The Src Homology 2 Domain of Rin1 Mediates Its Binding to the Epidermal Growth Factor Receptor and Regulates Receptor Endocytosis*

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Activated epidermal growth factor receptors (EGFRs) recruit intracellular proteins that mediate receptor signaling and endocytic trafficking. Rin1, a multifunctional protein, has been shown to regulate EGFR internalization (1). Here we show that EGF stimulation induces a specific, rapid, and transient membrane recruitment of Rin1 and that recruitment is dependent on the Src homology 2 (SH2) domain of Rin1. Immunoprecipitation of EGFR is accompanied by co-immunoprecipitation of Rin1 in a time- and ligand-dependent manner. Association of Rin1 and specifically the SH2 domain of Rin1 with the EGFR was dependent on tyrosine phosphorylation of the intracellular domain of the EGFR. The recruitment of Rin1, observed by light microscopy, indicated that although initially cytosolic, Rin1 was recruited to both plasma membrane and endosomes following EGF addition. Moreover, the expression of the SH2 domain of Rin1 substantially impaired the internalization of EGF without affecting internalization of transferrin. Finally, we found that Rin1 co-immunoprecipitated with a number of tyrosine kinase receptors but not with cargo endocytic receptors. These results indicate that Rin1 provides a link via its SH2 domain between activated tyrosine kinase receptors and the endocytic pathway through the recruitment and activation of Rab5a.

Ligand-induced internalization of epidermal growth factor receptors (EGFRs)1 is a highly regulated process and has served as a model system for studying clathrin-mediated endocytosis of tyrosine kinase receptors (RTKs) (2, 3). Upon ligand binding, activated cell surface RTKs regulate a wide variety of cellular processes from cell proliferation to apoptosis (4–8). Much of this regulation is mediated by specific components that are recruited to the RTKs. For example, the activation of the EGFR kinase and subsequent auto-phosphorylation of this receptor protein lead to the recruitment and/or activation of phospholipase γ-1 (9–13), Src (14–16), phosphatidylinositol-3 kinase via p85 (17), Eps15 (18, 19), Eps8 (20, 21), c-Cbl (22, 23), Grb2 (24, 25), and the adapter AP-2 (26, 27). Of the many cell systems that are impacted by EGFR signaling, the activation of the Ras-dependent extracellular-regulated kinase/microtubule-activated protein kinase cascade appears to have the most pronounced effect on the proliferative response to EGF (28, 29). Therefore, regulation of this EGF-activated pathway plays a critical role in the maintenance of cellular homeostasis (30).

Attachment of EGFR signaling is mediated by internalization and subsequent degradation of activated receptors (31, 32). However, there is increasing evidence that simple removal of the activated EGFR receptor from the plasma membrane does not lead to immediate signal attenuation (33). Rather, the signaling capacity of the receptor may be qualitatively or quantitatively changed due to the different effector molecules encountered by the receptor as it travels through the compartments or subcompartments of the endocytic pathway (34–39). Only upon the final delivery to the lysosome and/or some prelysosomal degradative compartment are the receptors completely inactivated. Following receptor internalization, Rab5a (a member of the small GTP-binding protein family) plays a key role in regulating the trafficking of the EGFR (2). Newly formed endocytic vesicles carrying the activated EGFR and the other cargo fuse with early endosomes and gain access to the endocytic pathway. Endosome fusion is a Rab5a-dependent process. Using an in vitro assay that measures early endosome fusion, activation of Rab5a was found to be rate limiting for this process (40–44). Overexpression of Rab5a and/or an activated allele of Rab5a has also been shown to stimulate both EGFR uptake and fluid-phase endocytosis (2). Furthermore, we have demonstrated that activation of EGFR regulates the nucleotide status of Rab5a, a process that was dependent on selected domains in the cytoplasmic tail of the EGFR and on the activation of Ras (1).

Overexpression of activated Ras has long been known to stimulate fluid-phase endocytosis (45–47), but the mechanism by which Ras exerted its influence on the endocytic pathway was not understood. Recently, we have demonstrated that Ras-activated endocytosis is facilitated by Rab5a, in part, by the ability of Ras to directly regulate the Rab5a nucleotide exchange activity of Rin1 (1). Rin1 contains an SH2 (Src homology 2) domain, a proline-rich domain, a Vps9p domain, and a...
region involved in the binding of activated Ras (48, 49). The Vps3p domain of Rin1 has been shown to serve as a Rab5α-specific guanine nucleotide exchange factor. Guanine nucleotide exchange factor activity of Rin1 is potentiated by the binding of activated H-Ras and also increases EGFR endocytosis when co-expressed with Rin1. The ability of activated Ras to increase EGFR endocytosis is mitigated when a mutant form of Rin1 that lacks Rab5α guanine nucleotide exchange factor activity is co-expressed. These studies clearly demonstrate that Rab5α, Rin1, and Ras play a coordinated role in regulating EGFR endocytosis.

In this study, we examined the role that the SH2 domain found in Rin1 plays in mediating EGFR endocytosis and describe the functional relationship between specific EGFR and Rin1 domains. Both in vitro and in vivo assays demonstrated that the SH2 domain is essential for interaction of Rin1 with the EGFR. Moreover, EGFR tyrosine auto-phosphorylation is required for this interaction. The association of Rin1 with the EGFR appears to be transient, and expression of the Rin1-SH2 domain has been shown to effectively compete EGFR internalization. Rin1 was also found to interact with several other well-characterized RTKs. The functional interaction between the SH2 domain of Rin1 and RTKs points to a novel signal transduction pathway by which Ras and Rab5α are directly linked to the EGFR via SH2 domain of Rin1.

EXPERIMENTAL PROCEDURES

Cell Lines

EGFR cell lines were kindly supplied by A. Sorkin (University of Colorado Health Sciences Center, Denver, CO), L. Beguinot (Laboratory of Molecular Oncology, Milan, Italy), A. Wells (University of Pittsburgh), and G. Carpenter (Vanderbilt University, Nashville, TN). CHO-insulin receptor (IR) and CHO-EGFR cell lines were kindly supplied by R. A. Roth (Stanford University School of Medicine) and J. E. Pessin (Mt. Sinai School of Medicine, New York, NY), respectively. HEK293 cells transfected with wild-type EGFR and stably transfected with EGFR were kindly supplied by Dr. Hiroshi Maruta from the Ludwig Institute for Cancer Research (London).

Plasmids and Viruses

Construction of GST-Rin1 Domains—Rin1-SH2 (amino acids 67-154) and Rin1-C (amino acids 413-730) were amplified by PCR using primers designed to produce a wild-type Rin1 vector using FuGENE 6 (Roche Applied Science) as described previously (46). The amplified fragments were digested with appropriate restriction enzymes and cloned into pGEX4T (Amersham Biosciences).

Construction of Recombinant Sindbis Viruses—Human Rab5α cDNA was subcloned into the unique XhoI restriction site of the Sindbis vector Totol000–223F (46). Rin1-WT, Rin1-C, and Rin1-N cDNA fragments were cloned by PCR using primers designed previously (1). The amplified fragments were ligated into pGEX4T vector (Amersham Biosciences) as described previously (46).

Whole Cell Lysates, Immunoprecipitation, and Western Blotting—

NR6 cells that stably express the human EGFR (NR6-EGFR) were serum-starved, cultured with 100 nM EGF for 1 h at 4°C, and then incubated at 37°C for the indicated times. Cell monolayers were washed with PBS-buffered saline containing 1 mM sodium orthovanadate and 5 mM β-glycerophosphate and lysed in ice-cold lysis buffer (1% Nonidet P-40, 10% glycerol, 50 mM Hepes, 100 mM NaCl, 1 mM sodium orthovanadate, 5 mM β-glycerophosphate, 5 mM EDTA, 1 mM NaF, 1 mM phenylmethylsulfonyl fluoride, 2 μg/ml pepstatin A, 2 μg/ml leupeptin, and 2 μg/ml aprotinin). Lysates were clarified by centrifugation at 16,000 × g for 15 min at 4°C. Cell proteins were measured with Bio-Rad procedure, and the EGFR and Rin1 were immunoprecipitated using polyclonal anti-EGFR (Santa Cruz Biotechnologies) antibodies as described previously (46). The immunoprecipitates were then separated by SDS-PAGE, blotted to nitrocellulose membrane, and probed with monoclonal antibodies to EGFR, Rab5α, and Rin1. Cell lysates from CHO-IR, NIH-3T3-platelet-derived growth factor receptor (PDGFR), 293T-EGFR, CHO-mannose receptor (MR), and CHO-transferrin receptor (TR) were prepared from stimulated or non-stimulated cells. Each cell line was stimulated with the appropriate ligands as indicated in each figure legend. The cell extract was then prepared as described above for the NR6-EGFR cell line.

Receptor Internalization Studies—Mouse EGFR (Invitrogen) was iodinated with 125I (PerkinElmer Life Sciences) using IODO-BEADS (Pierce) according to the manufacturer’s protocol. The specific activities of labeled ligands were typically 150,000 cpm/ng (600 Ci/mmol). NR6 monolayers were infected with the empty Sindbis vectors or Sindbis viruses as described previously (46). NR6 cell monolayers infected with Sindbis virus alone or with Rin1 recombinant virus for 6 h were serum-starved as indicated in each figure legend prior to activation with EGF for the indicated times. NR6 cells expressing the different Rin1 constructs was washed in binding buffer (α-minimum essential medium supplemented with 13 mM HEPES, pH 7.4, 0.3% fetal bovine serum, and 1 mg/ml bovine serum albumin) and incubated at 4°C for 1 h with 100 pM 125I-EGF. To assess internalization, the cells were incubated at 37°C in binding buffer lacking 125I-EGF in an air environment for the indicated times. Non-specific binding (−2%) was assessed in the presence of 200 nM unlabeled human EGF (Sigma) and subtracted from the total.

Confocal Microscopy—Cells grown on glass coverslips were examined by confocal microscopy in the absence or presence of 100 nM EGF as described previously (2). Confocal microscopy was carried out on a Bio-Rad MRC1024 confocal microscope.

GST Pull-down Assay—GST fusion proteins were expressed and purified as described previously (50). A431 and NR6-EGFR cell lines were serum-starved for at least 6 h and stimulated or not by EGF (100 ng/ml) for 10 min at 37°C. Cells were solubilized at 4°C in 20 mM Tris-Cl, pH 7.4, 150 mM NaCl, 5 mM EDTA, 1% Triton X-100, and a mixture of 0.1% (w/v) CHAPS, 0.5 mM sodium orthovanadate, 5 mM β-glycerophosphate, and 1% non-ionic detergent (Nustar, NJ) was added. Lysates were clarified (4°C, 16,000 g) three times, and bound protein was eluted by SDS sample buffer.Solubilized protein was separated by SDS-PAGE, transferred to nitrocellulose filters, and probed with specific antibodies.

RESULTS

Full-length Rin1 Interacts with the EGFR Receptor—Activated tyrosine kinase receptors are rapidly internalized and targeted to early endosomes. Recent work (1) has demonstrated that Rin1 operates as a linker protein by serving to couple the activation of the EGFR to the recruitment and activation of Rab5α, a requisite for access to early endosomes. To determine whether Rin1 interacts with the EGFR, co-immunoprecipitation experiments were carried out using lysates from HeLa cells, representative of cultured cells expressing “normal” levels of EGFR receptor (Fig. 1A) as well as cells overexpressing Rin1 and EGFR receptor (Fig. 1B). In Fig. 1A, right panel, HeLa cells were serum-starved overnight and then stimulated with EGF for 5 min. The cells were lysed, and the EGFR was immunoprecipitated under native conditions. Proteins that co-immunoprecipitated with the EGFR were separated by SDS-PAGE, and the presence of Rin1 and EGFR was determined by Western analysis. As seen in Fig. 1A, right panel, Rin1 was co-immunoprecipitated with the EGFR when the cells were...
stimulated with EGF. In the absence of added EGF, detectable but small amounts of Rin1 were observed in the immunoprecipitate. As a control, we used the p85 regulatory subunit of phosphatidylinositol 3-kinase, an SH2-containing protein known to interact with the EGFR. In Fig. 1A, left panel, the presence p85 was detected in the EGFR immunoprecipitates by Western blotting following stimulation with EGF. The amount of Rin1 and p85 immunoprecipitated with EGFR was estimated at 0.8 and 1.2%, respectively. We further examined the co-immunoprecipitation of p85 and Rin1 in cells overexpressing p85 and Rin1. CHO cells stably expressing EGFR were transiently transfected with pcDNA3-Rin1 or pcDNA3-p85. Following 6-h serum starvation, the cells were stimulated with 100 nM EGF for 5 min. Cell lysates were prepared, and EGFR was immunoprecipitated. As shown in Fig. 1B, Rin1 (right panel) and p85 (middle panel) co-immunoprecipitated with the EGFR in the presence of added EGF but not in unstimulated cells. To determine the time dependence of Rin1-EGFR association, NR6-EGFR cells were grown in serum-free media and incubated in the presence or absence of EGF (100 nM) for a period covering a total of 60 min. Rin1 was co-immunoprecipitated with the EGFR from NR6-EGFR lysates when the cells were incubated in the presence of EGF but not in its absence. After as little as 1-min exposure to EGF, the EGFR-Rin1 interaction could be detected. This response was maximal between 1 and 10 min with the strongest signal being observed at 5 min, trailing to small but detectable levels after 60-min post-EGF addition. The trailing off of the Rin1-EGFR signal (Fig. 1C) may be attributed to the loss of EGFR as shown in the bottom panel of Fig. 1C. These results suggest that Rin1 is preferentially associated with activated EGFR in a transient manner.

The SH2 Domain in the N-terminal Region of Rin1 Interacts with the EGFR—Rin1 has at least four clearly defined domains (1). The N-terminal region of the molecule contains a SH2 domain followed by a proline-rich domain, the Vps9p domain, and finally, the Ras association domain (Fig. 2). To identify the portion of Rin1 that interacts with the EGFR, we prepared a series of HA-tagged constructs that encode various domains of Rin1. Initially, N-terminal (Rin1-N) and C-terminal (Rin1-C) expression constructs (Fig. 2) were tested for their ability to interact with the EGFR (Fig. 3A). These constructs, as well as full-length Rin1, were expressed in EGF-NR6 cells using Sindbis virus for transient expression. After infection, the cells were stimulated with EGF (100 nM) for 5 min or left untreated. Immediately following this incubation, the cells were cooled, washed, and lysed and EGFR was immunoprecipitated. EGFR and its associated proteins were separated by SDS-PAGE and subjected to Western analysis. As shown in Fig. 3A, full-length Rin1 co-immunoprecipitated with the EGFR as did the N-terminal portion of Rin1 (i.e. Rin1-N). The C-terminal portion of Rin1 (Rin1-C) did not co-immunoprecipitate with the EGFR. Because the N-terminal portion of Rin1 contains both a putative SH2 domain as well as a proline-rich domain, the ability of the SH2 domain alone to bind to the EGFR was determined. In this case, GST fusion proteins that contained the SH2 domain (GST-Rin1-SH2) or the proline-rich domain (GST-Rin1-Pro) were used in addition to a C-terminal expression construct (GST-Rin1-C). GST alone was included as a control. A431 cells were serum-starved for 6 h and then treated with EGF (100 nM) for 10 min. Control cells did not receive EGF. Cell lysates were prepared and incubated with glutathione beads that had been preloaded with the GST fusion proteins or GST alone. After
Fig. 3. EGFR interacts with the N-terminal region of Rin1. A, the N terminus of Rin1 is required for EGFR-Rin1 interaction. EGFR-NR6 cell lines were infected with Sindbis vector encoding HA-Rin1-WT, HA-Rin1-N (SH2 and Pro-rich domains), or HA-Rin1-C (Vps9p and Ras domains). The cells were serum-starved for 2 h and then stimulated with 100 nM EGF for 5 min. The cells were lysed, and EGFR was immunoprecipitated as described under “Experimental Procedures.” The bound proteins were separated by SDS-PAGE and examined by immunoblotting with anti-HA and anti-EGFR antibodies. Molecular mass (MW) is indicated in kDa. The experiment was repeated twice with similar results. B and C, SH2 domain of Rin1 is required for Rin1-EGFR interaction. EGFR-stimulated or EGFR-non-stimulated cell lysates were prepared from A431 cells as described under “Experimental Procedures.” Cell extracts were incubated either in the presence of GST alone (5 μg/50 μl) or in the presence of the indicated GST-Rin1 fusion proteins (B) or in the presence of either GST-p85 or GST-Rin1-SH2 (C) as indicated for 1 h at 4 °C. After incubation, the beads were washed three times and the eluted proteins were separated by SDS-PAGE. EGFR was detected by Western blot using either anti-EGFR or anti-phospho-EGFR antibodies. Added EGFR and GST proteins are also shown. The experiment was repeated three times with similar results.

extensive washing, the proteins were eluted from the beads with GSH (reduced glutathione) and separated by SDS-PAGE and the presence of EGFR was determined by Western analysis. The relative amount of EGFR in each cell lysate used is shown in Fig. 3B. As seen in the Fig. 3B, top panel, only GST-Rin1-SH2 was effective in interacting with the EGFR. Neither GST-Rin1-Pro nor GST-Rin1-C nor GST alone interacted with the EGFR. In Fig. 3C, an experiment identical to that described in Fig. 3B was carried out to compare the relative EGFR pull-down capacities of GST-Rin1-SH2 and GST-p85. The data show that both Rin1-SH2 and p85 interact with the EGFR under these conditions to approximately the same extent.

A Motif on the Cytoplasmic Tail of EGFR Interacts with the SH2 Domain of Rin1—To define the site on the EGFR where Rin1 interacts, a series of C-terminal truncation constructs and point mutations of the EGFR were examined for their ability to bind Rin1. Three truncations of the EGFR were used including EGFR-C’1000, EGFR-C’991, and EGFR-C’973. In addition, we used a full-length “kinase dead” point mutant (K721M) construct and several well described EGFR constructs (EGFR-F1, EGFR-F2, EGFR-F3, EGFR-F4, and EGFR-F5) in which the major auto-phosphorylation sites were mutated. We also used an EGFR-Phe-992 point mutant. The EGFR cell lines were transfected with a Sindbis virus construct that encoded a full-length HA-tagged version of Rin1. The transfected cells were serum-starved for 4 h, EGF (100 nM) was added, and the cells were incubated for 5 min. The cells were lysed, and the EGFR was immunoprecipitated under native conditions. Co-immunoprecipitated proteins were eluted, separated by SDS-PAGE, and subjected to Western analysis using anti-EGFR or anti-HA antibodies. As shown in Fig. 4A, only the full-length EGFR interacted with Rin1. The EGFR-C’1000 truncation construct also interacted with Rin1 but at a very reduced level. Interestingly, the truncated mutant EGFR-C’973 (Fig. 4A) and the “kinase dead” EGFR mutant (K721M) (data not shown) were unable to interact with Rin1, indicating that receptor phosphorylation is required for Rin1 interaction. Consistent with this interpretation, the tyrosine point mutants of EGFR-F5, EGFR-F4, EGFR-F3, and EGFR-F2 all failed to interact with Rin1 (data not shown). Taken together, these observations led us to speculate that tyrosine residues 992 and 1173 would be important for EGFR and Rin1 interaction. The results in Fig. 4A indicate that these two EGFR mutants (Phe-992 and Phe-1173) fail to interact with Rin1. It is also important to note that when the Western blot from these two EGFR mutants were overexposed we were able to find very reduced levels of Rin1 that suggest that both residues are important in the EGFR-Rin1 interactions. To confirm that the EGFR-Rin1 interaction was mediated by the SH2 domain of Rin1, the GST-Rin1-SH2 fusion construct was used in a similar experiment. Cells expressing wild-type or mutant EGFR were treated with EGF (100 nM) for 10 min. Lysates were generated and incubated with glutathione beads preloaded with GST-Rin1-SH2. Consistent with the results obtained with full-length Rin1, the GST-Rin1-SH2 fusion protein interacted robustly with wild-type EGFR and minimally with the C’1000 truncated receptor. Both interactions were dependent on the activation of the receptor by ligand binding (Fig. 4B). EGFR-Phe-1173 and EGFR-Phe-992 point mutants and the EGFR-C’973 truncated mutant were unable to interact with Rin1. These results suggest that Rin1 is associated with activated EGFR directly or indirectly through residues 992 and 1173.

Indirect Fluorescence Microscopy: The Effect of EGFR Activation on Rin1 Localization in NR6 Cells—NR6-EGFR cells were infected with Sindbis virus encoding Rin1. The cells were
As a result, we predicted that expression of Rin1-SH2 domain of EGFR internalization. To test this hypothesis, we expressed Rin1-WT and the Rin1-SH2 domain in EGFR-NR6 cells by transient transfection and then quantified EGFR expression of Rin1-SH2 has no effect on the activation of Ras by EGFR.

**EGF Induces the Formation of a Complex Containing Rin1, Ras, and Rab5a**—Rin1 is known to bind to Ras/GTP via the Ras association domain located in the C-terminal region of Rin1 (48). The Vps9p domain of Rin1 has been shown to interact with Rab5a and to mediate guanine nucleotide exchange on Rab5a in a Ras/GTP-dependent manner (1). We now show that the SH2 domain of Rin1 interacts directly or indirectly with a phosphorylated tyrosine motif on the cytoplasmic tail of the EGFR, as expected, increased receptor-mediated internalization of EGF. The levels of expression of Rin1-WT and Rin1-SH2 are shown in the Fig. 6A, insert. In Fig. 6B, we examined the effect of Rab5a-WT and Rin1-SH2 as well as Rin1-WT on transferrin endocytosis. Cells were infected with Sindbis virus alone and with Sindbis encoding Rab5a, Rin1-WT, and Rin1-SH2. The cells were cooled and incubated with HRP-transferrin (6 μg/ml, 60 min on ice) and then warmed to 37 °C for 6 min to allow internalization to proceed. The cells were then cooled and acid-washed to remove surface-bound HRP-transferrin, and the amount of HRP internalized was measured in cell lysates. As expected, Rab5a expression enhanced HRP-transferrin internalization by nearly 2-fold. Rin1-SH2 and Rin1-WT expression had virtually no effect. Earlier work has shown that Rab5a expression enhanced the internalization of the EGFR in response to added EGF (2). To confirm this point, a second double transfection experiment was carried out to determine whether the effect of Rab5a expression on EGF internalization was indeed sensitive to Rin1-SH2. In Fig. 6C, cells were co-transfected with Rin1-SH2 and Rab5a-WT. Endocytosis of EGF was then quantified as described. Rab5a expression increased EGF internalization by a factor of two. Again, in both the control cells and the Rab5a-transfected cells, Rin1-SH2 substantially reduced the internalization of EGF. We conclude that Rin1-SH2 interferes with the interaction of Rin1 with the EGFR receptor. Does Rin1-SH2 expression interfere with the ability of activated EGFR to enhance guanine nucleotide exchange on Ras? To explore this point, NR6-EGFR cells expressing Rin1-SH2 were stimulated with EGF for (5 min) and cell lysates were prepared as described in Fig. 6D. We then used GST-Raf to pull down Ras/GTP, which was then quantified by Western blotting as described earlier (1). The results in Fig. 6E suggest that expression of Rin1-SH2 has no effect on the activation of Ras by EGFR.
Rin1 Interacts with Other Growth Factor Receptors Such as Insulin Receptor, Fibroblast Growth Factor Receptor, and PDGF Receptor but Not Cargo Receptors—The interaction of Rin1 with the EGFR, Rab5a, and H-Ras may provide a paradigm for linking the trafficking of activated tyrosine kinase receptors to the modulation of cell signaling cascades. To explore this possibility further, we examined the ability of Rin1 to bind three other well characterized tyrosine kinase receptors, IR, PDGFR, and FGFR-II. In addition, two non-tyrosine kinase receptors, TIR and MR, were also used in this analysis. A series of established cell lines expressing the various receptors were infected with Sindbis virus encoding Rin1-WT. These cell lines included EGFR-NR6, CHO-IR, CHO-FGFR-II, NIH-3T3-PDGFR, CHO-TIR, and CHO-MR. Cells were serum-starved and then incubated with their respective ligands for 5 min. The cells were lysed and prepared for immunoprecipitation under native conditions with the appropriate receptor antibody. The immunoprecipitated proteins were then resolved by SDS-PAGE, and the presence of Rin1 and the various receptors was determined by Western blot analysis. In the presence of the appropriate ligand, Rin1 co-immunoprecipitated with each of the activated tyrosine kinase receptors examined. On the other hand, Rin1 did not co-immunoprecipitate with the transferrin receptor (Fig. 8A). In a second set of experiments, we tested the ability of the SH2 domain of Rin1 (GST-Rin1-SH2) to interact with the insulin tyrosine kinase receptor as well as with the mannose receptor, a well characterized cargo receptor. The EGFR was included as a control. NR6-EGFR and CHO-IR were incubated with their respective ligands as described above, and cell lysates were prepared. The lysates were incubated with GST alone or GST-Rin1-SH2 coupled to glutathione beads. The beads were washed, and the eluted proteins were separated by SDS-PAGE, transferred, and blotted with anti-receptor antibodies. As shown in Fig. 8B, both EGFR and IR interacted with the GST-Rin1-SH2 from cell lysates incubated with ligand but not in the absence of ligand. The mannose receptor was not detected among the proteins in the pull-down assays with a specific anti-MR antibody.

DISCUSSION

Tyrosine kinase receptors, in general, and the EGFR, in particular, are known to be internalized following activation. The traditional view is that the internalization process plays an attenuation role by removing active receptors from the plasma membrane. However, more recent studies suggest a more substantial role for internalization, possibly influencing the quality and type of signal generated or facilitating access to different intracellular targets. Activation of the receptor kinase by ligand binding and auto-phosphorylation initiates a process essential for all subsequent receptor signaling and trafficking events. Multiple auto-phosphorylation sites on the receptor facilitate the selective and reversible recruitment of adapter...
proteins including Shc, Grb2, EPS8, Cbl, and lipid kinases whose presence directs the functional output of the receptor (33). The overall program for adapter recruitment is complex with some factors preferentially recruited to cell surface receptors and others selectively recruited to internalized receptors on the surface of endosomes (33).

Early work by Nesterov et al. (26) and Sorkin (25) first demonstrated that EGFR activation and internalization were coupled by the activation-dependent recruitment of AP-2 by receptor molecules. Recent work has implicated the adapter protein Shc (51), which may utilize motifs that interact with AP-2 proteins to facilitate EGFR accumulation in coated pits and internalization (52). Apart from utilization of an AP-2 binding mechanism, a key unresolved question is the relationship between receptor activation and receptor trafficking. Di Fiore and colleagues (53) have shown that RnTre, a molecule initially identified as a protein that bound to the SH3 domain of Eps8, is a Rab5a GTPase activation protein. They further suggested that the GTPase activation protein activity of RnTre serves to reduce the internalization of the EGF receptor and thereby influence the signaling capacity of the activated receptor. Recent work by Martinu et al. (54) indicates that RnTre is associated with Grb2 where it may provide a negative signal to Rab5a activation. Barbieri et al. (2) have shown that activation of the EGFR leads to increased accumulation of Rab5a in the GTP form, suggesting a coupling of EGFR activation and GDP/GTP exchange or GTP hydrolysis by Rab5a or both. An effect of EGFR on GDP/GTP exchange was favored because overexpression of the dominant-negative mutant of Rab5a leads to inhibition of EGFR internalization. Moreover, dominant-negative Rab5a expression substantially reduced the signal transduction properties of the EGFR.2 These and other findings suggested a strong linkage between the activation of the EGFR and the activation of mechanisms to enter and navigate the early endocytic pathway, which is Rab5a-dependent. The ef-

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2 M. A. Barbieri and P. D. Stahl, unpublished observation.

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We set out to determine whether full-length Rin1 interacts with the EGFR. An EGFR immunoprecipitation assay was employed using lysates from cultured cells such as HeLa cells expressing normal levels of EGFR and from transfected cells expressing high amounts of Rin1 and high amounts of the EGFR. The results using both endogenous Rin1 as well as transiently expressed Rin1 indicated that Rin1 did interact either directly or indirectly with the EGFR when EGF was added to the cells prior to cell lysis. The time course of the interaction was also consistent with the internalization and trafficking of the EGFR. To hone in on the specific site in Rin1 that actually engaged the EGFR, we prepared deletion constructs of Rin1, both N- and C-terminal deletions. The rationale for this approach was based on earlier work showing that the C-terminal half of Rin1 is biologically active with respect to Rab5α exchange (1). The experiments were carried out with cell lysates by immunoprecipitation of the EGFR and by using GST fusion proteins of the various domains found in Rin1. These results conclusively showed that the SH2 domain of Rin1 is principally the domain that interacts with the activated EGFR.

We compared the co-immunoprecipitation of the Rin1-EGFR complex with the co-immunoprecipitation of the p85-EGFR complex and found that they were similar in efficiency. Moreover, point mutants in Rin1-SH2 (Rin1-SH2-(R94A,Y121F)) chosen because of their similarity to residues in Src-SH2 (55) decreased the binding to the EGFR in pull-down assays. The other half of the problem, the site on the EGFR where Rin1 binds, was then approached with the aid of the numerous

3 M. A. Barbieri, C. Kong, P.-I. Chen and P. D. Stahl, unpublished observations.
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deletion and point mutants available for the EGFR. Initial experiments with two EGFR truncation constructs, C’973 and C’1000, revealed that whereas the former was unable to interact with Rin1, the larger truncation construct, EGFR C’1000, retained a small amount of functional Rin1 binding activity. This activity was abolished by a point mutation at Tyr-992 (i.e. C’1000/Fhe-992). In addition, we also found that a “kinase dead” EGFR mutant (K721M) and other previously characterized EGFRs with multiple substitutions including EGFR-F2–5 were unable to interact with Rin1. Two point mutants, the EGFR-F1 mutant (Phe-1173) and Phe-992, were negative. We conclude from these observations that the key residues required for Rin1 interaction with the EGFR are Tyr-992 and Tyr-1173. The apparent requirement for two phosphorylation sites (Tyr-992 and Tyr-1173) is interesting. It is possible that more than one phosphorylation site provides a “timing” mechanism for Rin1 binding. We are unable to conclude whether the interaction of Rin1 with the EGFR is direct or indirect or whether the above-mentioned point mutations produce a conformational change in the EGFR that precludes Rin1 interaction.

Confocal analysis of Rin1 in EGF-treated cells shows that it is present on plasma membrane and on endosomes. Ras displayed a similar pattern in EGF-treated cells (i.e. present on plasma membrane and endosomes). On the other hand, Rab5a was found to co-localize with Rin1 and Ras on endosomes but Rab5a was not found on the plasma membrane. Experiments with cells expressing dynamin K44A, a mutant that prevents clathrin-mediated internalization, restricted Rin1 to the plasma membrane (data not shown). We also demonstrated that internalized Rin1 did not co-localize with Rab7, a marker for late endocytic compartments (data not shown).

To test the hypothesis that the SH2 domain mediates the recruitment of Rin1 to the EGFR and that the interaction plays a key functional role, we expressed the SH2 domain of Rin1 in NR6 cells and then examined the internalization of the EGFR. Expression of Rin1-SH2 severely retarded the internalization of the EGFR following the addition of ligand. The effect of Rin1-SH2 appeared to be specific for “activated” receptor-mediated endocytosis since Rin1-WT and Rin1-SH2 expression did not appreciably affect TFR internalization. Rab5a expression, on the other hand, stimulated both TFR and EGFR internalization (2, 44). How Rab5a expression stimulates TFR internalization is unknown, but as shown in Fig. 6D, our data suggest that the stimulatory effect of Rab5a on EGFR internalization is dependent on Rin1. Is the SH2 domain of Rin1 necessary for EGFR internalization? The fact that Rin1-SH2 interacts with the EGFR via one or more phosphotyrosine sites and that expression of Rin1-SH2 blocks the internalization of EGFR and the stimulatory effects of Rab5a on EGFR internalization supports the conclusion that Rin1-SH2-EGFR interaction is required. We cannot completely rule out the possibility that Rin1-SH2 interacts with an unknown factor, which is titrated away from the EGFR, resulting in impaired EGFR internalization. A second experiment, using cells overexpressing all of the necessary components that interact with Rin1 (EGFR, Rin1, H-Ras, and Rab5a), showed that the EGFR can “pull down” a complex containing at least Ras, Rin1, and Rab5a from cell lysates. When the experiment was repeated following expression of all of the above but substituting an N-terminal deleten construct of Rin1 lacking the SH2 domain, neither H-Ras nor Rab5a was found in the immunoprecipitates. On the other hand, immunoprecipitation of Rin1-C from lysates of EGF-stimulated cells permitted the co-immunoprecipitation of a complex consisting of Rin1-C, Ras, and Rab5a, suggesting that the activation of Ras via the EGFR is sufficient to recruit Rab5a to a macromolecular complex with Rin1-C. Together, these findings support the idea that a complex containing the receptor, Ras, Rin1, and Rab5a is operative at some point in the Rin1 cycle.

Lastly, addressing the question of whether Rin1 is specific to the EGFR or whether other tyrosine kinase receptors interact with Rin1, we studied several well characterized tyrosine kinase receptors including the IR, FGFR, and the PDGFR. As controls, we included two well characterized “cargo” receptors, the transferrin receptor and the mannose receptor. The data show specific interaction between Rin1 and all of the tyrosine kinase receptors studied and no interaction with any of the cargo receptors. No functional experiments with the above-mentioned receptors have been examined, but this will be an important question in the future. The data presented here and the results from our previous work (1, 2) and the work of many others (56) indicate that there is a critical physiological link between tyrosine kinase receptor signaling and the trafficking of these activated receptors. It is clear that the phosphorylation of the EGFR cytoplasmic domain following ligand binding not only initiates cell signaling cascades but also serves an important role in the recruitment of the Rab5a effector Rin1. The binding of Rin1 (through its SH2 domain) to the activated receptor efficiently localizes a Rab5a guanine nucleotide exchange factor to newly internalized receptor complexes. This close proximity may allow for the recruitment and immediate activation of Rab5a, which in turn directs the receptor complexes through the early stages of the endocytic pathway. Rab5a activation is further regulated by Ras. Earlier work (1) shows that binding to Rin1 potentiated the Rab5a guanine nucleotide exchange activity of Rin1. The interaction between EGFR and Rin1 delineates a novel signal transduction pathway between EGFR and its effectors, Rin1, Rab5a, and Ras, which together coordinate and regulate both signaling and membrane trafficking.

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The Src Homology 2 Domain of Rin1 Mediates Its Binding to the Epidermal Growth Factor Receptor and Regulates Receptor Endocytosis

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