal degradative compartment that is monensin sensitive. Enhancement of uptake by Rab7 appears to function via an Rab5-sensitive pathway in oocytes since the stimulatory effect of Rab7 was blocked by co-injection of Rab5:S34N. Stimulation of uptake by Rab5 was blocked by Rab5:S34N but not by Rab7:T22N. Our results suggest that Rab7, while functioning downstream of Rab5, may be rate limiting for endocytosis in oocytes.

GTP binding proteins are versatile molecular switches (Bourne, 1988; Bourne et al., 1990), and during the past few years, there has been an explosion of interest in unraveling the role of these proteins, particularly low molecular weight GTPases, in membrane traffic (Balch, 1990; Barr et al., 1992). Evidence from a variety of sources, including the temperature-sensitive secretion mutants in yeast and in vitro reconstitution experiments in permeabilized and broken cell preparations, has established that at least two subfamilies of GTP binding proteins, namely the Rab and ADP ribosylation factor subfamilies, are required for membrane traffic (Pfeffer, 1994; Rothman, 1994). Heterotrimeric GTPases (Colombo et al., 1992) and ras (Barsagi and Feramisco, 1986) also appear to play significant roles.

Among the monomeric small GTPases, the Rab family is well characterized as regulators of intracellular trafficking during endocytosis and secretion (Zerial and Stenmark, 1993). Rab proteins are specifically localized to the cytoplasmic surface of the intracellular compartments that they subserve, such as ER (Rab1 and Rab2) (Chavrier et al., 1990; Tisdale et al., 1992), cis-Golgi (Rab1 and Rab2) (Chavrier et al., 1990; Tisdale et al., 1992), medial Golgi (Rab6) (Goud et al., 1990), trans-Golgi (Rab9) (Gravotta et al., 1990; Lombardi et al., 1993), early endosomes (Rab4 and Rab5) (Chavrier et al., 1990; van der Sluijs et al., 1991), late endosomes (Rab7 and Rab9) (Chavrier et al., 1990; Lombardi et al., 1993; Feng et al., 1995), and plasma membrane/endosomes (Rab5) (Chavrier et al., 1990).

In transient transfection experiments and in vitro fusion assays using enriched membrane preparations, Rab5 has been shown to play a role in the regulation of endocytosis (Grovel et al., 1991; Li and Stahl, 1993). Overexpression of Rab5 increases the rate of endocytosis and the recycling of the transferrin receptor. Moreover, Rab5-specific antiserum and Rab5 dominant-negative mutants specifically inhibit endosome fusion in vitro (Bucci et al., 1992; Li et al., 1994; Barbieri et al., 1994). While it has been shown that Rab5 regulates endocytosis at the level of early endosome fusion, the mechanism by which Rab5 exerts its function remains to be established. Moreover, transport of macromolecules along the endocytic pathway requires a series of highly coordinated and specific fusion events involving multiple cytosolic and membrane-bound factors including the N-ethylmaleimide–sensitive factor (NSF)\(^1\) (Diaz et al., 1988; Rothman, 1994) and a membrane-bound trypsin-sensitive factor (Colombo et al., 1991). Protein phosphorylation has also been shown to regulate the fusion of endosomal

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\(^{1}\) Abbreviations used in this paper: GST, glutathione-S-transferase; MBS, modified Barth’s saline; NSF, N-ethylmaleimide–sensitive factor; WT, wild type.
vesicles (Toumikoski et al., 1989). Certain lipids may also play a role in membrane docking and fusion. Both PLA₂ (Mayorga et al., 1993) and PI3 kinase (Li et al., 1995) are intimately involved in the regulation and/or action of Rab5. However, the exact relationship among all these factors in the overall mechanism of intracellular trafficking during endocytosis is largely unknown.

Both fluid phase and receptor-mediated endocytosis occur through vesicle fusion and transfer of endocytic materials from one compartment to another in a highly regulated fashion. Depending on the receptor system, ligands and receptors are internalized and sorted in the early endosomal compartment (Goldstein et al., 1985), often requiring acidic pH, and selected molecules are recycled back to the plasma membrane. Some are transported to the late endosomal compartment that may communicate with the trans-Golgi network or the lysosomal compartment (Yamashiro et al., 1984). The late endosome represents the convergence point of several pathways such as (a) the targeting of newly synthesized lysosomal enzymes and lysosomal membrane proteins to the lysosome (Griffiths et al., 1990; Riederer et al., 1994), (b) the killing of internalized microorganisms by phagocytes (Ludwig et al., 1991; Raft et al., 1990; Riederer et al., 1994), (c) the processing and presentation of antigens where major histocompatibility complex class II molecules are recycled to the plasma membrane after the formation of peptide–major histocompatibility complex class II complex in the late endosomal compartment (Cresswell, 1994; Germain, 1994; Qui et al., 1994). It is now clear that multiple Rab proteins, including Rab7, Rab9, and Rab24, and perhaps others to be identified, are associated with the late endosomal compartment (Chavrier et al., 1990; Lombardi et al., 1993; Ollkonen et al., 1993). Rab9 has been shown to regulate the traffic from the late endosome to the TGN (Lombardi et al., 1993; Riederer et al., 1994), and Rab7 has been shown to regulate the traffic from early to late endosomes (Wichmann et al., 1992; Feng et al., 1995) or from late endosomes to vacuoles (Schimmoller et al., 1993; Hass et al., 1995). Considerable progress has been made toward understanding the regulation of transport by specific RabS, each with its own family of effector proteins. However, the relation of one Rab to another and the regulation of intracellular pathways made up of a series of relays is poorly understood. In the present investigation, we have exploited the Xenopus oocyte as a model system to study the regulation of endocytosis by multiple RabS. Our data suggest that the action of one Rab may influence the function of a second Rab acting downstream.

Materials and Methods

mAbs used in this study include: 4F11, a mouse IgG2a mAb that recognizes mouse Rab5, generously provided by A. Wandinger-Ness (Northwestern University, Evanston, IL), and A46, a mouse IgM mAb that recognizes native NSF, kindly provided by J.E. Rothman (Sloan-Kettering Memorial Hospital, New York). Irrelevant IgG and IgM mAbs were purchased from Sigma Chemical Co. (St. Louis, MO) and used as control. Recombinant NSF wild-type and mutant proteins were generously provided by S.W. Whiteheart (University of Kentucky, Lexington). HRP-conjugated antibodies and enhanced chemiluminescence detection reagents were from Amersham Corp. (ECL, Arlington Heights, IL). All other reagents were from Sigma Chemical Co.

Preparation of Oocytes

Oocytes were dissected from adult female Xenopus laevis (Nasco, Fort Atkinson, WI) anesthetized with 3-aminobenzoic acid ethyl ester (1 g/liter) in ice water. The ovaries were agitated gently for 2 h in modified Barth’s saline (MBS) containing 10 calcium and 2 mg/ml type 1 collagenase at room temperature (Swick et al., 1992). After collagenase treatment, the oocytes were washed six times in MBS containing 5% BSA. Cells obtained in this manner were incubated overnight in MBS with BSA and gentamycin (50 μg/ml) at 4°C.

Expression and Purification of Recombinant Rabs and Mutants

The Rab5 fusion proteins used in this study including Rab5:Q79L, Rab5: S34N, and Rab5:ΔC4 were described previously (Li and Stahl, 1993; Li et al., 1994). Rab7 wild type (WT) and Rab7:T22N were expressed and purified as glutathione-S-transferase (GST) fusion protein as described for Rab5 (Li and Stahl, 1993; Li et al., 1994). To prepare fusion proteins, cDNAs were amplified by 25 cycles of PCR and subcloned into unique BamH1/EcoR1 restriction sites of the bacteria expression vector pGEX-3X (Pharmacia Biotech Inc., Piscataway, NJ). Recombinant proteins were expressed in large quantities as GST fusion proteins in Escherichia coli strain JM101. GST fusion proteins were affinity purified with glutathione–Sepharose.

Microinjection of Test Proteins in Xenopus Oocytes and HRP Uptake

Before microinjection, healthy oocytes (stage V or VI) were visually selected. Oocytes were injected with the indicated concentrations of test molecules in 50 nl of buffer A (20 mM Hepes, pH 7.4, containing 20 mM NaCl, 1 mM MgCl₂, and 100 μM ATP), and then allowed to recover in MBS at 18°C for 2 h. Finally, healthy oocytes were selected for HRP uptake. The injected oocytes were incubated in the presence of HRP (2 mg/ml) for 1 h at 18°C. HRP uptake by the oocytes was stopped by washing the cells three times with MBS containing 5% BSA. Individual oocytes were then lysed in 200 μl PBS containing 0.1% Triton X-100 and centrifuged, and the lysate was assayed for HRP. The enzyme assay was conducted in a 96-well plate (Costar Corp., Cambridge, MA) using o-phenylenediamine as the chromogenic substrate (Wolters et al., 1976). Briefly, the reaction was initiated by adding 5 μl of the lysate to 100 μl of 0.05 N sodium acetate buffer, pH 5.0, containing o-phenylenediamine (0.75 mg/ml) and H₂O₂ (0.006%). After incubation (5 min at room temperature), the reaction was stopped with 0.1 N H₂SO₄. The products were quantified by measuring the OD 490 nm in a microplate reader (Bio Rad Laboratories, Hercules, CA). Results were expressed as ng of HRP uptake per oocyte.

Western Blot Analysis of Rab5 and Mutants

Oocytes were injected with Rab5:Q79L or Rab5:ΔC4 (100 ng in 50 nl of buffer A) and incubated for 2 h at 18°C. The cells were then lysed, and crude membrane and cytosolic fraction were prepared by centrifuging at 70,000 rpm for 15 min in a TL-100 ultracentrifuge (Beckman Instruments, Inc., Palo Alto, CA). Each fraction (5 μg) was separated by SDS-PAGE and transferred onto nitrocellulose membranes, and Western blot analysis was carried out using polyclonal anti-Rab5 antibody and developed by enhanced chemiluminescence.

Degradation Assay Using Preloaded HRP in Oocytes

To monitor the inactivation/degradation of the marker protein, oocytes were preloaded with HRP (2 mg/ml) in MBS for 1 h at 18°C. Subsequently, cells were washed three times with ice-cold MBS to remove unbound HRP. HRP-preloaded cells were injected with the test proteins and incubated for 2 h at 18°C. The amount of HRP activity present after the incubation was determined after lysis of the oocytes.

To follow degradation, oocytes were incubated with 125I-HRP (500 μg/ml; 2 × 10⁶ cpm/μg) for 1 h at 18°C. The cells were then washed to remove unbound radioactivity. The preloaded cells were injected either with control buffer or Rab proteins and incubated in MBS for 2 h at 18°C, the media were collected, and the cells were lysed. TCA-soluble and -precipitable radioactivity both in the cell lysates and in the medium was determined by addition of 10% TCA followed by centrifugation.
For confocal microscopy, oocytes were injected with Rab7 or mutant proteins and allowed to recover for 1 h at 18°C. The cells were then incubated with HRP-FITC for 1 h at room temperature under slow rotation in the dark. Subsequently, the cells were washed three times with MBS and incubated in the dark for one additional hour. The labeled oocytes were then observed with an MRC 600 laser confocal microscope (Bio Rad Microscience, Cambridge, MA) using a ×63 plan apochromat oil immersion objective (Carl Zeiss, Inc., Thornwood, NY).

Results

Role of GTPases in Endocytosis in Xenopus Oocytes
To determine the function of GTPases in the endocytic pathway, we used HRP as a marker to follow endocytosis and transport from the cell surface to a lysosomal compartment. The results presented in Fig. 1 show that HRP uptake is linear with time when the cells are incubated with HRP at 2 mg/ml. All subsequent uptake experiments were carried out for 1 h with 2 mg/ml HRP. Many experiments were also carried out at a higher ligand concentration (5 mg/ml) with identical results.

To determine the role of GTPases in intracellular trafficking, we measured HRP uptake by oocytes after injection of GTPγS, a nonhydrolyzable analogue of GTP. About 80% inhibition of HRP uptake was obtained when GTPγS (20 μM; 50 nl per cell) was injected, suggesting that one or more GTPases are involved in endocytosis in oocytes (data not shown). HRP uptake was also inhibited by aluminum fluoride (data not shown) but was not significantly affected when the cells were microinjected with equivalent concentrations of aluminum sulfate or potassium fluoride. These results provide evidence for a role for heterotrimeric GTPases in endocytosis in oocytes (Mayorga et al., 1989; Chabre, 1990; Colombo et al., 1994).

Effect of Injected Rab GTPases on Oocyte Endocytosis
To explore the role of Rab GTPases in endocytosis in Xenopus oocytes, we microinjected different Rab proteins (100 ng) and followed HRP uptake. Both Rab5 and Rab7 significantly stimulated HRP uptake by oocytes by 1.5- to twofold (Fig. 2). This result is consistent with the finding that Rab5 stimulates endocytosis during transient expression in vivo and endosome–endosome fusion in vitro (Li and Stahl, 1993; Bucci et al., 1992; Li et al., 1994; Barbieri et al., 1994; Stenmark et al., 1994). The stimulation of HRP uptake by Rab7 was an unexpected but interesting observation. Injection of Rab4 had no effect on HRP endocytosis. We studied four different constructs of Rab5: Rab5: S34N, a dominant-negative mutant (Li and Stahl, 1993; Grovel et al., 1991; Burstein et al., 1992); Rab5:ΔC4 where the isoprenylation motif is deleted; Rab5:Q79L, a GTPase-defective mutant (Li et al., 1994; Burstein et al., 1992); and Rab5:WT and Rab5:Q79L enhanced uptake of HRP. Rab5:S34N, on the other hand, markedly inhibited HRP uptake by the oocytes (Fig. 3 a). Rab5:ΔC4 failed to associate with mem-

Figure 1. Time-dependent uptake of HRP by the oocytes. Oocytes were incubated with 2 mg/ml of HRP for different periods of time. The cells were washed three times with MBS and each oocyte was lysed in 200 μl of lysis buffer. Aliquots of the lysate were used to measure the amount of HRP present in each cell. The results, expressed as ng of HRP per oocyte, represent the average of eight independent observations.

Figure 2. Effect of different Rab proteins on HRP uptake in oocytes. Oocytes were injected with 100 ng of the indicated Rabs in 50 nl of microinjection buffer and incubated for 2 h at 18°C. The cells were then incubated with HRP (2 mg/ml) for 1 h at 18°C to determine the uptake rate. Results were expressed as percentage of uptake per oocyte with respect to control (220 ng per oocyte) ± SD (average of eight experiments).
branes and did not significantly affect HRP uptake. Rab5: DC4 was present exclusively in the cytosolic fraction (Fig. 3b). These results strongly suggest that frog oocytes have the ability to carry out the required posttranslational modification of Rab proteins, and that COOH-terminal isoprenylation is required for the binding of the Rab GTPase to the target membrane and for biological activity. Moreover, we have also found that Rab proteins are readily prenylated in vitro using oocyte cytosol in the presence of labeled substrate (data not shown). Rab5 protein was localized using polyclonal Rab5-specific antibodies (Alvarez-Dominguez et al., 1996).

Role of NSF in Rab5-Stimulated Endocytosis of HRP

NSF is required for in vitro endosome fusion (Diaz et al., 1989). To delineate the role of NSF in endocytosis in oocytes, we injected anti-NSF antibody into the cells and measured HRP uptake. The results presented in Fig. 5a demonstrate that anti-NSF antibody inhibits the stimulation of HRP uptake in cells injected with Rab5:Q79L when anti-NSF antibodies and Rab5:Q79L were coinjected. Injection of anti-NSF antibodies in control oocytes resulted in a modest reduction in uptake (data not shown). To confirm the participation of NSF in Rab5 function, we coinjected oocytes with Rab5:Q79L and His$_6$ NSF wild-type protein. The results in Fig. 5b show that Rab5:Q79L-stimulated HRP uptake is enhanced in the presence of NSF protein. NSF is a heterotrimer whose polypeptide subunits are made up of three distinct domains: an amino-terminal domain and two homologous ATP (D1 and D2) binding domains (Whiteheart et al., 1994). The ability of the D1 domain to hydrolyze ATP is required for NSF-mediated membrane fusion. The D2 domain is required for trimerization, but its ability to hydrolyze ATP is not absolutely required for NSF function. Two distinct mutations in the first ATP binding site (D1), i.e., D1-K266A and D1-E329Q, are known to affect ATP binding and hydrolysis, resulting in inactive forms of NSF (Whiteheart et al., 1994). To determine the role of the ATP binding domains of NSF in Rab5-mediated endocytosis, we coinjected Rab5:Q79L and different NSF mutant proteins into oocytes and followed HRP uptake. The results in Fig. 5b demonstrate that His$_6$ D1-E329Q and His$_6$ D1-K266A significantly inhibit HRP uptake mediated by Rab5:Q79L, indicating that ATP hydrolysis and binding are required for NSF activity and Rab5-mediated endocytosis.
Figure 5. Effect of NSF on HRP uptake by oocytes. (a) Cells were injected with either 100 ng of Rab5:Q79L with or without anti-NSF (200 ng) antibody. Uptake was carried out as described in Materials and Methods. Results were expressed as percentage of control uptake (180 ng/oocyte) from the average of eight experiments. (b) Oocytes were injected with 75 ng of Rab5:Q79L alone or with 100 ng of NSF and its mutants as indicated. Uptake was determined as described, and the results are expressed as percentage of control (220 ng per oocyte) uptake.

Figure 6. Rab7-mediated uptake of HRP by oocytes. Oocytes were injected with 100 ng of the indicated proteins and uptake was carried out with HRP-FITC for 1 h in the dark (1 mg/ml). Subsequently, the cells were washed three times with MBS, incubated in the dark for another 1 h, and then observed under the confocal microscope.
Effect of Rab7 and Its Mutants on HRP Uptake

As indicated above, injection of Rab7:WT (100 ng) into oocytes produced a marked stimulation in HRP uptake (Fig. 2). To characterize the effect of Rab7 on endocytosis, we examined the effect of Rab7 on the uptake HRP-FITC by confocal microscopy. The results presented in Fig. 6 show substantial increases in fluorescence when the cells were injected with Rab7:WT protein. Cells injected with Rab7:T22N appeared very similar to control cells. In fact, injection of Rab7:T22N showed significant inhibition of HRP-FITC uptake (data not shown). In control and Rab7:T22N injected cells, most of the staining was observed near the plasma membrane, presumably in early endosomal compartments. In Rab7:WT-injected cells, it is evident that HRP-FITC is localized to larger vesicles and much deeper into the cells, possibly reflecting the transport of the marker proteins into later compartments. To further confirm the effect of Rab5 and Rab7 on the endocytic pathway at the EM level, oocytes were injected with Rab5:WT and Rab7:WT, and then allowed to internalize WGA–gold particles for 60 min. The cells were then fixed and prepared for EM. Oocytes injected with Rab5 had substantially more early endosome–like vesicular structures, whereas a significant labeling of large multivesicular endosomes (1–1.5 μm) deeper inside the cells was observed when cells were injected with Rab7:WT. These data suggest that Rab7 induced the transport to a late endosome/prelysosome compartment. About 180–200 large multivesicular compartments were observed per square millimeter only in oocytes injected with Rab7:WT protein (data not shown).

Differential Kinetics of Rab5 and Rab7 on Endocytosis in Oocytes

Rab5 is an early endosomal Rab that regulates endosome fusion, whereas Rab7 is localized to the late endosomal compartment (Wichmann et al., 1992; Schimmoller et al., 1993; Feng et al., 1995). Our data indicate that both proteins stimulate HRP uptake in oocytes, suggesting an effect of both proteins on the early endocytic pathway. To check the effect of Rab5 and Rab7 on later events, we developed a more direct biochemical assay to measure the degradation of internalized HRP after injection of the test proteins, as a measure of transport to a degradative or late compartment. In this assay, oocytes were preloaded with HRP for 1 h at 18°C. HRP activity was then followed over time after injecting Rab5 or Rab7 proteins. The results presented in Fig. 7 show that ~80% of the HRP activity is lost within 2 h when the cells were injected with Rab7:WT protein. In contrast, most of the HRP activity is retained when the cells were injected with Rab5:WT protein, much like control cells. Thus, Rab7 not only promotes the internalization of HRP but also mediates apparent rapid degradation possibly by transferring internalized mole-
cules to a late compartment. Moreover, when the Rab7:WT-injected/HRP-preloaded cells were incubated in the presence of monensin (100 μM), ~80% of the HRP inactivation was prevented. We speculate that monensin prevents the inactivation and degradation of HRP either by blocking the transport to the later compartment or by increasing the pH of the lysosome-like compartment, which in turn inhibits the inactivation and/or degradation of HRP.

To examine the degradation of internalized HRP, oocytes were incubated with 125I-HRP for 60 min, after which they were injected with control buffer or Rab7. The data presented in Fig. 7b show the distribution of internalized 125I-HRP in oocytes after injecting Rab7:WT protein into the cells. 43% of the HRP was degraded by the Rab7:WT protein-injected cells. In contrast, only 25% of the pre-loaded HRP was degraded when the cells were injected with control buffer. In both sets of cells, the same amount of the TCA-precipitable radioactivity (50%) was recovered in the medium, suggesting that a substantial amount of recycling occurs in this preparation. However, Rab7 did not induce or enhance recycling or regurgitation of the HRP to the medium.

Effects of Rab5- and Rab7-negative Mutants on Rab5-stimulated Endocytosis

We next examined the effects of the dominant-negative mutants of Rab5 and Rab7 to determine which acts first in the sequence. Injection of Rab5:WT into oocytes stimulated HRP uptake (Fig. 8a) by about twofold. Coinjection of the Rab7 dominant-negative mutant (Rab7:T22N) with Rab5 had no effect on the Rab5-mediated stimulation of uptake. However, coinjection of the Rab5 dominant-negative mutant (Rab5:S34N) with Rab5:WT significantly at-
tenuated the Rab5:WT effect. On the other hand, injection of Rab7:WT produced a threefold increase in uptake, as shown in Fig. 8 b, and coinjection of the Rab7 dominant-negative mutant with Rab7:WT blocked the Rab7:WT effect. Interestingly, Rab5:S34N inhibited Rab7:WT-induced endocytosis. Moreover, Rab7:T22N and Rab5:S34N inhibited uptake when injected alone. Lastly, we examined the additive effects of Rab5 and Rab7 on endocytosis when coinjected. Injection of Rab5 and Rab7 separately increased endocytosis. Coinjection of Rab5 and Rab7 produced an additive effect on uptake of HRP (Fig. 9).

Discussion

The Xenopus laevis oocyte is an excellent cell model for investigating the mechanisms and the regulation of endocytosis (Opresko et al., 1980). Several characteristics of receptor-mediated endocytosis have been explored in the frog oocyte (Wall and Patel, 1987), and ultrastructural and biochemical studies have shown that intracellular transport of internalized ligands occurs via fusion between early endocytic vesicles and consecutively enlarged endosomes (Busson et al., 1989). In brief, these studies show that vitellogenin, the precursor to stored yolk protein, is taken up by receptor-mediated endocytosis. Internalized vitellogenin receptor–ligand complexes are selectively protelyzed and sequestered from the normal endocytic pathway with transport to yolk platelets. Selective targeting to the storage pathway appears to be dependent both on receptor occupancy and on selective ligand proteolysis (Opresko and Karpf, 1987). Fluid phase markers, on the other hand, in the absence of vitellogenin, are routed to the degradative pathway. In the work described in this paper, we have used HRP as a fluid phase marker.

In the last few years, it has become apparent that GTP binding proteins play an important role in endocytosis, and that a series of GTPases act in concert to effect transport from cell surface to lysosomes or the TGN. A large number of studies have shown that GTPases impair transport by interfering with budding or the fusion events, consistent with the conclusion that a large repertoire of GTPases is required for efficient endocytosis (Gomperts and Fernandez, 1985; Mayorga et al., 1989; Goda and Pfeffer, 1988; Damke et al., 1995).

Numerous reports indicate that several small GTPases regulate endocytosis. Most of these studies have employed transient overexpression of proteins using recombinant virus encoding the proteins of interest. This approach has been extremely useful in studying transport but is somewhat limited because usually only one variable can be changed at a time. We have developed an in vivo assay by microinjection of purified proteins into Xenopus oocytes followed by endocytosis of HRP. This approach takes advantage of the high endocytic capacity of oocytes and allows one to examine the effect of one or more proteins that can be injected simultaneously. These studies have led to some unexpected findings.

Two experiments, one with GTPyS and another with aluminum fluoride, indicate that one or more GTPases are involved in oocyte endocytosis and that GTP hydrolysis by at least one GTPase is required. Aluminum fluoride is a potent and reversible activator of the heterotrimeric GTP binding proteins (Chabre, 1990; Colombo et al., 1994), although recent experiments (Mittal et al., 1996) indicate that, under certain circumstances, low molecular weight GTPases can be affected by AlF4. Heterotrimeric GTPases have been implicated in transport along the secretory pathway (Burgoyne, 1992) and in endocytosis (Colombo et al., 1992).

Rab4 and Rab5 are associated with early endosomes, and Rab5, in particular, has been shown to play a role in the regulation of endocytosis. As shown in Fig. 3 a, Rab5:Q79L, a GTPase-defective mutant, enhances HRP uptake. This result suggests that the GTP form of Rab5 is the molecular switch for endocytosis in oocytes as reported for other cells (Barbieri et al., 1994; Stenmark et al., 1994). Rab5:Q79L has been shown to induce HRP uptake in BHK cells (Li et al., 1994) and to enhance fusion between early endosomes in an in vitro reconstitution assay (Stenmark et al., 1994; Barbieri et al., 1994). Thus, enhanced uptake of HRP by oocytes is probably due to enhanced fusion between early endosomes. In contrast, Rab5:S34N, the dominant-negative mutant, inhibited HRP uptake. This is in agreement with the observation that Rab5:S34N inhibits both endocytosis, when overexpressed in mammalian cells, and in vitro endosome fusion. The inhibition of HRP uptake by Rab5:S34N may be due to competition for fusion between early endosomes. Rab5:S34N did not significantly affect HRP uptake in oocytes. This is probably due to the fact that this protein is not prenylated and unable to bind to target membranes. This was further confirmed by the Western blot analysis (Fig. 3 b). A band corresponding to Rab5 was detected in the membrane preparation of oocytes injected with Rab5:Q79L but not Rab5:ΔC where most of the protein was detected in the cytosol. In vitro prenylation experiments confirmed that oocyte cytosol has the capacity, in the presence of added substrate, to prenylate Rab GTPases (data not shown). These results indicate that the oocyte has the ability to carry out prenylation, and that COOH-terminal isoprenylation is required for the binding of the Rab proteins and for their biological activity.

To further characterize endocytosis in microinjected oocytes, we examined NSF. NSF fusion protein is required for efficient fusion of endosomes in vitro. Mutants of NSF that fail to bind or to hydrolyze ATP are effective inhibitors of endosome fusion in vitro (Colombo et al., 1996). NSF and associated SNAP molecules (Wilson et al., 1992; Sollner et al., 1993) are required in multiple intracellular vesicle fusion events (Becker et al., 1989; Diaz et al., 1989; Sztul et al., 1993; Sogaard et al., 1994). The results presented in Fig. 5 a show that anti-NSF antibody inhibits Rab5:Q79L-induced HRP uptake in oocytes. (Western blot experiments not shown indicate that the mAb used recognizes a frog protein of the same apparent molecular weight...
as NSF.) Injection of anti-NSF into control cells produced a modest decrease in uptake. The role of NSF was further confirmed by coinjecting wild-type NSF or NSF mutants (D1E-Q, D1K-A) along with Rab5-Q79L. The results show that NSF activates endocytosis of HRP mediated by Rab5 and suggest that NSF may be rate limiting in Rab5-sensitive pathways. This is the first report of activation of a transport pathway by NSF in vivo. To confirm the role of NSF, we used two NSF mutants that are known to block NSF function. These mutant proteins inhibited uptake, suggesting that ATP binding and hydrolysis by NSF is required for function in oocytes as in mammalian cells (Whiteheart et al., 1994). However, why anti-NSF antibodies effectively block Rab-induced endocytosis and yet only modestly inhibit control uptake is unclear. It is likely that endogenous NSF and endogenous Rab GTPases are more efficient than the injected mammalian counterparts. This might explain why antibody inhibition of uptake is only modest in control cells since the antibody would be competing with endogenous factors for binding to NSF. Similarly, exogenous Rabs might be more easily competing for interaction with the docking and fusion machinery than endogenous Rabs by the anti-NSF antibody.

Recently, it has been shown in mammalian cells that Rab7 regulates transport between the early and late endosomes (Feng et al., 1995). Similar results were also obtained in yeast using Ypt7, a yeast homologue of mammalian Rab7. Schimmoller and Riezmann (1995) have shown that Ypt7 regulates the transport between the late endosome to the vacuole. Moreover, it has been shown that Ypt7 is also involved in the homotypic fusions between the vacuole in the yeast (Hass et al., 1995; Mayer et al., 1996). All these studies have shown that Rab7 in mammalian cells or Ypt7 in yeast initiates the degradation of the marker proteins by enhancing transport to a late endosome/prelysosome or lysosomal compartment. Our data demonstrate that injected Rab7 WT stimulates HRP uptake. Rab7:T22N, the dominant-negative mutant of Rab7 (Feig et al., 1988; Nuoffer et al., 1994; Riederer et al., 1994), was found to be mildly inhibitory for HRP uptake when injected alone but was fully able to block the stimulatory effect of Rab7. As outlined above for inhibition by anti-NSF antibodies, it is likely that the exogenous mammalian Rab7-negative mutant would more effectively antagonize injected mammalian wild-type Rab7 than the corresponding endogenous Rab. The results presented in Fig. 7a show that when Rab7 was injected into HRP preloaded cells, only 20% of the injected HRP activity was recovered at the indicated times in Rab7:WT-injected cells compared with controls. This rapid inactivation of HRP may be due to loss of enzymatic activity, actual degradation, or both. In Fig. 7b, we show that Rab7-injected cells degrade 125I-HRP more rapidly than control cells, but the degradation was not commensurate with the loss of HRP activity in Rab7-injected cells. Thus, the loss of HRP activity in Rab7-injected cells is probably due to inactivation and degradation of the marker protein. The results in Fig. 7b also confirm that the loss of activity in Rab7-injected cells is not due to the recycling or regurgitation of HRP. Rab7 probably mediates the rapid degradation of the marker protein by stimulating transport to a late endosome/prelysosomal compartment (Fig. 7). Thus, kinetically, Rab7 function is different from that of Rab5 because the latter does not stimulate inactivation. The rapid inactivation of HRP in Rab7-injected cells is effectively blocked by monensin. Monensin may block the transport of the marker protein from an early compartment to the degradative compartment, or it may inhibit the degradation of the ligand by increasing the pH of the lysosome-like compartment. The latter is consistent with the finding that monensin blocks the transport of markers to a later compartment described by Opresko and Karpf (1987). An effect of Rab7 on transport to late compartments is further strengthened by localization of WGA–gold in large multivesicular endosomes deep inside the oocyte when the cells were injected with Rab7:WT. These data are consistent with that of Wall and colleagues (Wall and Patel, 1987), who demonstrate that multivesicular bodies are found along the endocytic pathway. One issue that remains unresolved is whether the degradation of the marker protein occurs in late endosomes or lysosomes. GTPyS has been shown to block the transport from late endosomes to lysosomes, implicating GTPases in this process (Mullock et al., 1994). It is also possible that Rab7 is involved in both processes. Interestingly, homotypic fusion of the vacuole has been shown to require Ypt7 (Hass et al., 1995), and recently Rab7 was found, in part, to be localized in lysosomes (Meresse et al., 1995).
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