Rin1 Interacts with Signal-transducing Adaptor Molecule (STAM) and Mediates Epidermal Growth Factor Receptor Trafficking and Degradation*\\[5]

Chen Kong, Xiong Su, Pin-I Chen, and Philip D. Stahl\(^1\)

From the Department of Cell Biology and Physiology, Washington University School of Medicine, St. Louis, Missouri 63110

---

Rin1, the prototype of a new family of multidomain Rab5 exchange factors, has been shown to play an important role in the endocytosis of the epidermal growth factor receptor (EGFR). Herein, we examined the role of Rin1 in the down-regulation of EGFR following EGF stimulation. We observed that overexpression of Rin1 accelerates EGFR degradation in EGF-stimulated cells. In concordance, depletion of endogenous Rin1 by RNA interference resulted in a substantial reduction of EGFR degradation. We showed that Rin1 interacts with signal-transducing adaptor molecule 2 (STAM2), a protein that associates with hepatocyte growth factor-regulated substrate and plays a key role in the endosomal sorting machinery. Green fluorescent protein (GFP)-Rin1 co-localizes with hemagglutinin (HA)-STAM2 and with endogenous hepatocyte growth factor-regulated substrate. Furthermore, wild type STAM2, but not a deletion mutant lacking the SH3 domain, co-immunoprecipitates with endogenous Rin1. This interaction is dependent on the proline-rich domain (PRD) of Rin1 as Rin1ΔPRD, a mutant lacking the PRD, does not interact with STAM2. Moreover, EGFR degradation was not accelerated by expression of the Rin1ΔPRD mutant. Together these results suggest that Rin1 regulates EGFR degradation in cooperation with STAM, defining a novel role for Rin1 in regulating endosomal trafficking.

The epidermal growth factor receptor (EGFR)\(^2\) plays a central role in cell proliferation, differentiation, survival, and migration (1, 2) and has served as a prototype in growth factor receptor trafficking. Following activation, EGFR with intrinsic tyrosine kinase activity is rapidly internalized by clathrin-coated pits (3), sorted through early endosomes, and eventually transported to and degraded within multivesicular bodies (MVB) and lysosomes (4, 5). The process is generally known as receptor down-regulation and is considered to be an important cellular strategy for signal attenuation (6, 7). Alterations in receptor trafficking and signal attenuation have been associated with carcinogenesis (5, 8) and certain developmental processes, which has stimulated interest in the molecular mechanisms that regulate EGFR trafficking and degradation.

EGFR targeted for lysosomal degradation is delivered to the MVB by a highly specialized process that begins with receptor ubiquitination and sequestration by elements of the ESCRT (endosomal sorting complex required for transport) complex on the surface of the early endosomes (9, 10). Invagination of the endosomal membrane and delivery of sequestered receptors into the lumen of the MVB is accompanied by receptor deubiquitination and disassembly of the ESCRT complex (11, 12). The ESCRT machinery, first identified in yeast as class E Vps (vacuolar protein sorting) mutants (13) and highly conserved among eukaryotic cells (14), is important in targeting EGFR into the lumen of the MVB (15, 16). Substantial progress has been made in identifying the molecular mechanisms involved (12, 17). Among the early acting factors are Hrs (hepatocyte growth factor-regulated tyrosine kinase substrate) and STAM (signal transducing adaptor molecule) proteins, which tightly associate with each other (18, 19) and together are key players in the sorting of ubiquitinated EGFR into the MVB pathway (20, 21). STAM1 and STAM2 have 53% amino acid sequence identity and display the same overall domain structure (22): an N-terminal Vsp27/Hrs/STAM domain and ubiquitin-interacting motif, a Src homology 3 (SH3) domain, and a coiled-coil region (18). The STAMs appear to have redundant functions (23). Fibroblast cell lines derived from STAM1 and STAM2 double knock-out mice display delayed EGFR degradation (24). Hrs-STAM complex is required for the recruitment of ESCRT-1 (16, 25), an early event in MVB formation (26).

Rin1 (Ras interaction/interference 1) was originally identified as a Ras effector protein based on its ability to block Ras-induced cell death (27). Rin1 was found to bind GTP-Ras, Bcr-Abl, and 14–3–3 (27, 28). It is composed of several functional domains: SH2 and proline-rich (29) domains in the N-terminal region and Vps9 and Ras association domains in the C-terminal region. Recent studies have shown that through its interaction with Abl tyrosine kinase, Rin1 mediates actin cytoskeleton remodeling associated with migration and adhesion of epithelial cells (30). Our previous work suggested that Rin1 is an exchange factor for small GTPase Rab5, whose overexpression...
stimulates EGF-mediated endocytosis (31). The Rin family now has at least four members, all of which have active Rab5 guanine nucleotide exchange factor domains (32–35). Subsequent work showed that Rin1 is recruited to the EGF via its SH2 domain and that it regulates EGF-induced signal transduction (36, 37). Identification of additional Rin1-interacting partners is critical to understanding the dynamic of EGF trafficking. The presence of a PRD suggests that Rin1 interacts with SH3-containing proteins involved in EGFR trafficking. In this study, we identified the interaction between Rin1 and STAM and studied its function in EGFR trafficking and degradation.

EXPERIMENTAL PROCEDURES

Antibodies and Reagents—Human epidermal growth factor and monoclonal anti-EGFR (Ab5) antibody were obtained from Calbiochem. Rabbit polyclonal anti-Rin and mouse monoclonal anti-EEA1 antibodies were purchased from BD Biosciences. Mouse monoclonal anti-HA and rabbit polyclonal anti-EGFR 1005 antibodies were from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Monoclonal anti-β-tubulin antibody was from Sigma. Monoclonal anti-V5 antibody was from Invitrogen. Rabbit polyclonal Hrs antibody was a gift of Dr. Tim McGraw (Cornell University).

Cell Culture and Transfection—HeLa, HEK293 and Cos7 cells were cultured in Dulbecco’s modified Eagle’s medium (Invitrogen) supplemented with 10% bovine growth serum (HyClone Laboratories, Logan, UT). 100 units/ml penicillin G sodium, and 100 μg/ml streptomycin sulfate at 37 °C with 5% CO2. NR6-RGFR-Rin1 and NR6-RGFR-vector stable cell lines were described in Ref. 36. Cos7 or HeLa cells were transfected using FuGENE 6 (Roche Diagnostics). HeLa or HEK293 cells were transfected using Lipofectamine 2000 (Invitrogen) according to manufacturer’s instruction. 48 h after transfection, the cells were used for immunoblot and immunocytochemical analysis.

Immunolabeling—Cells grown on coverslips were fixed with 3% paraformaldehyde (Electron Microscope Sciences, Hatfield, PA) for 20 min and quenched for 10 min with 50 mM ammonium chloride. Cells were permeabilized with 0.1% Triton X-100 for 10 min, blocked with 2% goat serum and 1% bovine serum albumin for 1 h, and incubated with primary antibodies for 1 h followed by Alexa Fluor 594- or 488-goat anti-mouse or rabbit secondary antibodies (Invitrogen/Molecular Probe) for 30 min at room temperature. The coverslips were mounted with fluorescent mounting medium (DakoCytomation, Carpinteria, CA). Images were collected using a MRC1024 laser scanning confocal microscope equipped with a ×63 objective (Bio-Rad Laboratories).

RESULTS

Overexpression of Rin1 Enhances EGF Degradation—Previous reports show that Rin1 interacts with the EGF through its SH2 domain and that the interaction modulates EGF endocytosis (37). The interaction between Rin1 and EGF following internalization may be sustained and may mediate the trafficking of the EGF to the MVB for degradation. To examine this possibility, murine NR6 cells stably expressing both hEGFR and Rin1 were incubated with primary antibodies overnight at 4 °C and then incubated with protein A- or G-Sepharose (Sigma) for 2 h at room temperature followed by incubation with horseradish peroxidase-conjugated goat anti-rabbit or anti-mouse IgG (Jackson ImmunoResearch, West Grove, PA), and proteins were visualized by enhanced chemiluminescence detection reagents (Pierce). Immunoblot data were quantified by AlphaEaseFC 4.0 software (Alpha Innotech Corp., San Leandro, CA). For immunoprecipitation, the clarified cell lysates were incubated with primary antibodies overnight at 4 °C and then incubated with protein A- or G-Sepharose (Sigma) for an additional 1 h at 4 °C. The beads were washed extensively with STE buffer (100 mM NaCl, 20 mM Tris-HCl, pH 7.5, 1 mM EDTA) and solubilized in SDS sample loading buffer.
Rin1 Mediates EGFR Degradation

To further evaluate the effects of Rin1 expression on endogenous EGFR trafficking, we transfected HeLa cells with GFP-Rin1 construct and followed both endogenous EGFR and GFP-Rin1 by light microscopy. Transfected HeLa cells were serum-starved for 3 h and preincubated with EGF (100 ng/ml) at 4 °C for 1 h. The cells were then warmed to 37 °C in the absence of ligand for 10 and 20 min. Localization of EGFR and Rin1 was then examined by confocal microscopy. Prior to warm up, EGFR is localized almost exclusively to the plasma membrane (Fig. 1B, top panel). Overexpression of Rin1 had no apparent effect on the distribution of EGFR. After 10 min of stimulation, the receptor was clustered and internalized into small punctate structures that probably correspond to early endosomes (Fig. 1B, middle panel). Although Rin1 expression is known to increase EGFR internalization (37), the amounts of internalized EGFR between transfected cells and untransfected cells were similar at 10 min. However, after 20 min, cells expressing Rin1 contained significantly less EGFR than untransfected cells (Fig. 1B, bottom panel). Interestingly, overexpressed GFP-Rin1 also shows a nuclear localization in addition to endosomes in cells overexpressing the fusion protein (Fig. 1B). It has been difficult to identify endogenous Rin1 by immunocytochemical studies in part because the commercially available antibodies detect nonspecific targets. Thus, we set out to examine the localization of endogenous Rin1 using standard cellular fractionation methods. As shown in the supplemental data (supplemental Fig. S1), endogenous Rin1 was enriched in both cytosol and endosome/lysosome fractions and was also detected in membrane/nuclear fractions. There are several nuclear localization signal motifs in Rin1. Why endogenous Rin1, as detected by immunofluorescence, is not detected in nuclei is not clear. It is possible that endogenous Rin1 is retained in the cytosolic compartment via intermolecular interactions with effector proteins or binding partners and that following overexpression, such interactions are saturated and insufficient to prevent nuclear localization. Together, these results indicate that overexpression of Rin1 accelerates the degradation of EGFR.

Suppression of Rin1 Delays EGFR Degradation—To provide stronger evidence that Rin1 accelerates EGFR degradation, we employed RNA interference to suppress endogenous Rin1. HeLa cells were depleted of Rin1 by transfection with two small interfering RNA (siRNA) duplexes with sequences specific to Rin1 or a nonspecific scrambled siRNA. Cells were then serum-starved and stimulated with EGF in the presence of cycloheximide. At various times of incubation, whole cells lysates were prepared and examined by Western blot analysis. As shown in Fig. 2A, Rin1 was efficiently depleted by specific siRNAs (typically more than 90% knockdown). In control cells, EGFR degradation was virtually completed within 60 min. However, in HeLa cells depleted of Rin1, EGFR degradation was substantially delayed (Fig. 2, A and B). We have observed that t₁⁄₂ (the time required to degrade 50% of the amounts of EGFR at time 0) of EGFR degradation depends upon the amount of receptors at steady state. The higher the EGFR level is, the longer it takes. That is why endogenous EGF receptors in HeLa cells are degraded much faster than overexpressed receptors in NR6/EGFR cell line (Fig. 1A).

To extend the analysis of the effect of Rin1 knockdown on EGFR trafficking, we examined Rin1-depleted cells by immunocytochemistry. In both control and Rin1-depleted cells, most of the EGFR was located at or near the cell surface before stimulation. After the addition of EGF for 10 min, the EGFR was found in early endosomes (mainly co-localized with EEA1, an early endosome marker, data not shown) in both control and Rin1-depleted cells. In control cells, the overall intensity of the EGFR signal was significantly weakened by 30 min, consistent with the Western blot data. However, in Rin1-depleted cells, the EGFR signal at 30 min remained strong and showed a punctate distribution throughout the cytoplasm (Fig. 2C). Collectively, we have shown by different approaches that Rin1 regulates EGFR degradation.

Rin1 Associates with STAM2—Delivery of the EGFR to the MVB is initiated by the ESCRT-0 components STAM and Hrs (26). We set out to determine whether the effect of Rin1 on EGFR degradation involved or required STAM and possibly other components of endosomal sorting machinery. Cos7 cells co-transfected with constructs encoding GFP-Rin1 and either
HA-STAM2 or Hrs display extensive co-localization of Rin1 with both STAM2 and Hrs in endosomes (Fig. 3A). We reasoned that Rin1 might interact with the SH3 domain of STAM2 given the fact that Rin1 possesses a proline-rich domain. To address this question, HeLa cells were transfected with HA-tagged wild type STAM2 or a STAM2 mutant lacking the SH3 domain (STAM2\textsubscript{WT}/H9004\textsubscript{SH3}). Co-immunoprecipitations were carried out using an anti-Rin1 antibody, and precipitates were immunoblotted to reveal Rin1 and the STAM2 proteins. We found that HA-STAM2\textsubscript{WT}, but not HA-STAM2\textsubscript{WT}/H9004\textsubscript{SH3}, co-immunoprecipitated with endogenous Rin1 (Fig. 3B). Since STAM and Hrs are forming complex in endosomes, our co-immunoprecipitation experiment showed that GFP-Rin1 was also able to pull down both Hrs and STAM (data not show). These results suggested that Rin1 binds STAM2 and Hrs on endosomes, possibly through the SH3 domain of STAM2 and proline-rich domain of Rin1. There are many proteins contain-

**FIGURE 2.** Knockdown of endogenous Rin1 delayed EGFR degradation in HeLa cells. A, HeLa cells were transfected with 20 nm scrambled negative control (NC) or Rin1-specific siRNA for 48 h. The cells were then stimulated with 100 ng/ml EGF in the presence of 25 μg/ml cycloheximide for indicated times. 10 μg of total proteins from each of the cell lysates was subjected to SDS-PAGE and immunoblotting (IB) with antibodies to EGFR, Rin1, and β-tubulin to monitor the degradation of EGFR, the efficiency of knock-down, and the total protein levels, respectively. B, Western blot signals were quantified using AlphaEaseFC 4.0 software. The data represent the mean of three independent experiments. C, suppression of Rin1 delays the trafficking and degradation of the EGFR. 20 nm scrambled negative control or Rin1-specific siRNA transfected HeLa cells were starved, treated with 100 ng/ml EGF for 1 h at 4 °C, and then allowed to endocytose EGF for 0, 10, and 30 min at 37 °C. At the end of each time point, the cells were fixed and immunostained with antibody to EGFR. Scale bar represents 10 μm. Data represented at least three independent experiments.

**FIGURE 3.** Rin1 associates with STAM2. A, co-localization of GFP-Rin1 with HA-STAM2 and endogenous Hrs. Cos7 cells were transfected with GFP-Rin1 and HA-STAM2 (upper panel) or GFP-Rin1 alone (bottom panel). 24 h after transfection, cells were fixed, immunostained with anti-HA (upper panel) or anti-Hrs antibodies (bottom panel), and imaged by confocal microscopy. The single cell image represented the results from at least 30 cells of three experiments. Scale bar represents 5 μm. B, endogenous Rin1 in HeLa cells binds to HA-STAM2 but not HA-STAM2\textsubscript{DSH3}. 200 μg of extracts from HeLa cells transiently transfected with HA-STAM2 or HA-STAM2\textsubscript{DHS3} was subjected to immunoprecipitation (IP) with a polyclonal anti-Rin1 antibody followed by immunoblotting (IB) with anti-Rin1 and anti-HA antibodies. Whole cell lysates (WCL) were also analyzed by immunoblotting.

**FIGURE 3.** Rin1 associates with STAM2. A, co-localization of GFP-Rin1 with HA-STAM2 and endogenous Hrs. Cos7 cells were transfected with GFP-Rin1 and HA-STAM2 (upper panel) or GFP-Rin1 alone (bottom panel). 24 h after transfection, cells were fixed, immunostained with anti-HA (upper panel) or anti-Hrs antibodies (bottom panel), and imaged by confocal microscopy. The single cell image represented the results from at least 30 cells of three experiments. Scale bar represents 5 μm. B, endogenous Rin1 in HeLa cells binds to HA-STAM2 but not HA-STAM2\textsubscript{DHS3}. 200 μg of extracts from HeLa cells transiently transfected with HA-STAM2 or HA-STAM2\textsubscript{DHS3} was subjected to immunoprecipitation (IP) with a polyclonal anti-Rin1 antibody followed by immunoblotting (IB) with anti-Rin1 and anti-HA antibodies. Whole cell lysates (WCL) were also analyzed by immunoblotting.

HA-STAM2 or Hrs display extensive co-localization of Rin1 with both STAM2 and Hrs in endosomes (Fig. 3A). We reasoned that Rin1 might interact with the SH3 domain of STAM2 given the fact that Rin1 possesses a proline-rich domain. To address this question, HeLa cells were transfected with HA-tagged wild type STAM2 or a STAM2 mutant lacking the SH3 domain (STAM2\textsubscript{DHS3}). Co-immunoprecipitations were carried out using an anti-Rin1 antibody, and precipitates were immunoblotted to reveal Rin1 and the STAM2 proteins. We found that HA-STAM2\textsubscript{WT}, but not HA-STAM2\textsubscript{DHS3}, co-immunoprecipitated with endogenous Rin1 (Fig. 3B). Since STAM and Hrs are forming complex in endosomes, our co-immunoprecipitation experiment showed that GFP-Rin1 was also able to pull down both Hrs and STAM (data not show). These results suggested that Rin1 binds STAM2 and Hrs on endosomes, possibly through the SH3 domain of STAM2 and proline-rich domain of Rin1. There are many proteins contain-
Rin1 Mediates EGFR Degradation

FIGURE 4. Rin1 interacts with STAM2 through its proline-rich domain.
A, scheme of Rin1 and STAM2 constructs used in this study. RA, Ras association domain; VHS, Vsp27/Hrs/STAM domain; UIM, ubiquitin-interacting motif; SSM, STAM-specific motif. aa, amino acids. B, HEK293 cells were co-transfected with HA-STAM2 and Rin1-V5 or Rin1ΔPRD-V5. 150 μg of cell lysates was immunoprecipitated (IP) with anti-V5 antibody. The recovered immunocomplex was immunoblotted (IB) with anti-HA (top) and anti-V5 (middle). The whole cell lysates (WCL) were immunoblotted with anti-HA (bottom) to show the expression level of HA-STAM2. C, HEK293 cells were co-transfected with Rin1-V5 or Rin1ΔPRD-V5 together with HA-STAM2 or HA-STAM2ΔSH3. STAMs were immunoprecipitated with anti-HA antibody from cells and immunoblotted with anti-V5 (top) and anti-HA (middle) antibodies. The expression level of Rin1 and Rin1ΔPRD was assessed by Western blot of the whole cell lysates with anti-V5 antibody (bottom).

EGF Stimulation Increases the Association of EGFR with Both Rin1 and STAM—Previous studies showed that Rin1 binds EGFR through the SH2 domain of Rin1 (37). Given the results in Fig. 4 showing that Rin1 interacts with STAM2, we examined whether the EGFR can be found as part of a Rin1-STAM2 complex. To test this possibility, HEK293 cells were transfected with constructs encoding GFP-EGFR, HA-STAM2, and Rin1-V5. The cells were serum-starved for 3 h and then incubated with or without EGF for 10 min. Whole cell lysates were prepared and immunoprecipitated with EGFR antibody. The resulting immunoprecipitates were subjected to immunoblot analysis using antibodies against EGFR, Rin1, or HA. As shown in Fig. 5A, EGFR has a significant increase binding with both Rin1 and STAM2 after the EGF stimulation, indicating the formation of EGF-dependent EGFR-Rin1-STAM complex.

To address whether internalized EGFRs traffic through the Rin1-containing endosomes and Rin1 also co-localizes with STAM upon the stimulation, Cos7 cells were transfected with EGFR-GFP and Rin1-V5 or GFP-Rin1 and HA-STAM. Following incubation with EGF for 10 min, the cells were then stained with V5 or HA antibodies. This analysis showed that most of the EGFR-containing endosomes were positive for Rin1 (supplemental Fig. S2) in agreement with our previous report (37). Furthermore, without EGF stimulation (Fig. 5B, upper panel), Rin1 was primarily cytosolic with partially a few plasma membrane localizations. After incubation with EGF (Fig. 5B, lower panel), Rin1 co-localized with STAM on endosomes, and those STAM-containing endosomes were also labeled with EGFR (supplemental Fig. S3). These light microscopy studies are consistent with the result in Fig. 5A, suggesting that EGFR together with Rin1 is recruited to STAM-positive compartments in a EGF-dependent manner.

Rin1 Mutant without Proline-rich Domain (Rin1ΔPRD) Fails to Promote EGFR Degradation—To investigate the functional role of the PRD-mediated Rin1-STAM interaction, we examined the effect of expressing the Rin1ΔPRD mutant on EGFR degradation. Rin1WT or Rin1ΔPRD was co-expressed with EGFR in HEK293 cells. The cells were stimulated with EGF for up to 2 h. Immunoblot analysis of the total cell lysates was carried out with anti-EGFR and anti-Rin1 antibodies. Again, EGF receptor degradation was substantially accelerated in cells expressing Rin1 WT (as compared with vector alone Fig. 6A). EGF receptor degradation in cells expressing the Rin1ΔPRD mutant was slightly accelerated. However, the difference in

ing the SH3 domain, and STAM may not be the only protein that is recruited to Rin1. However, it is possible that the interaction between Rin1 and STAM is required for Rin1-mediated EGFR degradation.

The Proline-rich Domain of Rin1 Is Required for STAM2 Binding—To test whether the proline-rich domain of Rin1 is required for association with STAM2, a V5-tagged Rin1 proline-rich domain deletion construct (Rin1ΔPRD-V5) was made and co-expressed with HA-STAM2 or HA-STAM2ΔSH3 in HEK293 cells. The scheme of Rin1 and STAM2 constructs used was shown in Fig. 4A. 24 h later, the cells were lysed, and immunoprecipitation was carried out with a V5 antibody. Immunoblot analysis was carried out with anti-HA and anti-V5 antibodies. As shown in Fig. 4B, Rin1WT-V5, but not Rin1ΔPRD-V5, was able to pull down HA-STAM2. When the cell lysates were immunoprecipitated with HA antibody (Fig. 4C), the interaction between the HA-STAM2 and Rin1WT-V5 was confirmed (lane 1), whereas the interaction between HA-STAM and Rin1ΔPRD-V5 was substantially reduced (lane 2). This experiment also examined the interaction between HA-STAM2ΔSH3 and Rin1WT-V5 (lane 3) or HA-STAM2ΔSH3 and Rin1ΔPRD-V5 (lane 4). Although HA-STAM2 efficiently co-precipitated Rin1WT-V5, HA-STAM2ΔSH3 failed to pull down Rin1WT-V5. These data indicate that Rin1 and STAM interact via the PRD of Rin1 and the SH3 domain of STAM.
EGFR degradation between transfected and control cells was not statistically significant (the means ± S.E. of the relative EGFR levels are shown in Fig. 6A). To determine whether the lack of any effect of the Rin1ΔPRD mutant on EGFR degradation was due to some alteration in the interaction between Rin1 and Rab5, we examined the interaction between Rin1ΔPRD and Rab5a. We showed in the supplemental data (supplemental Fig. S4) that both Rin1 and Rin1ΔPRD mutant are able to bind Rab5: S34N, the dominant negative form of Rab5a. Moreover, previous studies showed that a Rin1 construct lacking the N-terminal SH2 domain and the proline-rich domain (but including the vps9 domain and Ras binding domain) tested positively for Rab5 exchange activity (31). These findings indicated that the PRD was not essential for Rab5 exchange activity (31). Together, the finding that the PRD mutant on EGFR degradation is due to some alteration in the interaction between Rin1 and Rab5, we examined the interaction between Rin1ΔPRD and Rab5a. We showed in the supplemental data (supplemental Fig. S4) that both Rin1 and Rin1ΔPRD mutant are able to bind Rab5: S34N, the dominant negative form of Rab5a. Moreover, previous studies showed that a Rin1 construct lacking the N-terminal SH2 domain and the proline-rich domain (but including the vps9 domain and Ras binding domain) tested positively for Rab5 exchange activity (31). These findings indicated that the PRD was not essential for Rab5 exchange activity (31). Together, the data in Fig. 6 indicate that although Rin1 expression accelerates EGFR degradation, Rin1 lacking the PRD domain is largely unable to promote EGF-induced EGFR degradation. This provides further support for a functional role for Rin1-STAM interaction during EGFR trafficking.

**DISCUSSION**

Our results define a novel role for Rin1 in regulating the degradation of the EGF receptor. At the outset of experiments leading to this report, our hypothesis was built around the concept of Rin1 as a mediator of EGF signal transduction. Two paradoxical findings changed the focus of our work. 1) When we overexpressed Rin1, we blocked EGF–triggered activation of the Raf kinase pathway and the proliferative response to EGF. Our results define a novel role for Rin1 in regulating the activity of the Raf kinase pathway and the proliferative response to EGF. 2) When we overexpressed Rab5a, we enhanced the signal-transducing properties of EGF including cell proliferation (36). We queried how it could be that overexpression of Rin1, an activator of Rab5, suppressed EGF signaling, whereas overexpression of Rab5, the target of the guanine nucleotide exchange factor activity of Rin1, enhanced EGF signaling. This led us to use RNA interference to suppress Rin1 expression to better understand its function and determine whether the negative effects of Rin1 in EGF signaling are due to a specific effect on EGFR degradation. In this report, we have used complementary overexpression and RNA interference knock-down approaches to demonstrate the role of Rin1 in EGF trafficking and degradation. We observed that although endogenous Rin1 is present in the cytosolic fraction, substantial amounts are present in membrane-associated pools similar to Hrs and STAM. Like STAM and Hrs, Rin1 may cycle from membrane to cytosol during cycles of EGFR targeting to the MVB.

As a component of the endosome sorting machinery, STAM binds Hrs via their respective coiled-coil regions (19, 40). Hrs possesses a FYVE domain that binds to phosphatidylinositol 3-phosphate that is enriched on endosomes (13, 41) and that recruits the Hrs-STAM complex to the endosome. Our double immunofluorescence analysis showed that Rin1 co-localized with both overexpressed and endogenous Hrs on endosomes. Endogenous Rin1 was able to pull down wild type STAM2 but not a STAM2-SH3 deletion mutant, and overexpressed STAM binds full-length Rin1 but not Rin1 lacking a proline-rich domain. These two independent approaches provided further evidence that Rin1 associates with the STAM-Hrs complex. Previous studies have shown that the SH2 domain of Rin1 interacts, directly or indirectly, with the EGF receptor. In this...
Rin1 Mediates EGFR Degradation

report, we show that Rin1ΔPRD, a mutant lacking the proline-rich domain, retains binding to the EGFR but fails to associate with STAM. It is likely that Rin1 performs its regulatory function with the EGFR by recruiting the receptor into the STAM-Hrs complex through its proline-rich domain. The mutually exclusive interaction of STAM and of EGFR with Rin1 indicated that Rin1ΔPRD would interfere with the ability of STAM-Hrs to recruit EGFR to the MVB pathway. Supporting this view, overexpression of Rin1 wild type in HEK293 cells showed an accelerated EGFR degradation, whereas Rin1ΔPRD expression had only minimal effects on EGFR degradation.

Ubiquitination has been established as a key component of the targeting mechanism directing EGFR to the MVB pathway. Deubiquitination of membrane proteins targeted for degradation also appears to be an essential element. Two ubiquitin isopeptidases or deubiquitinases, ubiquitin-specific protease Y (UBPY) and associated molecule with the SH3 domain of STAM (AMSH), have been identified as hydrolases that deubiquitinate EGFR (26, 42, 43). Both enzymes have been shown to bind to STAM via a proline-rich motif on the deubiquitinase and the SH3 domain of STAM. Based upon these data, a model for the role of Rin1 in EGFR trafficking and degradation can be envisaged. EGFR can enter the MVB pathway by multiple routes, one of which is regulated by Rin1. Rin1, by interacting with STAM, enhances the recruitment of EGFR into a functional STAM-Hrs complex and perhaps stabilizes the recruitment of one or more deubiquitinases. Disruption of this association by suppression of Rin1 would then decrease the availability of EGFR to the ESCRT complex, disrupt lysosomal targeting of EGFR, and impair EGFR down-regulation. It remains unclear how the interaction of Rin1 with STAM2 facilitates or competes with AMSH or UBPY recruitment, which clearly required further experiments.

Rin1 was initially identified by Colicelli and colleagues (27) as a Ras-interacting protein. They showed that Rin1 interacts with GTP-Ras and thereby competes with Raf1 (28). More recent work from the same group indicates that Rin1 interacts with the tyrosine kinase Abl and that via phosphorylation of CRK and CRKL, Rin1 plays a role in actin remodeling and cell migration in epithelial cells and neurons (30). Abl binds Rin1 through the PRD and SH2 domains of Rin1, the same domains that we have shown to interact with STAM2 and EGFR, respectively. Thus, it would seem that Rin1 plays dual roles, one facilitating cytoskeletal-dependent cellular movement and a second facilitating vesicle trafficking and receptor degradation. These two roles have some common features, however, both requiring interactions with the cytoskeleton. Our preliminary studies indicate that Rin1 is potentially active in vesicle motility.3 Future work will need to focus on bringing these two themes, actin remodeling and vesicle motility, together.

What is the physiological function of Rin1? Rin1 is selectively expressed in various tissues and cells (27). Some cultured cells have high levels of expression. Others appear to be Rin1-negative. Rin1 may serve specialized functions in cells where motility or signal attenuation might be important possibly as a tumor suppressor or as a mediator of some developmental or physiological process.

Acknowledgments—We thank Dr. Sylvie Urbe for the STAM constructs and other related reagents. We thank Audra Charron, Didier Hodzic, and Marisa Wainsztein for critical reviews of the manuscript and for helpful suggestions.

REFERENCES

1. Stetak, A., Hoier, E. F., Croce, A., Cassata, G., Di Fiore, P. P., and Hajnal, A. (2006) EMBO J. 25, 2347–2357
2. Ullrich, A., and Schlessinger, J. (1990) Cell 61, 203–212
3. Lakadamalyi, M., Rust, M. J., and Zhuang, X. (2006) Cell 124, 997–1009
4. Felder, S., Miller, K., Moehren, G., Ullrich, A., Schlessinger, J., and Hopkins, C. R. (1990) Cell 61, 623–634
5. Katzmann, D. J., Odorizzi, G., and Emr, S. D. (2002) Nat. Rev. Mol. Cell Biol. 3, 893–905
6. Dikic, I., and Giordano, S. (2003) Curr. Opin. Cell Biol. 15, 128–135
7. Husnjak, K., and Dikic, I. (2006) Nat. Cell Biol. 8, 787–788
8. Sorkin, A., and Waters, C. M. (1993) BioEssays 15, 375–382
9. Gruenberg, J., and Stenmark, H. (2004) Nat. Rev. Mol. Cell Biol. 5, 317–323
10. Hoeller, D., Crosetto, N., Blagoev, B., Raiborg, C., Tikkanen, R., Wagner, S., Kowanez, K., Breitling, R., Mann, M., Stenmark, H., and Dikic, I. (2006) Nat. Cell Biol. 8, 163–169
11. Bowers, K., Piper, S. C., Edeling, M. A., May, S. R., Owen, D. J., Lehner, P. J., and Luzio, J. P. (2006) J. Biol. Chem. 281, 5094–5105
12. Hurley, J. H., and Emr, S. D. (2006) Annu. Rev. Biophys. Biomol. Struct. 35, 277–298
13. Raymond, C. K., Howald-Stevenson, I., Vater, C. A., and Stevens, T. H. (1992) Mol. Biol. Cell 3, 1389–1402
14. Babst, M. (2005) Traffic 6, 2–9
15. Bache, K. G., Raiborg, C., Mehlum, A., and Stenmark, H. (2003) Mol. Biol. Cell 14, 2513–2523
16. Zheng, B., Lavoie, C., Tang, T. D., Ma, P., Meier, T., Beas, A., and Farquhar, M. G. (2004) Mol. Biol. Cell 15, 5538–5550
17. Stahl, P. D., and Barbieri, M. A. (2002) Science 298, 848–856
18. Komada, M., and Kitamura, N. (2005) J. Biol. Chem. 280, 12513–12521
19. Mizuno, E., Kawahata, K., Okamoto, A., Kitamura, N., and Komada, M. (2004) J. Biochem. 135, 385–396
20. Bache, K. G., Raiborg, C., Mehlm, A., and Stenmark, H. (2003) J. Biol. Chem. 278, 12513–12521
21. Bilodeau, P. S., Urbanowski, J. L., Winistorfer, S. C., and Piper, R. C. (2002) Nat. Cell Biol. 4, 534–539
22. Pandey, A., Fernandez, M. M., Steen, H., Blagoev, B., Nielsen, M. M., Roche, S., Mann, M., and Lodish, H. F. (2000) J. Biol. Chem. 275, 38633–38639
23. Yamada, M., Ishii, N., Asao, H., Murata, K., Kanazawa, C., Sasaki, H., and Sugamura, K. (2002) Mol. Cell Biol. 22, 8648–8658
24. Kanazawa, C., Morita, E., Yamada, M., Ishii, N., Miura, S., Asao, H., Yoshimori, T., and Sugamura, K. (2003) Biochem. Biophys. Res. Commun. 309, 848–856
25. Lu, Q., Hope, L. W., Brasch, M., Reinhard, C., and Cohen, S. N. (2003) Proc. Natl. Acad. Sci. U. S. A. 100, 7626–7631
26. Cliquet, M. J., and Urbe, S. (2006) Trends Cell Biol. 16, 551–559
27. Han, L., Wong, D., Dhaka, A., Abar, D., White, M., Xie, W., Herschman, H., Witte, O., and Colicelli, J. (1997) Proc. Natl. Acad. Sci. U. S. A. 94, 4954–4959
28. Wang, Y., Waldron, R. T., Dhaka, A., Patel, A., Riley, M. M., Rozengurt, E., and Colicelli, J. (2002) Mol. Biol. Cell 22, 916–926
29. Hierro, A., Sun, J., Rusnak, A. S., Kim, J., Prag, G., Emr, S. D., and Hurley, J. H. (2004) Nature 431, 221–225
30. Hu, B., Bliss, J. M., Wang, Y., and Colicelli, J. (2005) Curr. Biol. 15, 815–823
31. Tall, G. G., Barbieri, M. A., Stahl, P. D., and Horazdovsky, B. F. (2001) Dev.

3 C. Kong and P. D Stahl, unpublished data.
32. Kajiho, H., Saito, K., Tsujita, K., Kontani, K., Araki, Y., Kurosu, H., and Katada, T. (2003) *J. Cell Sci.* **116**, 4159–4168
33. Saito, K., Murai, J., Kajiho, H., Kontani, K., Kurosu, H., and Katada, T. (2002) *J. Biol. Chem.* **277**, 3412–3418
34. Topp, J. D., Gray, N. W., Gerard, R. D., and Horazdovsky, B. F. (2004) *J. Biol. Chem.* **279**, 24612–24623
35. Kimura, T., Sakisaka, T., Baba, T., Yamada, T., and Takai, Y. (2006) *J. Biol. Chem.* **281**, 10598–10609
36. Barbieri, M. A., Fernandez-Pol, S., Hunker, C., Horazdovsky, B. H., and Stahl, P. D. (2004) *Eur. J. Cell Biol.* **83**, 305–314
37. Barbieri, M. A., Kong, C., Chen, P. I., Horazdovsky, B. F., and Stahl, P. D. (2003) *J. Biol. Chem.* **278**, 32027–32036
38. Deleted in proof
39. Su, X., Lodhi, I. J., Saltiel, A. R., and Stahl, P. D. (2006) *J. Biol. Chem.* **281**, 27982–27990
40. Takata, H., Kato, M., Denda, K., and Kitamura, N. (2000) *Genes Cells* **5**, 57–69
41. Gaullier, J. M., Simonsen, A., D’Arrigo, A., Bremnes, B., Stenmark, H., and Aasland, R. (1998) *Nature* **394**, 432–433
42. McCullough, J., Clague, M. J., and Urbe, S. (2004) *J. Cell Biol.* **166**, 487–492
43. Row, P. E., Prior, I. A., McCullough, J., Clague, M. J., and Urbe, S. (2006) *J. Biol. Chem.* **281**, 12618–12624