Uncoupling of Membrane Ruffling and Pinocytosis during Ras Signal Transduction

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Activation of Ras stimulates cell surface membrane ruffling and pinocytosis. Although seen as coupled events, our study demonstrates that membrane ruffling and pinocytosis are regulated by distinct Ras signal transduction pathways. Ras controls membrane ruffling via the small GTPase Rac. In BHK-21 cells, expression of the constitutively active Rac(G12V) mutant, via a Sindbis virus vector, resulted in a dramatic stimulation of membrane ruffling without affecting the uptake of horseradish peroxidase. Expression of Ha-Ras(G12V), an activated Ras mutant, stimulated both membrane ruffling and horseradish peroxidase uptake. The Ha-Ras(G12V)-stimulated pinocytosis but not membrane ruffling was abolished by either wortmannin or co-expression with a dominant negative mutant of Rab5, Rab5(S34N). Expression of the activated Rab5(Q79L) mutant mimics the stimulatory effect of Ha-Ras(G12V) on pinocytosis but not membrane ruffling. Our data indicate that Ha-Ras(G12V) separately activates Rab5-dependent pinocytosis and Rac1-dependent membrane ruffling.

Effectors of Ras include Ras-GAP (GTPase-activating protein) (15),Ral-GDS (GDP dissociation stimulator) (16, 17), and phosphatidylinositol-3-OH kinase (PI-3-kinase) (18). It has been suggested that multiple Ras functions contribute to mammalian cell proliferation (19).

Parallel to the activation of nuclear gene expression, Ras-GTP also triggers profound changes in the cytoplasm. The Ras signaling cascade involving the small GTPase Rac1 plays an important role in regulating the actin cytoskeleton and cell surface membrane ruffles (20). Ras activation also stimulates pinocytosis (21) and transport to endosomes and lysosomes. This process involves the small GTPase, Rab5 (22, 23). Because cell surface ruffling and pinocytosis both involve organized movements of the plasma membrane, and because Ras stimulates both processes, the two processes have long been thought to be linked (21). We have found that membrane ruffling and pinocytosis are regulated by distinct Ras signal transduction pathways. Whereas Ras-stimulated ruffling depends on Rac1 and is independent of PI-3 kinase and Rab5, Ras-stimulated pinocytosis depends on Rab5 and probably PI-3 kinase and is independent of Rac1.

Materials and Methods

Cells and Wortmannin Treatment—BHK-21 cells were grown as described previously (23). Wortmannin (Sigma) in dimethyl sulfoxide at 1 mg/ml (stock solution) was freshly diluted with α-MEM and added to previously rinsed cell monolayers. The wortmannin treatment (15 min, 37 °C) was followed by horseradish peroxidase (HRP) uptake. Dimethyl sulfoxide (<0.1% after dilution) had no effect on pinocytosis or membrane ruffling (data not shown).

Construction of Recombinant Sindbis Viruses—cDNAs of Ha-Ras, Rac1, and Rab5 were subcloned into the unique XbaI restriction site of the Sindbis vector Toto1000:3 (23, 24). The plasmid was then linearized by Xhol digestion and used as a template for in vitro transcription with SP6 RNA polymerase. The resulting RNA transcripts were used for transfection of confluent BHK-21 cell monolayers using a Lipofectin-mediated procedure (Life Technologies, Inc.). Cells were maintained at 37 °C, and the media containing released viruses were harvested 40 h after transfection. Virus titers were generally between 10^8 and 10^9 plaque-forming units per ml. Viral stocks were aliquoted and kept frozen at −80 °C before use.

Immunoblot Analysis of Protein Expression—Cell lysates (5 μl) were analyzed by SDS-polyacrylamide gel electrophoresis (12% acrylamide), and the proteins were transferred to an Immobilon-P membrane (Millipore) using a Bio-Rad semi-dry transfer apparatus. The membrane was probed with an Ha-Ras specific monoclonal antibody (AB1, from Oncogene Science), and the immunoblot was developed using the ECL reagents from Amersham Corp.

HRP Uptake Assay—Confuent BHK-21 monolayers were mock-infected or infected with the vector or recombinant viruses as described (23). At 4 h post-infection or otherwise indicated, cells were washed twice with serum-free α-MEM, and HRP uptake was initiated by adding HRP (5mg/ml) in α-MEM (1ml) containing 0.2% bovine serum albumin. Cell lysates were assayed for HRP activity as described (23).

Electron Microscopy of Early Endosomal Structures—At 4 h post-infection, early endosomes were marked by a 10-min HRP uptake. Cells were rinsed four times with ice-cold PBS, fixed in 2% glutaraldehyde (in PBS) for 30 min, and washed four more times with PBS. HRP reaction was developed for 10 min by incubating with 50 ml Tris-HCl, pH 7.4, containing 2 mg/ml diaminobenzidine (Sigma) and 0.01% H2O2 (Sigma). Cells were then processed for PolyBed (Polysciences, Inc.) embedding, semi-thick section (≈200 nm) preparation, and electron microscopy.

Fluorescence Microscopy of Actin Architecture and Membrane Ruffling—

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fles—At 4 h post-infection, cells were fixed with 2% paraformaldehyde (in PBS) for 30 min, permeabilized, and quenched with PBS containing 0.05% Triton X-100, 0.1N NH₄Cl, and 0.2% gelatin, followed by staining with rhodamine-phalloidin (25). The coverslips were mounted in 70% glycerol (in PBS), and the actin localization and cell edge membrane ruffles were visualized by a Zeiss axiovert microscope and a Bio-Rad confocal scanning imaging system.

RESULTS AND DISCUSSION

Pinocytosis is regulated by serum growth factors but the molecular mechanisms and biological significance are not well understood. Many growth factors also stimulate cell surface membrane ruffling via rearrangement of actin filaments (20). It has long been thought that membrane ruffling contributes to increased pinocytosis (reviewed in Ref. 26). One example is oncogenic Ras-stimulated pinocytosis and membrane ruffling (21). To understand the molecular basis of the two processes, we used a combination of reagents that specifically alter pinocytosis and membrane ruffling and found, to our surprise, that pinocytosis and membrane ruffling are independent cellular processes regulated by distinct Ras signal transduction pathways. To determine the effect of Ras activation on pinocytosis, we transiently expressed a constitutively active Ras mutant, Ha-Ras(G12V), in cultured BHK-21 cells (23, 24) and monitored pinocytosis of HRP (23). Fig. 1A shows immunoblot analysis of Ha-Ras(G12V) expression at different times after virus infection. At 4 h after infection, abundant Ha-Ras(G12V) expression was detected. Thereafter, the protein accumulated throughout virus infection (Fig. 1A). Endogenous Ras protein was not detected. HRP accumulation increased dramatically in cells expressing Ha-Ras(G12V) (Fig. 1B). At 4 h after infection, cells expressing Ha-Ras(G12V) showed a 3-fold stimulation of HRP uptake compared with the control cells (Fig. 1B). Ha-Ras(G12V) also increased the rate of HRP uptake (Fig. 1C). By electron microscopy, HRP was identified in intracellular endosomes (Fig. 2), which were significantly enlarged in cells expressing Ha-Ras(G12V) (Fig. 2), suggesting increased endosome activity.

Ha-Ras(G12V) interacts with multiple effector molecules via its effector domain (residues 32–40) (10–19, 27, 28). To identify the Ras pathway(s) leading to the stimulation of pinocytosis, we expressed an effector domain mutant of Ha-Ras(G12V), Ha-Ras(G12V,D38A), and tested the effect on HRP uptake. Ras(G12V,D38A) completely abolished the ability of Ha-Ras(G12V) to stimulate HRP uptake (Fig. 3A). Since the D38A mutation can abolish the interaction between Ha-Ras(G12V) and its effector molecules including PI 3-kinase (18), this result demonstrated the existence of a responsible Ras signal transduction pathway. Expression of the constitutively active Raf and RalB mutants (the C-terminal 344 residues of human c-Raf (29) and RalB(G23V), respectively), putative Ras effectors, failed to mimic the stimulatory effect of Ha-Ras(G12V) on HRP uptake (data not shown). Wortmannin, a potent PI 3-kinase inhibitor (30, 31), completely blocked the stimulation of HRP uptake (23).

Fig. 1. Stimulation of HRP uptake by Ha-Ras(G12V). Confluent BHK-21 cell monolayers were infected with recombinant virus as described under “Materials and Methods.” A, immunoblot analysis to determine Ha-Ras(G12V) expression. B, HRP uptake assay. The HRP uptake results are the means of triplicate samples. C, HRP uptake kinetics. Cells infected with either the vector virus or the recombinant virus were incubated at 37 °C for 4 h, followed by HRP uptake at 37 °C for the times indicated. The data are presented as the means of triplicate samples.

Fig. 2. Electron microscopy of early endosomal structures. Expression of Ha-Ras(G12V) or Rab5(Q79L) leads to the formation of enlarged endosomal structures (B and C, respectively) that are not seen in control cells (A). Arrows indicate HRP-marked endosomes. N denotes nucleus. Magnification 1:12,800. Bar = 1 cm = 730 nm.
uptake by Ha-Ras(G12V) (Fig. 3B). Wortmannin (100 nM) also inhibited HRP uptake in control cells (Fig. 3B) (32, 33).

Early endosome fusion immediately follows the internalization step at the plasma membrane and appears to be rate-limiting (22, 23). Wortmannin inhibits a function required for the activation of Rab5 (33), a GTPase essential for early endosome fusion. Expression of the constitutively active Rab5 mutant, Rab5(Q79L), leads to increased early endosome fusion and increased pinocytosis while expression of the dominant negative Rab5(S34N) mutant results in decreased early endosome fusion and decreased pinocytosis (23, 34). The positive correlation between early endosome fusion activity and cellular pinocytic activity led us to suggest that Ha-Ras(G12V) sequentially activates a wortmannin-sensitive function (possibly PI 3-kinase) and Rab5, leading to an increase in early endosome fusion and pinocytosis. We found that Rab5(Q79L) mimicked the stimulatory effect of Ha-Ras(G12V) on HRP uptake, and this stimulation could be blocked neither by wortmannin (33) nor by the dominant negative Ha-Ras(S17N) mutant (Fig. 3C). Co-expression of the activated Rab5(Q79L) and Ha-Ras(G12V) mutants did not lead to a synergistic stimulation of HRP uptake (Fig. 3C). Furthermore, like cells expressing Rab5(Q79L) (Fig. 2C), cells expressing Ha-Ras(G12V) exhibited enlarged endosomal structures (Fig. 2B), indicative of enhanced endosome fusion. Finally, co-expression of the dominant negative Rab5(S34N) mutant completely blocked the stimulatory effect of Ha-Ras(G12V) on HRP uptake (Fig. 3D).

The pioneering work of Hall and colleagues (20) has partially defined the Ras signal transduction pathway regulating membrane ruffling. Our study begins to shed light on this important...
aspect of Ras signal transduction. We show that oncogenic Ras-stimulated pinocytosis is sensitive to wortmannin and is dependent on Rab5 function. Because Ras can directly activate a PI 3-kinase (18) and PI 3-kinase activity can stimulate Rab5-dependent early endosome fusion in vitro (33), we suggest that Ras sequentially activates PI 3-kinase and Rab5, leading to a quantitative increase in endosome fusion and pinocytosis. Ridley et al. (20) previously characterized a Ras signal transduction pathway that promotes membrane ruffling via activation of the small GTPase Rac1. To further examine the relationship of Ras-stimulated membrane ruffling and pinocytosis, we expressed the constitutively active and dominant negative Rac1 mutants. As shown in Fig. 3E, expression of Rac1(G12V) did not stimulate HRP uptake while under the same conditions Ha-Ras(G12V) stimulated HRP uptake 3-fold. Fig. 3F shows the co-expression of constructs used in this study. These data immediately suggested that membrane ruffling and pinocytosis are controlled by distinct Ras signal transduction pathways. We then examined membrane ruffling (Fig. 4). Consistent with previous reports (20, 25), expression of Rac1(G12V) resulted in a dramatic production of cell surface membrane ruffles (Fig. 4C). Under the same conditions, expression of Ha-Ras(G12V) also stimulated membrane ruffling (Fig. 4D). Cells expressing the activated Rac5(Q79L) mutant, a strong stimulator of pinocytosis, did not show membrane ruffling (Fig. 4E), nor did the vector virus-infected (Fig. 4B) or mock-infected control cells (Fig. 4A). In agreement with Nobes et al. (25), we found that wortmannin (100 nM) failed to block Rac1(G12V)-induced or Ha-Ras(G12V)-induced membrane ruffling (data not shown), under conditions where Ha-Ras(G12V)-stimulated pinocytosis was completely inhibited (Fig. 3B). From these data, we conclude that the Ras signal transduction pathway leading to the activation of Rac1 and membrane ruffling does not contribute to the stimulation of pinocytosis.

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