GRIF1 binds Hrs and is a new regulator of endosomal trafficking

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Summary

Endosomal sorting of internalized cell surface receptors to the lysosomal pathway plays a crucial role in the control of cell signaling and function. Here we report the identification of GABA$_A$ receptor interacting factor-1 (GRIF1), a recently discovered protein of unknown function, as a new regulator of endosome-to-lysosome trafficking. Yeast two-hybrid screen and co-immunoprecipitation analysis reveal that GRIF1 interacts with hepatocyte growth factor-regulated tyrosine kinase substrate (Hrs), an essential component of the endosomal sorting machinery. We have mapped the binding domains of GRIF1 and Hrs that mediate their association and shown the colocalization of GRIF1 with Hrs on early endosomes. Like Hrs, both overexpression and siRNA-mediated depletion of GRIF1 inhibit the degradation of internalized epidermal growth factor receptors and block the trafficking of the receptors from early endosomes to the lysosomal pathway. Our results indicate, for the first time, a functional role for GRIF1 in the regulation of endosomal trafficking. Interestingly, overexpression of full-length GRIF1, but not the Hrs- or kinesin-interacting GRIF1 deletion mutants, causes a perinuclear clustering of early endosomes. Our findings suggest that GRIF1 may also participate in microtubule-based transport of early endosomes by acting as an adaptor linking Hrs-containing endosomes to kinesin.

Key words: Endosome, Hrs, GRIF1, Trafficking, Kinesin, Lysosome

Introduction

Endocytic trafficking is critical to the development and function of all eukaryotic cells. Receptors on the cell surface are internalized, either constitutively or in response to binding of their ligands, for example growth factors. The internalized ligand-receptor complexes are delivered to the early endosome, where a sorting decision has to be made between recycling back to the cell surface or transport to the lysosome for degradation. Cargo proteins, such as epidermal growth factor receptor (EGFR), destined for the lysosome are incorporated into vesicles that bud into the lumen of the endosome, leading to the formation of a multivesicular body (MVB). Subsequent fusion of mature MVBs (also known as late endosomes) with lysosomes results in delivery of the internal vesicles into the lysosome lumen, thus allowing the degradation of entire transmembrane proteins (Katzmann et al., 2002; Mullins and Bonifacino, 2001; van Dam et al., 2002). Endosome-to-lysosome trafficking is a key step in the endocytic pathway that not only controls protein degradation, but also determines the potency and duration of intracellular signaling (Katzmann et al., 2002; Waterman and Yarden, 2001). Given the importance of endosome-to-lysosome trafficking in cell physiology, it is not surprising that aberrant changes in endosomal trafficking have been associated with a number of diseases. For example, endosomal abnormalities are among the earliest pathological features of several neurodegenerative disorders, including Alzheimer's disease, Neimann-Pick type C, and Down syndrome (Nixon, 2005). Furthermore, abnormal endosomal trafficking has been linked to cancer (Polo et al., 2004). Elucidation of the molecular mechanisms that control endosomal sorting and trafficking is thus crucial to our understanding of both normal physiology and the pathogenesis of neurodegenerative disorders and cancer.

Hepatocyte-growth-factor-regulated tyrosine kinase substrate (Hrs) is an evolutionarily conserved endosomal protein that has emerged as a key player in the control of endosome-to-lysosome trafficking (Clague and Urbe, 2003). Studies in yeast, *Drosophila*, mouse and various mammalian culture systems have demonstrated that Hrs is required for cargo trafficking from the early endosome to the lysosomal pathway (Chin et al., 2001; Kanazawa et al., 2003; Komada and Soriano, 1999; Lloyd et al., 2002; Piper et al., 1995). Depletion of Hrs results in enlarged early endosomes, impaired MVB formation, and cargo accumulation on the limiting membrane of the MVB (Bache et al., 2003a; Kanazawa et al., 2003; Komada and Soriano, 1999; Lloyd et al., 2002; Piper et al., 1995). Furthermore, overexpression of Hrs has a dominant negative effect on endosome-to-lysosome trafficking (Chin et al., 2001; Hislop et al., 2004; Li et al., 2002). Hrs is believed to coordinate the sorting 'decision' at the early endosome by clustering ubiquitinated cargo proteins underneath a clathrin micro-domain and by recruiting the endosomal sorting complexes required for transport (ESCRTs) (Clague and Urbe, 2003). Although this basic framework has been proposed for Hrs-mediated endosomal sorting, the precise mechanisms regulating the specificity and progression of this process remain elusive.

In order to understand the mechanism of action and regulation of Hrs, we performed yeast two-hybrid screens for Hrs binding partners. We report here the identification and

characterization of γ-amino-*n*-butyric acid A (GABA$_A$) receptor interacting factor-1 (GRIF1) as a new Hrs-interacting protein. GRIF1 is a newly discovered protein of unknown function initially identified in a yeast two-hybrid screen for proteins that bind to the intracellular loop of the GABA$_A$ receptor (GABA$_A$R) β2 subunit (Beck et al., 2002). GRIF1, also known as OGT-interacting protein (OIP98), with a molecular mass of 98 kDa, was independently identified as both an interactor and a substrate for the enzyme β-*N*-acetylglucosaminyl transferase (OGT) (Iyer et al., 2003). Biochemically, GRIF1 has been shown to interact with kinesin (Brickley et al., 2005). However, the functional role of this interaction has not yet been examined. In mammals, there is a GRIF1/OIP98 homologue, called OIP106, which is a distinct gene product and shares 47% amino acid identity with the sequence of GRIF1 (Iyer et al., 2003). Interestingly, a spontaneous mutation in the mouse OIP106 gene (recently renamed *Trak1*) was found to cause hypertonia, a neurological disorder characterized by stiff gait, hunched posture, jerky movements and slight tremor (Gilbert et al., 2006). Furthermore, mutations in the *Drosophila* Trak1 orthologue, called Milton, results in synaptic transmission defects (Gorska-Andrzejak et al., 2003; Stowers et al., 2002). Despite the genetic evidence indicating their importance in neuronal physiology, the cellular localization and biological function of the GRIF1 family of proteins remain unknown.
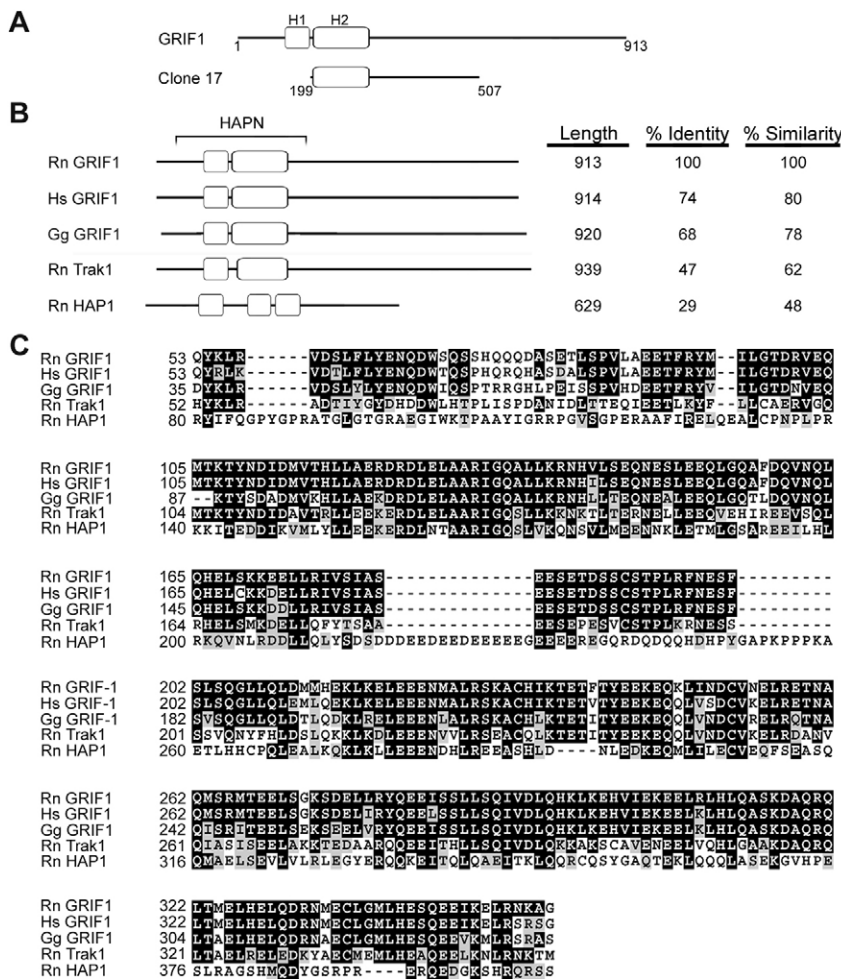
In this study, we characterized the association of GRIF1 with Hrs and defined the structural requirement underlying this new interaction. Moreover, we determined the subcellular localization of GRIF1 and investigated the functional role of GRIF1 in various steps of endocytic trafficking. Our results reveal that GRIF1 interacts and colocalizes with Hrs on the early endosome and that GRIF1 plays an essential role in the regulation of endosome-to-lysosome trafficking. Moreover, our data suggest that GRIF1 may also be involved in microtubule-based transport of early endosomes through its interactions with Hrs and kinesin.

Fig. 1. The identified Hrs interactor, GRIF1, is a member of a new family of coiled-coil domain proteins. (A) Domain structure of GRIF1 (top) and the Hrs-interacting clone (clone 17) isolated from the yeast two-hybrid screen (bottom). (B) Domain structure of GRIF1 and its homologues. Accession numbers for the sequences are as follows: *Rn* GRIF1, NP_598244; *Hs* GRIF1, NP_055864; *Gg* GRIF1, XP_421937; *Rn* Trak1, XP_236715; and *Rn* HAP1, P54256. The amino acid identity and similarity of each protein relative to the protein sequence of rat GRIF1 are indicated. Each protein contains two or three predicted coiled-coil domains shown as white boxes. *Rn* GRIF1: 132-188, 202-354; *Hs* GRIF1: 132-177, 198-354; *Gg* GRIF1: 113-159, 187-334; *Rn* Trak1: 130-173, 206-354; *Rn* HAP1: 169-205, 262-305, 328-370. The bracket indicates the location of the HAPN domain. (C) Alignment of the HAPN domain in GRIF1 and related proteins. Numbers indicate the position of the HAPN domain in each sequence. Rn, *Rattus norvegicus*; Hs, *Homo sapiens*; Gg, *Gallus gallus*.

Results

Identification of GRIF1 as a Hrs-interacting protein

To identify new Hrs binding partners, we screened a rat brain hippocampal/cortical cDNA library using yeast two-hybrid selection with full-length rat Hrs as bait (Chin et al., 2001; Li et al., 2002). A positive clone (clone 17) isolated from the screen encodes the central region (residues 199-507) of GRIF1 (Fig. 1A), a new protein recently discovered from its ability to bind the β2 subunit of the GABA$_A$R (Beck et al., 2002) and the OGT enzyme (Iyer et al., 2003). The specificity of the interaction between GRIF1 and Hrs was confirmed by yeast two-hybrid analysis demonstrating the selective interaction of GRIF1 with Hrs, but not with the coiled-coil domain-containing protein SNAP-25 or the proline-rich cytoplasmic region of synaptophysin (data not shown).

Cloning of the full-length rat GRIF1 cDNA revealed that GRIF1 is a 913-amino-acid protein with two predicted coiled-coil domains, H1 and H2 (Fig. 1A). Database searches showed that GRIF1 orthologues are present in human, dog, mouse and chicken, but not in *C. elegans* or yeast. Sequence analysis indicated that human and chicken GRIF1 share 74% and 68% overall amino acid identity with rat GRIF1, respectively (Fig. 1B). Moreover, rat GRIF1 exhibits 47%

overall amino acid identity to rat Trak1 (Fig. 1B), a 939-amino-acid-long, new protein. Mutation of this new protein has been identified as a genetic defect responsible for hypertonia (Gilbert et al., 2006). GRIF1 is more distantly related to Huntingtin-associated protein-1 (HAP1), a 629-amino-acid protein, for which the binding affinity for huntingtin is enhanced by the polyglutamine expansion associated with Huntington's disease (Li et al., 1995). Rat HAP1 shares 29% overall amino acid identity with rat GRIF1 (Fig. 1B). Interestingly, all three proteins, GRIF1, Trak1 and HAP1, contain a conserved 301- to 324-amino-acid HAP1 N-terminal homologous domain (HAPN) that includes two or three putative coiled-coil regions (Fig. 1B,C). Although the function of the HAPN domain is unknown, this domain is likely to mediate protein-protein interactions. In mammals, GRIF1, Trak1 and HAP1 are the only proteins that contain the HAPN domain. Thus, these three proteins constitute a new protein family, which is characterized by the presence of the HAPN domain. The importance of this protein family in neuronal function is highlighted by recent findings that mutations in the members of this family or their interactor lead to hypertonia and Huntington's disease (Gilbert et al., 2006; Li et al., 1995).

GRIF1 mRNA and protein are enriched in brain and heart

The expression and distribution of the recently identified GRIF1 protein are poorly characterized and remain controversial. Previous northern blot analyses showed a ubiquitous expression of GRIF1 mRNA in all tissues (Beck et al., 2002; Hadano et al., 2001), whereas western blot analysis revealed a preferential expression of GRIF1 protein in brain and heart (Beck et al., 2002; Hadano et al., 2001). The discrepancy between GRIF1 mRNA and protein expression could either be a real phenomenon or an artifact due to the cross-hybridization of the GRIF1 cDNA probe with the ubiquitously expressed mRNA transcripts of the closely related protein Trak1 (Brickley et al., 2005; Gilbert et al., 2006; Iyer et al., 2003). To clarify GRIF1 tissue distribution, we performed northern blot analysis of GRIF1 mRNA expression in various rat tissues under high-stringency hybridization conditions (Fig. 2A). We found a single GRIF1 transcript of 7.0 kb, which is abundantly expressed in heart and brain and moderately expressed in kidney. In addition, we observed a testis-specific GRIF1 transcript of 3.6 kb (Fig. 2A). Longer exposure of the same blot revealed that the 7.0 kb GRIF1 transcript is present at very low levels in all tissues except testis (data not shown).

For characterization of GRIF1 protein, we generated two distinct rabbit polyclonal anti-GRIF1 antibodies, GF1 and GF2, against different regions of rat GRIF1. To investigate the specificity of the GRIF1 antibodies, immunoblot analysis was performed using cell lysates prepared from untransfected PC12 and HeLa cells as well as transfected HeLa cells expressing HA-tagged GRIF1 (Fig. 2B). We found that both GF1 and GF2
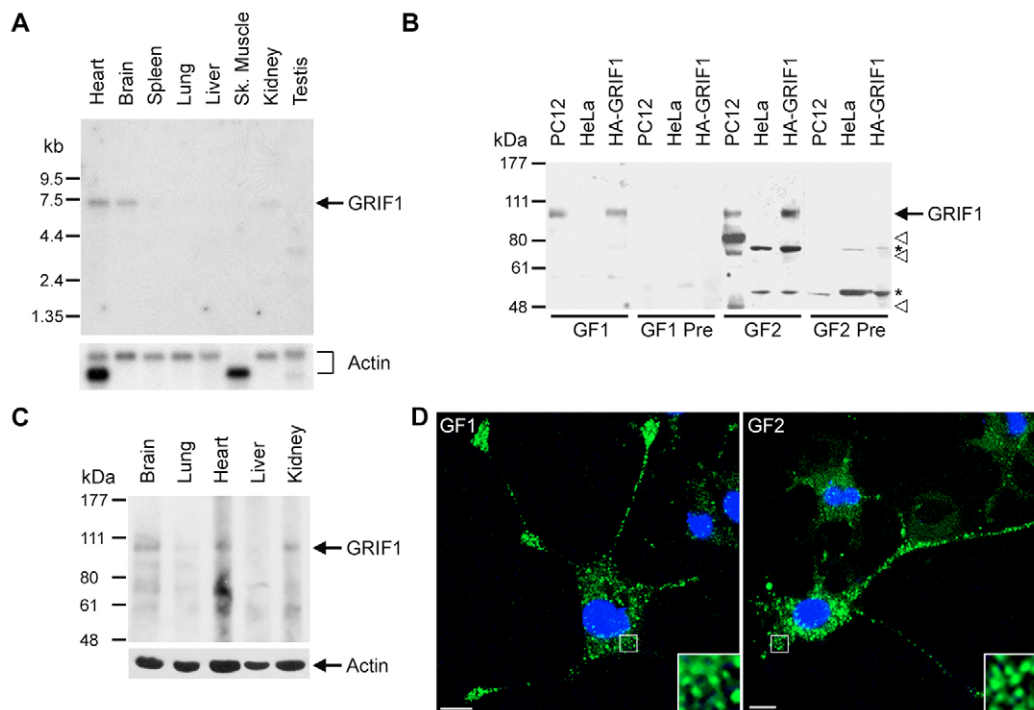
Fig. 2. Preferential expression of GRIF1 mRNA and protein in brain and heart. (A) Northern blot analysis of GRIF1 mRNA expression. A rat multi-tissue northern blot was hybridized with a ^{32}P-labeled partial cDNA probe of GRIF1 (top). Equal loading was confirmed by using a β-actin cDNA probe (bottom). Sk., Skeletal. (B) Specificity of anti-GRIF1 antibodies, GF1 and GF2. Western blot analysis of cell lysates from untransfected PC12 and HeLa cells, and pCHA-GRIF1-transfected HeLa cells using anti-GRIF1 antibodies GF1, GF2, or the corresponding pre-immune serum. The asterisks indicate non-specific immunoreactive bands, and the open arrowheads indicate bands that appear specific to the GF2 antibody. (C) Equal amounts of homogenates (100 μg) from the indicated rat tissues were analyzed by immunoblotting using anti-GRIF1 (GF1) and anti-β-actin antibodies. (D) NGF-differentiated PC12 cells were immunostained using purified anti-GRIF1 antibodies GF1 or GF2 (green); Hoechst-stained nuclei (blue). Insets: enlarged views of boxed region showing punctate staining. Bars, 10 μm.

antibodies specifically recognized recombinant GRIF1 protein in transfected HeLa cells, but not in untransfected HeLa cells. Furthermore, both antibodies recognized the 105 kDa endogenous GRIF1 protein in PC12 cells (Fig. 2B). By contrast, the GF1 and GF2 pre-immune sera did not show any immunoreactivity to the recombinant or endogenous GRIF1 protein, confirming the specificity of our anti-GRIF1 antibodies (Fig. 2B). The lower molecular mass bands (Fig. 2B, arrowheads) seen only in the immunoblots using the GF2 antibody, appear specific and probably represent GRIF1 degradation products. The non-specific bands (Fig. 2B, asterisks), seen when the GF2 antibody or corresponding pre-immune serum was used, disappeared upon GF2 antibody purification (data not shown). In agreement with our northern blot analysis (Fig. 2A), western blot analysis revealed that GRIF1 protein is expressed in rat brain and heart, but not in lung and liver (Fig. 2C). The abundant expression of GRIF1 in brain and heart suggests the involvement of GRIF1 in brain and heart function. The presence of GRIF1 at low levels in other tissues suggests it might also have a broad role in cellular function.

Previous studies have shown that, in transfected HEK293 and COS-7 cells, FLAG- or CFP-tagged, as well as untagged, recombinant GRIF1 protein exhibited a punctate staining pattern (Beck et al., 2002; Brickley et al., 2005; Pozo and Stephenson, 2006), suggesting the presence of GRIF1 on vesicular structures. However, the identity of these vesicular structures has not yet been determined. Moreover, the subcellular localization of endogenous GRIF1 remains uncharacterized. To determine whether our anti-GRIF1 antibodies could be used for immunocytochemistry, we used immunofluorescence confocal microscopy to determine the intracellular distribution of endogenous GRIF1 in nerve growth factor (NGF)-differentiated PC12 cells using the affinity-purified GF1 and GF2 antibodies. Both antibodies revealed a similar punctate staining pattern of endogenous GRIF1 distributed throughout the cell body and neurites (Fig. 2D). No immunoreactivity was observed when the GF1 and GF2 pre-immune sera were used or when primary antibodies were omitted, confirming that the GRIF1 staining is specific (data not shown). The punctate staining pattern of endogenous GRIF1 is consistent with the pattern observed with recombinant GRIF1 and suggests a vesicular localization for GRIF1, which was further characterized as described later in this section.

Hrs and GRIF1 associate in vivo
To determine if the Hrs-GRIF1 interaction detected in the yeast two-hybrid screens actually takes place in vivo, we first performed co-immunoprecipitation experiments using lysates of HeLa cells co-transfected with pEGFP-GRIF1 and pCHA-Hrs or pEGFP-GRIF1 and pCHA vector (Fig. 3A). Immunoprecipitation of the lysates with an anti-HA antibody revealed that GFP-GRIF1 was specifically co-immunoprecipitated with HA-Hrs, but was not co-immunoprecipitated in HA-vector transfected control cells (Fig. 3A). In reciprocal co-immunoprecipitation experiments, anti-HA antibody was able to co-immunoprecipitate GFP-Hrs with HA-GRIF1 (data not shown). These results confirmed a specific interaction of GRIF1 with Hrs in transfected cells.

We then performed additional co-immunoprecipitation
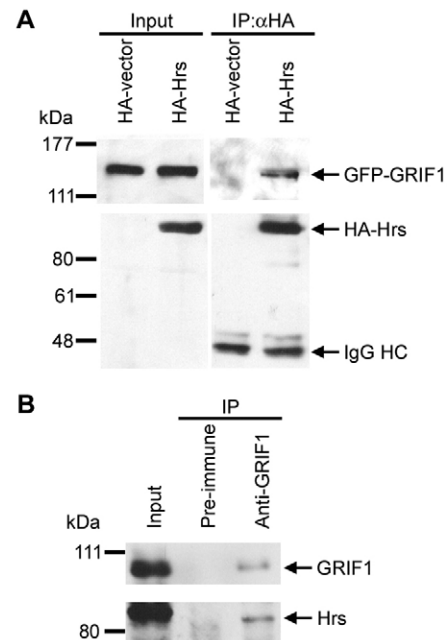
Fig. 3. GRIF1 and Hrs associate in vivo. (A) Co-immunoprecipitation of GRIF1 with Hrs in transfected HeLa cells. HeLa cells were co-transfected with pEGFP-GRIF1 in combination with pCHA-Hrs or pCHA-vector. Lysates were immunoprecipitated with anti-HA antibody, followed by immunoblotting with anti-GFP and anti-HA antibodies. (B) Co-immunoprecipitation of endogenous Hrs with GRIF1 in PC12 cells. PC12 cell lysates were immunoprecipitated with anti-GRIF1 antibody or pre-immune serum, followed by immunoblotting for Hrs and GRIF1.

experiments to examine the association of endogenous GRIF1 and Hrs in PC12 cells (Fig. 3B). The anti-GRIF1 antibody, but not the pre-immune serum, was able to co-immunoprecipitate GRIF1 and Hrs from PC12 cell lysates, demonstrating the existence of an endogenous Hrs-GRIF1 complex (Fig. 3B).

Identification of the binding domains mediating the association between Hrs and GRIF1
To understand the structural requirements for the interaction between Hrs and GRIF1, we used deletion analysis to map the specific domains of Hrs and GRIF1 required for their association. The Hrs-interacting clone (GRIF1Δ1) isolated in the yeast two-hybrid screen encodes residues 199-507 of GRIF1 (Fig. 4A), indicating that the N- (residues 1-198) and C-(residues 507-913) terminal regions of GRIF1 are not required for binding Hrs. To further map the Hrs-binding domain of GRIF1, we made two GRIF1 deletion mutants, GRIF1Δ2 and GRIF1Δ3 (Fig. 4A), and tested their ability to interact with GFP-tagged full-length Hrs by co-immunoprecipitation analysis in transfected HeLa cells. The results showed that the central region of GRIF1 (residues 359-507) was involved in binding Hrs, whereas the coiled-coil domain H2 (residues 199-363) was not required for the Hrs-GRIF1 interaction (Fig. 4B).

To define the specific domain of Hrs responsible for the association with GRIF1, we analyzed the interaction of various HA-tagged Hrs deletion mutants (Fig. 4C) with GFP-tagged full-length GRIF1 by co-immunoprecipitation analysis (Fig.
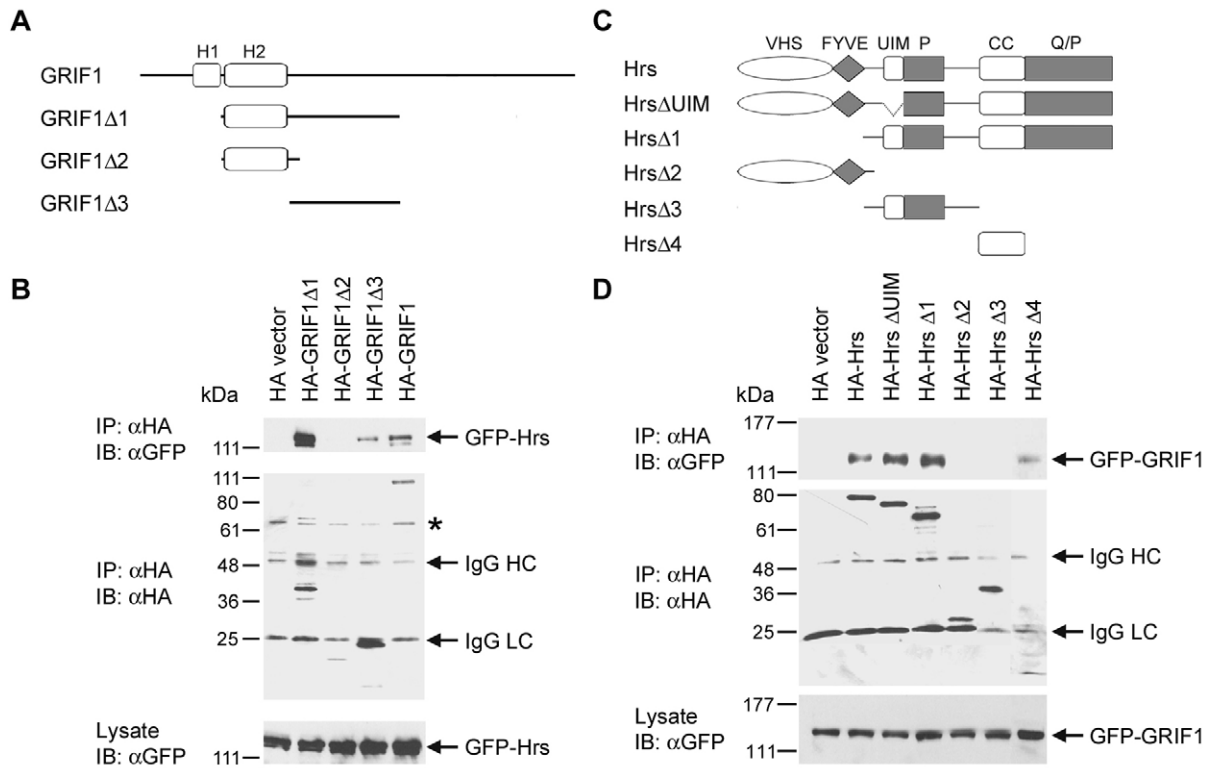
Fig. 4. Identification of Hrs and GRIF1 interaction domains by deletion analysis. (A) Domain structure of GRIF1 and its deletion mutants encoded by HA-tagged cDNA constructs. (B) Interaction of full-length and truncated GRIF1 proteins with Hrs. HeLa cells were co-transfected with the indicated HA- and GFP-tagged constructs. Lysates were immunoprecipitated with anti-HA antibody, followed by immunoblotting with anti-GFP and anti-HA antibodies. The asterisk indicates a non-specific band that probably represents undissociated IgG. (C) Schematic illustrating Hrs and its deletion mutants encoded by HA-tagged cDNA constructs. The following domains are indicated: VHS (Vps27p, Hrs and STAM), FYVE (Fab1b, YOTB, Vac1p and EEA1), UIM (ubiquitin-interacting motif), P (proline-rich), CC (coiled-coil), and Q/P (glutamine- and proline-rich region). (D) Interaction of full-length and truncated Hrs proteins with GRIF1. Co-immunoprecipitation experiments were performed as in B.

4D). We found that the coiled-coil domain (residues 443-541) of Hrs was both necessary and sufficient for interacting with GRIF1, whereas the VHS, FYVE, UIM, P-rich, and Q/P-rich domains were dispensable for the association of Hrs with GRIF1.

Endogenous GRIF1 colocalizes with Hrs on the early endosome

During the course of our antibody characterization, we observed that endogenous GRIF1 exhibited a punctate staining pattern suggestive of a vesicular localization (Fig. 2D). To investigate the identities of the vesicular structures labeled by GRIF1, we performed double immunofluorescence experiments to compare the intracellular distribution of endogenous GRIF1 with Hrs and various markers of intracellular membrane compartments in NGF-differentiated PC12 cells (Fig. 5). We observed a significant overlap between GRIF1 and Hrs immunoreactivity (Fig. 5A), indicating that at least a subpopulation of endogenous GRIF1 colocalizes with Hrs in PC12 cells. Since it is well established that Hrs is primarily localized to the early endosome (Bache et al., 2003b; Chin et al., 2001; Komada et al., 1997; Raiborg et al., 2001b), the colocalization of GRIF1 with Hrs suggests that the GRIF1-positive vesicular compartments may be early endosomes. To examine this possibility, we compared the distribution of

GRIF1 with that of early endosome antigen 1 (EEA1). We found that a subpopulation of endogenous GRIF1 is localized on EEA1-positive early endosomes (Fig. 5B). By contrast, no colocalization was observed between the distribution of GRIF1 and that of other proteins examined, such as the endoplasmic reticulum marker KDEL (Fig. 5C), β-tubulin (Fig. 5D) and SNAP-25 (data not shown).

GRIF1 is recruited to enlarged early endosomes induced by Hrs overexpression

Given the previous reports that Hrs overexpression induces the formation of enlarged early endosomes (Chin et al., 2001; Doyotte et al., 2005; Komada et al., 1997; Mizuno et al., 2004; Raiborg et al., 2001a), we next investigated whether GRIF1 could be recruited to these aberrant endosomes. We observed that, when expressed at low levels, HA-tagged GRIF1 exhibited a vesicular staining pattern (Fig. 6A) that was similar to that observed for endogenous GRIF1 (Fig. 5). Moreover, HA-GRIF1 staining partially overlaps with that of endogenous Hrs (Fig. 6A). The extent of colocalization of HA-GRIF1 with Hrs was significantly increased in cells overexpressing Hrs (Fig. 6B) compared to cells with only endogenous Hrs (Fig. 6A). Furthermore, HA-GRIF1 was found to be present on enlarged early endosomes induced by Hrs overexpression (Fig. 6B). These results provide additional evidence supporting an

in vivo association of GRIF1 with Hrs and suggest that GRIF1 may be recruited to early endosomes through interaction with Hrs.

Overexpression of GRIF1 causes a clustering of early endosomes in the perinuclear region

Next, we performed overexpression experiments to determine whether GRIF1 overexpression could lead to enlargement of early endosomes similar to that observed in cells overexpressing Hrs. Surprisingly, we found that GRIF1 overexpression did not cause early endosome enlargement, but rather, it induced a clustering of EEA1-positive early endosomes in the perinuclear region (Fig. 7A).

Recent evidence indicates that the intracellular localization of early endosomes is achieved by regulation of the plus-end, kinesin-dependent and the minus-end, dynein-dependent microtubule-based movement (Hoepfner et al., 2005; Murray and Wolkoff, 2003). Given the reported interaction between GRIF1 and kinesin (Brickley et al., 2005), we investigated whether the perinuclear clustering of early endosomes is a consequence of the GRIF1 interaction with kinesin. We made a GRIF1 deletion construct, GRIF1Δ4, which encodes the N-terminal region (residues 1-363) of GRIF1 that encompasses the published kinesin-binding domain (Brickley et al., 2005)

but excludes the Hrs-binding region. As shown in Fig. 7E, overexpression of GRIF1Δ4 did not lead to the perinuclear clustering of early endosomes. Instead, this GRIF1 deletion mutant protein formed perinuclear and peripheral aggregates that did not colocalize with EEA1.

We also examined the distribution of EEA1-positive early endosomes in cells overexpressing other GRIF1 deletion mutants, namely GRIF1Δ1, GRIF1Δ2 and GRIF1Δ3. We found that none of these deletion mutants could cause the perinuclear clustering of early endosomes (Fig. 7B-D). The results of the deletion analysis (Fig. 7B-E) indicate that binding to kinesin or Hrs alone is insufficient to induce the early endosome clustering and suggest that the interaction of GRIF1 with both kinesin and Hrs is required for the observed phenotype.

Interestingly, deletion analysis revealed that only the GRIF1 deletion mutants (GRIF1Δ1 and GRIF1Δ3) that retained the Hrs-binding domain showed colocalization with EEA1 (Fig. 7B,D). By contrast, the GRIF1Δ2 and GRIF1Δ4 deletion mutants that lack the Hrs-binding domain are not localized on EEA1-positive early endosomes (Fig. 7C,E). Together, these data demonstrate that the early endosome localization of GRIF1 is dependent upon its interaction with Hrs.

Overexpression of GRIF1 inhibits ligand-induced EGFR degradation without affecting endocytosis

EGF-induced degradation of the EGFR is a widely used model for studying endocytic trafficking. Binding of EGF to the EGFR at the cell surface triggers rapid internalization of the EGF-EGFR complex and subsequent sorting at the early endosome for lysosomal degradation (Mellman, 1996a; Mellman, 1996b; Morino et al., 2004). The role of Hrs in regulating endosomal trafficking of EGFR has been well established; both overexpression and knockdown of Hrs inhibit ligand-induced EGFR degradation (Chin et al., 2001; Kanazawa et al., 2003). The observed interaction and colocalization of GRIF1 with Hrs thus raise the possibility that
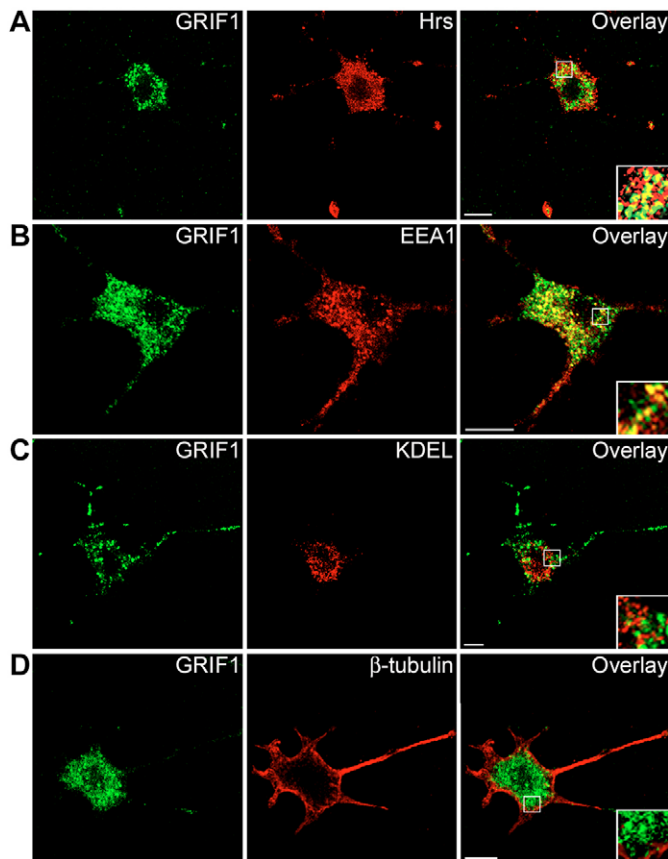
Fig. 5. Colocalization of GRIF1 with Hrs on early endosomes. NGF-differentiated PC12 cells were double immunostained using purified anti-GRIF1 antibody GF2 (green; A-D) and anti-Hrs (red; A), anti-EEA1 (red; B), anti-KDEL (red; C), and anti-β-tubulin (red; D) antibodies. Bars, 10 μm.
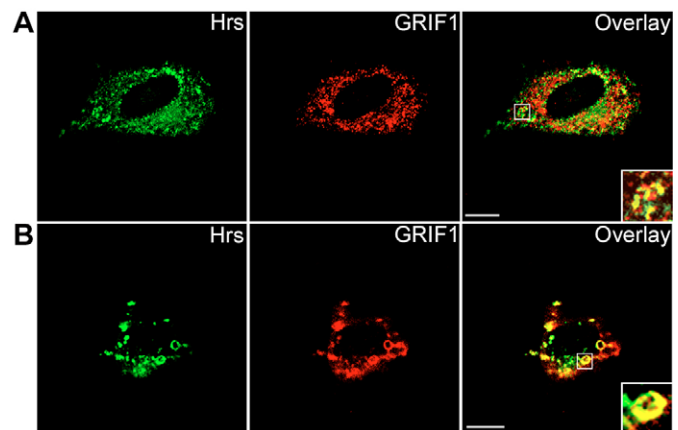

Fig. 6. GRIF1 is recruited to enlarged early endosomes in Hrs-overexpressing cells. HeLa cells were transfected with pCHA-GRIF1 alone (A) or co-transfected with pEGFP-Hrs (B). HA-GRIF1 was detected using an anti-HA antibody (red). Endogenous Hrs was detected using an anti-Hrs antibody (green; A), and GFP-Hrs was visualized by the green fluorescence emitted by the GFP tag (green; B). Bars, 10 μm.

GRIF1 may participate in regulation of EGFR degradation. To test this possibility, we examined the effect of overexpressing GRIF1 on EGF-induced EGFR degradation. For overexpression experiments, we transfected HeLa cells with pCHA-GRIF1 using the Lipofectamine 2000 reagent (Invitrogen) and achieved a transfection efficiency of >90%, which is in agreement with the value (~94%) reported by the manufacturer. Consistent with previous reports (Chin et al., 2001; Li et al., 2002; Lu et al., 2003), in vector-transfected control cells, treatment with EGF for 1 hour led to the degradation of 76.3±8.6% (n=3) of EGFR proteins compared to the corresponding untreated control cells (Fig. 8). In comparison, in cells overexpressing GRIF1, the EGFR degradation was significantly decreased to 25.6±9.7% (n=3; P<0.05) over the same period (Fig. 8). The extent of inhibition of EGFR degradation by GRIF1 overexpression is comparable to the decrease in EGFR degradation caused by overexpression of Hrs, HAP1 or Tsg101 (Chin et al., 2001; Li et al., 2002; Lu et al., 2003).

To determine if the effect of GRIF1 overexpression on EGFR degradation was due to altered endocytosis, HeLa cells overexpressing GRIF1 were tested for their ability to internalize Texas Red conjugated-EGF (TR-EGF) or TR-transferrin. We found that GRIF1 overexpression had no significant effect on constitutive or regulated endocytosis, as cells overexpressing GRIF1 internalized a similar amount of TR-transferrin (Fig. 9A) or TR-EGF (Fig. 9B) compared with untransfected cells. This conclusion was further supported by the results of ^{125}I-EGF internalization experiments, which showed that overexpression of GRIF1 or of its deletion mutants has no statistically significant effect on ^{125}I-EGF endocytosis (Fig. 9D).

## GRIF1 regulates EGFR trafficking from early endosomes to late endosomes

The lack of an effect on EGF-induced EGFR endocytosis (Fig. 9) suggests that the inhibition of EGFR degradation by GRIF1 overexpression (Fig. 8) was probably due to altered trafficking of internalized EGFR from early endosomes to the lysosomal
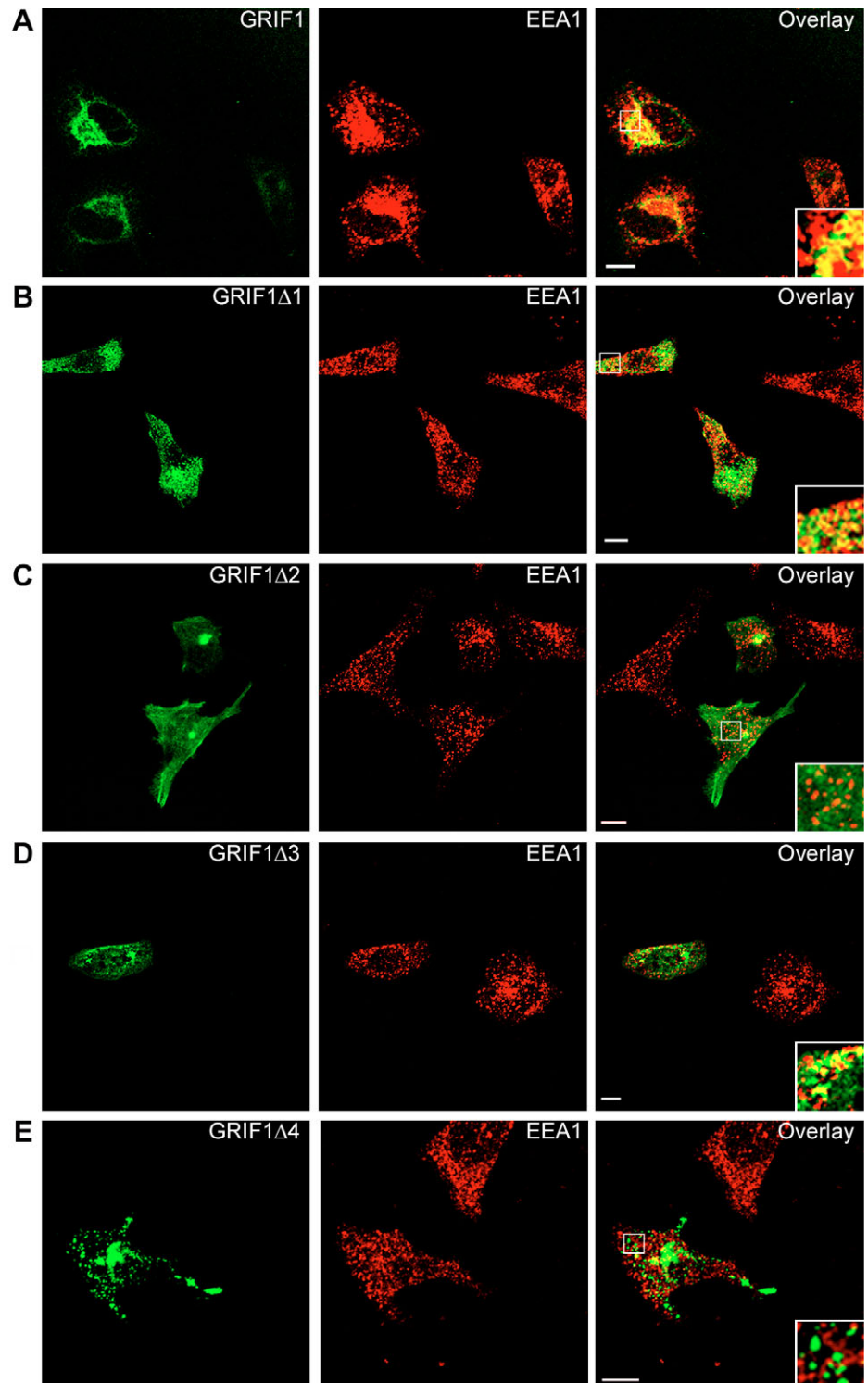
Fig. 7. GRIF1 overexpression alters the distribution of early endosomes. HeLa cells were transiently transfected with pEGFP-GRIF1 (A), pEGFP-GRIF1Δ1 (B), pEGFP-GRIF1Δ2 (C), pEGFP-GRIF1Δ3 (D), or pEGFP-GRIF1Δ4 (E). GFP-GRIF1 was visualized by the green fluorescence emitted by the GFP tag (green), and endogenous EEA1 was detected using an anti-EEA1 antibody (red). Bars, 10 μm.

pathway. To assess the effect of GRIF1 overexpression on endosome-to-lysosome trafficking, we used a 'pulse-chase' trafficking assay (Li et al., 2002). In this assay, HeLa cells were
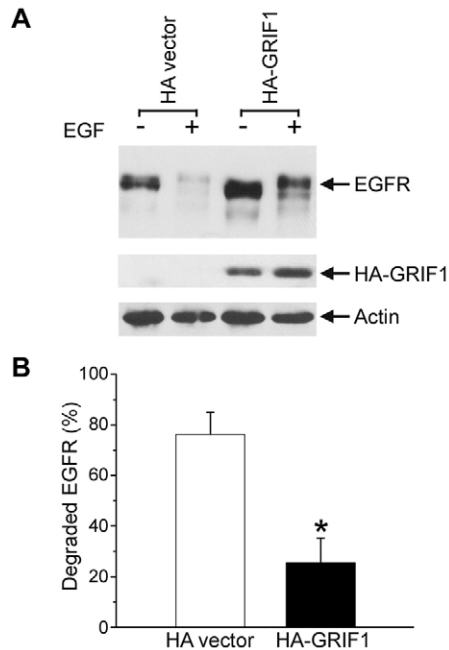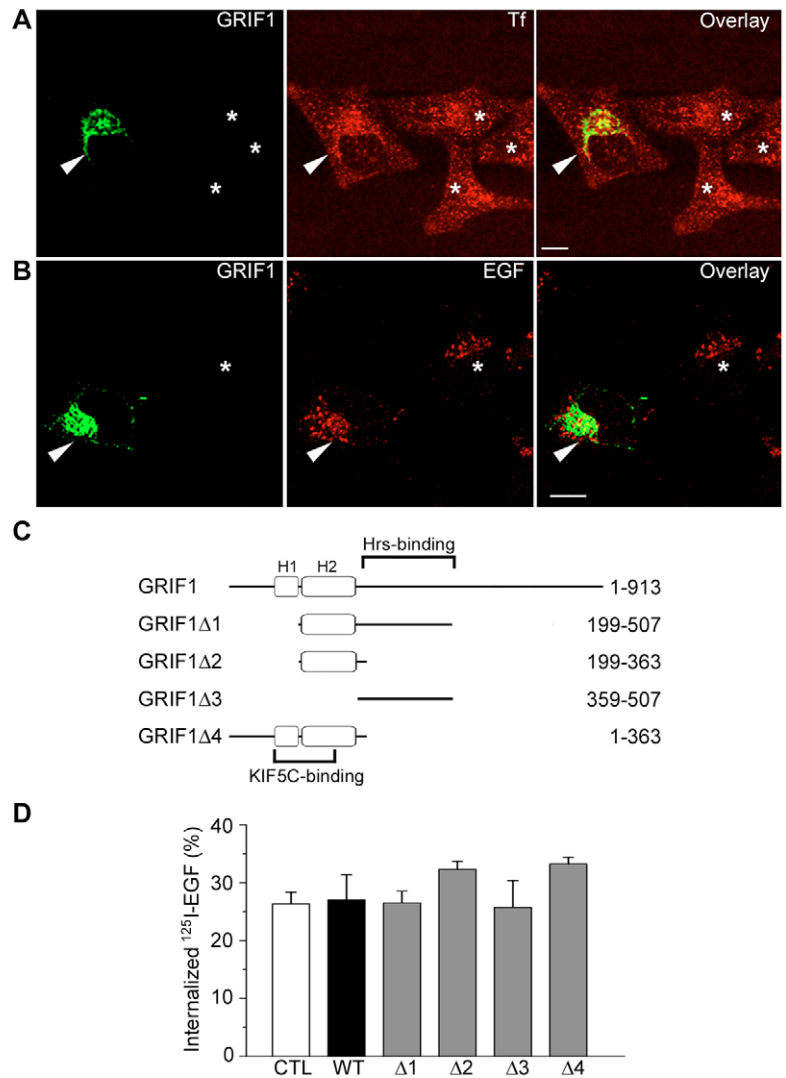
Fig. 8. Overexpression of GRIF1 inhibits EGF-induced EGFR degradation. (A) HeLa cells transfected with pCHA-GRIF1 or pCHA vector were incubated in the presence or absence of 100 ng/ml EGF for 1 hour at 37°C. Equal amounts of proteins from cell lysates were analyzed by immunoblotting with antibodies against EGFR (top), HA (middle), or actin (bottom). (B) The degraded EGFR is expressed as a percentage of the EGFR level in the corresponding untreated cells. Data represent mean ± s.e.m. from three independent experiments. The asterisk indicates a statistically significant difference ($P<0.05$) from the vector-transfected cells.

Fig. 9. Overexpression of GRIF1 does not affect the endocytosis of EGF or transferrin. (A,B) HeLa cells transfected with pEGFP-GRIF1 (green) were treated with TR-transferrin for 30 minutes (red; A) or with TR-EGF for 10 minutes (red; B) at 37°C. Cells were then examined by immunofluorescence microscopy. Arrowheads and asterisks indicate transfected and untransfected cells, respectively. Bars, 10 μm. (C) Schematic representation of the GRIF1 deletion mutants used in the ^{125}I-EGF endocytosis assays. The Hrs-binding domain (residues 359-507) and the kinesin-binding domain (residues 128-283) are indicated by brackets. (D) HeLa cells were transiently transfected with empty vector (CTL) or the following GRIF1 constructs: WT (GRIF1), Δ1 (GRIF1Δ1), Δ2 (GRIF1Δ2), Δ3 (GRIF1Δ3), Δ4 (GRIF1Δ4). Cells were incubated with ^{125}I-EGF for 10 minutes at 37°C. The internalized ^{125}I-EGF is expressed as a percentage of the initially bound ^{125}I-EGF. Values are mean ± s.e.m. from three independent experiments.

allowed to internalize TR-EGF for 10 minutes, and the fate of internalized TR-EGF was monitored after a 3-hour 'chase' period. Untransfected cells did not exhibit any detectable red fluorescence after the 3-hour chase, indicating that the internalized TR-EGF had been degraded (Fig. 10A, asterisks). In comparison, cells overexpressing GRIF1 retained a significant amount of the internalized TR-EGF after the same 3-hour chase period (Fig. 10A, arrowheads). Furthermore, immunostaining analysis revealed that at least a subset of internalized EGF remained in EEA1-positive early endosomes after the 3-hour chase (Fig. 10A). Our results suggest that GRIF1 overexpression impedes trafficking from the early endosome to the lysosomal degradation pathway, resulting in the accumulation of TR-EGF in cells. These findings are consistent with our biochemical data (Fig. 8) showing a significant inhibition of ligand-induced EGFR degradation by GRIF1 overexpression.

The action of GRIF1 regulating ligand-induced EGFR degradation depends on its interaction with Hrs
We next assessed the potential involvement of the GRIF1-Hrs and GRIF1-kinesin interaction in regulation of EGF-induced EGFR degradation by analyzing the functional consequences

of overexpressing GRIF1 deletion mutants. For comparison of the effects of these deletion mutants, we used a quantitative ^{125}I-EGF degradation assay. In this assay, cells were allowed to internalize ^{125}I-EGF for 10 minutes. After washing, to remove extracellular and surface-bound ^{125}I-EGF, cells were chased at 37°C for 1 hour to allow degradation. As shown in Fig. 10B, in vector-transfected cells, 66.8±2.8% (n=3) of internalized ^{125}I-EGF was degraded after the 1-hour chase.
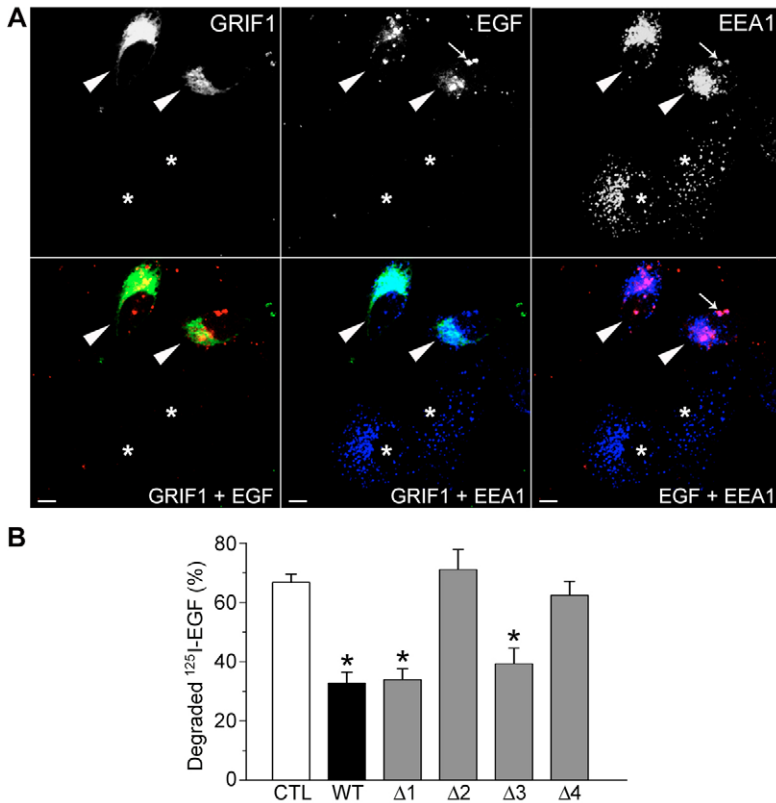
Fig. 10. GRIF1 overexpression inhibits trafficking of EGF-EGFR complexes from early endosomes to the lysosomal pathway. (A) HeLa cells transfected with pEGFP-GRIF1 were allowed to internalize TR-EGF for 10 minutes and then chased for 3 hours at 37°C. Cells were stained with a primary antibody against EEA1 and a CY5-conjugated secondary antibody. Arrowheads and asterisks indicate transfected and untransfected cells, respectively. Small arrows indicate EEA1-positive early endosomes that contain EGF. Merged images (bottom) compare the labeling of GRIF1 (green) with EGF (red), GRIF1 (green) with EEA1 (blue), and EGF (red) with EEA1 (blue). Bars, 10 μm. (B) HeLa cells transfected with the indicated GRIF1 constructs or the empty vector were allowed to internalize ^{125}I-EGF for 10 minutes and then chased for 1 hour at 37°C. The degraded ^{125}I-EGF is expressed as a percentage of the initially internalized ^{125}I-EGF. Values are mean ± s.e.m. from three independent experiments. The asterisks indicate a statistically significant difference ($P<0.05$) from the vector-transfected cells.

Overexpression of full-length GRIF1 significantly decreased the degradation of ^{125}I-EGF to 32.8±3.7% ($n=3$; $P<0.05$) in agreement with the results obtained using other types of assays (Fig. 8 and Fig. 10A). ^{125}I-EGF degradation was inhibited to a similar degree in cells overexpressing GRIF1 deletion mutants that contain the Hrs-binding domain, namely GRIF1Δ1 (33.9±3.7%, $n=3$; $P<0.05$) and GRIF1Δ3 (39.3±5.3%, $n=3$; $P<0.05$). By contrast, the amount of degraded ^{125}I-EGF in cells overexpressing the non-Hrs-, non-kinesin-interacting GRIF1 deletion mutant GRIF1Δ2 (71.1±6.7%, $n=3$) or the kinesin-binding domain-containing, non-Hrs-interacting mutant GRIF1Δ4 (62.4±4.7%, $n=3$) was not significantly different from that in the vector-transfected control cells. Together, these data indicate that the GRIF1-Hrs interaction, but not the GRIF1-kinesin interaction, is required for the inhibitory effect of GRIF1 on EGF-induced EGFR degradation.

GRIF1 is essential for ligand-induced degradation of EGFR

To provide further evidence supporting the role of GRIF1 in regulation of EGFR trafficking, we examined the effect of small-interfering RNA (siRNA)-mediated knockdown of GRIF1 expression on EGF-induced EGFR degradation in PC12 cells. For selective depletion of endogenous GRIF1, we used two distinct siRNA duplexes, GRIF1 siRNA-1 and GRIF1 siRNA-2, which specifically target different regions of the GRIF1 mRNA but show no homology to the OIP106/Trak1 mRNA. Western blot analysis confirmed that GRIF1 siRNA-1 and GRIF1 siRNA-2 both specifically inhibited the expression of endogenous GRIF1 but not actin (Fig. 11A). Unfortunately, there is no commercial anti-OIP106/Trak1 antibody available to confirm that OIP106/Trak1 expression is unaffected by

GRIF1 siRNA transfection. There was a relatively weak EGFR band from PC12 cells (Fig. 11A) compared with the EGFR band from HeLa cells (Fig. 8A). This could reflect a cell-type-specific difference in expression levels of endogenous EGFR in these two cell lines. Another factor that may contribute to the observed difference is that the rabbit anti-EGFR antibody (1005, Santa Cruz) used in these experiments was generated against human EGFR, which may bind endogenous human EGFR in HeLa with a higher affinity than endogenous rat EGFR in PC12 cells.

We found that, in both the control siRNA transfected and untransfected PC12 cells, treatment with EGF for 1 hour led to the degradation of 70.4±9.6% ($n=6$) and 69.2±12.1% ($n=6$), respectively, of EGFR proteins compared to the corresponding untreated control cells (Fig. 11B). By contrast, the EGFR degradation was significantly decreased to 19.8±3.7% ($n=6$; $P<0.05$) in GRIF1 siRNA-1 and to 23.4±9.2% ($n=6$; $P<0.05$) in GRIF1 siRNA-2 transfected cells (Fig. 11B).

To complement the results of the EGF-induced EGFR degradation assay, we performed ^{125}I-EGF internalization and degradation assays to assess the effects of GRIF1 knockdown. We found that depletion of GRIF1 by GRIF1 siRNA-1 or GRIF1 siRNA-2 had no statistically significant effect on ^{125}I-EGF internalization (data not shown). As shown in Fig. 11C, we observed a statistically significant ($P<0.05$) decrease in ^{125}I-EGF degradation in both GRIF1 siRNA-1 (42.4±4.5%, $n=3$) and GRIF1 siRNA-2 (44.4±6.7%, $n=3$) transfected PC12 cells compared with the untransfected controls (60.7±0.3%, $n=3$). Together, these data provide strong evidence supporting a functional role for GRIF1 in regulation of the trafficking of internalized EGF-EGFR complexes to the lysosome for degradation.
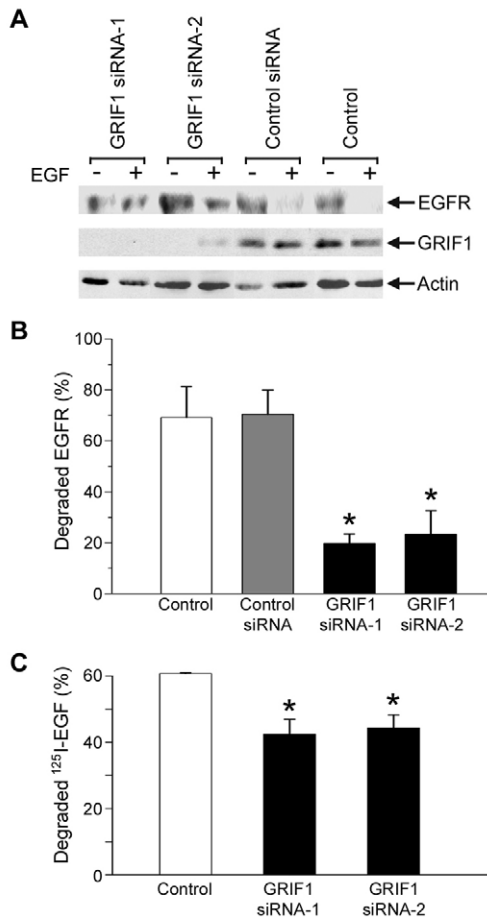
Fig. 11. GRIF1 knockdown inhibits EGF-induced EGFR degradation. (A) PC12 cells transfected with the indicated siRNAs or untransfected controls were incubated in the presence or absence of 100 ng/ml EGF for 1 hour at 37°C. Equal amounts of protein from whole cell lysates were analyzed by immunoblotting with antibodies against EGFR (top), GRIF1 (middle), or actin (bottom). (B) The degraded EGFR is expressed as a percentage of the EGFR level of the corresponding untreated cells. (C) PC12 cells transfected with the indicated siRNAs or untransfected controls were allowed to internalize ^{125}I-EGF for 10 minutes and then chased for 1 hour at 37°C. The degraded ^{125}I-EGF is expressed as a percentage of the initially internalized ^{125}I-EGF. Values are mean ± s.e.m. from three (C) or six (B) independent experiments. The asterisks indicate a statistically significant difference ($P<0.05$) from untransfected control cells.

Discussion

Although endosome-to-lysosome trafficking plays a critical role in normal physiology and disease, the molecular machinery and biochemical mechanisms that control this trafficking process remain poorly understood. Recent evidence indicates that the evolutionarily conserved protein Hrs plays a central role in the control of endosomal sorting and trafficking of membrane cargo to the lysosomal pathway (Clague and Urbe, 2003). In a yeast two-hybrid screen for Hrs binding partners, we identified an interaction between Hrs and GRIF1, a recently discovered protein of unknown function. Our characterization of GRIF1 and its interaction with Hrs reveals that GRIF1 is a new regulator of

endosome-to-lysosome trafficking of membrane cargo, such as EGFR.

Hrs is a well-characterized early endosomal protein whose localization is mediated through the binding of its FYVE domain to phosphatidylinositol 3-phosphate on the endosomal membrane (Raiborg et al., 2001b). By contrast, very little is known about the subcellular localization of GRIF1. Previous studies have relied on overexpression or crude subcellular fractionation, which localizes GRIF1 to both membrane-associated and cytosolic fractions (Beck et al., 2002; Brickley et al., 2005; Pozo and Stephenson, 2006). However, the membrane compartments containing GRIF1 remain unknown. Using two different anti-GRIF1 antibodies, we have shown that in PC12 cells, endogenous GRIF1 is associated with early endosomes and a significant percentage of GRIF1 is colocalized with Hrs. Furthermore, we found that GRIF1 is recruited to the enlarged early endosomes induced by Hrs overexpression. Our deletion analysis reveals that the early endosomal localization of GRIF1 depends on its interaction with Hrs. These findings suggest that GRIF1 is appropriately localized to influence Hrs-mediated endosomal sorting and trafficking events. Consistent with this notion, our functional studies reveal that overexpression of GRIF1 inhibits the ligand-induced degradation of EGFR by blocking the trafficking of endocytosed EGFR from early endosomes to late endosomes. Moreover, GRIF1 siRNA experiments demonstrate that GRIF1 is required for ligand-induced degradation of EGFR, providing direct evidence supporting a functional role for GRIF1 in the regulation of endosome-to-lysosome trafficking of EGFR.

Database searches and sequence analyses show that GRIF1 belongs to a new family of coiled-coil domain proteins characterized by the presence of the HAPN domain. This protein family has only three members, GRIF1, Trak1 and HAP1. GRIF1 is closely related to Trak1 (47% overall amino acid identity and 62% similarity), but more distantly related to HAP1 (29% overall amino acid identity and 48% similarity). The present study has revealed that GRIF1 is a functional partner of Hrs in the regulation of endosomal trafficking of EGFR. By using deletion analysis, we have mapped the Hrs-binding domain of GRIF1 to the central region (residues 359-507) immediately downstream of the HAPN domain. This Hrs-binding domain is well conserved in Trak1, suggesting that Trak1 may also interact with Hrs and participate in the regulation of endosomal trafficking of EGFR. Our previous work has shown that HAP1 binds Hrs and regulates endosome-to-lysosome trafficking of EGFR (Li et al., 2002). This functional role of HAP1 is further supported by the analysis of HAP1 knockout mice (Li et al., 2003). Thus, this newly described family of proteins seems to share a similar function as a regulator of endosome-to-lysosome trafficking of EGFR.

In addition to regulating EGFR trafficking, this family of proteins may have a general role in regulation of endosome-to-lysosome trafficking of a variety of membrane cargo. In support of this hypothesis, all three members of this protein family have been shown to bind GABA$_A$R subunits (Beck et al., 2002; Gilbert et al., 2006; Kittler et al., 2004), and HAP1 has recently been implicated in the regulation of endosome-to-lysosome trafficking of the GABA$_A$R (Kittler et al., 2004) and the NGF receptor TrkA (Rong et al., 2006). Moreover, Trak1 mutant mice with hypertonia were reported to exhibit altered GABA$_A$R levels (Gilbert et al., 2006). Thus, it is possible that

GRIF1 may also participate in the regulation of endosomal trafficking of other receptor tyrosine kinases such as TrkA and/or other cell surface receptors such as GABA$_A$R.

The precise mechanisms by which GRIF1 and its homologues regulate endosome-to-lysosome trafficking remain to be elucidated. Monoubiquitination of endocytosed cell surface receptors, such as EGFR, has been shown to act as an endosomal sorting signal for targeting the receptors to the lysosomal pathway (Hicke, 2001; Katzmann et al., 2002). Hrs contains an ubiquitin-interacting motif (UIM) and a clathrin-binding motif. The interactions mediated by these motifs have been proposed to allow Hrs to sort the ubiquitinated receptors into clathrin-coated domains of early endosomes (Urbe et al., 2003). Based on evidence obtained in yeast and mammalian cells, Hrs has been further assumed to recruit the ESCRT-I to the endosomal membrane and somehow hand over the ubiquitinated receptors to the ESCRT-I (Babst, 2005; Hurley and Emr, 2006). The ubiquitinated receptors are then sequentially delivered to the ESCRT-II and ESCRT-III, which drive the incorporation of the receptors into lumenal vesicles of MVBs (Babst, 2005; Hurley and Emr, 2006). We speculate that GRIF1 might act as an adaptor protein, working together with Hrs to facilitate the cargo sorting and retention at early endosomes. Interestingly, the GRIF1-binding domain of Hrs identified in the present study overlaps with that of the known Hrs-binding partners HAP1 (Li et al., 2002) and STAM/Hbp (Asao et al., 1997), both of which have been linked to endosomal cargo sorting (Bache et al., 2003b; Kittler et al., 2004; Mizuno et al., 2003). This raises the possibility that these Hrs-binding proteins may share a common mechanism for regulating Hrs-mediated endosomal sorting.

Recently, GRIF1 and other members of this protein family have been shown to associate with the plus-end microtubule motor protein kinesin (Brickley et al., 2005; McGuire et al., 2006). Furthermore, HAP1 can also interact with the p150(Glued) subunit of dynactin, an essential component of the minus-end microtubule motor complex (Engelender et al., 1997; Gauthier et al., 2004; Li et al., 1998). It has become increasingly clear that bidirectional, microtubule-based endosome motility plays an important role in endosomal sorting and trafficking (Hoepfner et al., 2005; Lakadamyali et al., 2006; Murray and Wolkoff, 2003). Our deletion analysis indicates that Hrs binds to a region of GRIF1 that is distinct from the reported kinesin-binding domain (Brickley et al., 2005), raising the possibility that GRIF1, through its interaction with Hrs and kinesin, may regulate the motility of early endosomes, and thereby modulate endosomal trafficking. Our findings that overexpression of full-length GRIF1, but not the Hrs- or kinesin-interacting GRIF1 deletion mutants, induced a perinuclear clustering of early endosomes supports a role of GRIF1 in regulation of the motility and intracellular localization of early endosomes by acting as an adaptor linking Hrs-containing early endosomes to kinesin. Our observations that overexpression of full-length GRIF1, as well as the Hrs-interacting GRIF1 fragments, but not kinesin-interacting GRIF1 deletion mutants, caused an inhibition of EGFR endosomal trafficking suggests that the role of GRIF1 in the regulation of microtubule-based transport of early endosomes may be independent of its function in the control of endosomal trafficking. Further investigation of the mechanisms of action of GRIF1 and other members of this new protein family should not only advance our understanding of the complex process of endosomal sorting and trafficking, but may also provide insight into the pathogenesis of hypertonia, Huntington's disease, and related neurological disorders.

Materials and Methods
Yeast two-hybrid screen
The bait plasmid, pPC97-Hrs, was generated by subcloning the entire rat Hrs open reading frame into the pPC97 vector (Chin et al., 2001). The yeast strain CG-1945 (Clontech) was sequentially transformed with pPC97 Hrs and a rat hippocampal/cortical cDNA library (Chin et al., 2001; Li et al., 2002). Positive clones were selected on a 3-aminotrizole-containing medium lacking leucine, tryptophan and histidine, and confirmed by a filter assay for β-galactosidase activity. Prey plasmids from positive clones were rescued and re-transformed into fresh yeast cells with the Hrs bait or various control baits to confirm the specificity of the interaction (Chin et al., 2001; Li et al., 2002).

Cloning and sequence analysis
For cloning of full-length GRIF1, a partial GRIF1 cDNA probe (clone 17; Fig. 1A) was used to screen a rat hippocampal cDNA library in λZAPII (Stratagene) as described previously (Li et al., 2001). The rat GRIF1 protein sequence (accession number NP_598244) was used in a BLASTP search for homologous proteins and ClustalW was used for multiple sequence alignment (Chenna et al., 2003). Prediction of coiled-coil domains was carried out using pair coil (Berger et al., 1995) and coils (Lupas et al., 1991) analysis.

Antibodies
Polyclonal rabbit antibodies, GF1 and GF2, were generated against the GRIF1 peptide sequence RLSLRRQNYLSEKQ (residues 485-499) and CTTSPKMGILKED (residues 902-913), respectively. Antibodies were affinity purified as previously described (Kwong et al., 2000). Other primary antibodies used in this study include: anti-Hrs (Kwong et al., 2000); anti-EEA1 (BD Transduction Laboratories); anti-EGFR 1005 and anti-GFP B2 (Santa Cruz Biotechnologies); anti-KDEL (Stressgen); anti-actin C4 and anti-β-tubulin (Chemicon); anti-SNAP-25 (SMI 81, Sternberger Monoclonals, Inc.); and the mouse monoclonal anti-HA (12CA5) antibody. Horseradish peroxidase-conjugated secondary antibodies and fluorescein isothiocyanate (FITC)-, CY5- or Texas Red (TR)-conjugated secondary antibodies (Jackson Immunoresearch Laboratories) were used for immunoblotting and immunostaining, respectively.

Expression constructs
Conventional molecular biological techniques were used to generate the following expression constructs with N-terminal GFP (pEGFP) or HA (pCHA) tags: GRIF1 (1-913), GRIF1Δ1 (199-507), GRIF1Δ2 (199-363), GRIF1Δ3 (359-507), GRIF1Δ4 (1-363), Hrs (1-776), HrsΔUIM (1-776, Δ258-277), HrsΔ1 (225-776), HrsΔ2 (1-237), Hrs Δ3 (225-449) and Hrs Δ4 (443-541) (Chin et al., 2001; Kwong et al., 2000; Li et al., 2002). The full-length and deletion constructs generated for Hrs and GRIF1 are also diagrammed in Fig. 4 and Fig. 9.

Northern and western blot analyses
Northern blot analysis of GRIF1 mRNA expression was performed on a rat multiple tissue northern blot (Clontech), using a ^{32}P-labeled GRIF1 cDNA fragment from clone 17 (Fig. 1A) as the probe. Loading of poly(A)$^+$ RNA in each lane was confirmed by hybridization of the same blot with a ^{32}P-labeled β-actin cDNA probe (Chin et al., 2002). For western blot analysis, rat tissues were homogenized in 1% SDS and subjected to SDS-PAGE. The proteins were transferred onto nitrocellulose membranes and probed with anti-GRIF1 and anti-actin antibodies. Antibody binding was detected by using the enhanced chemiluminescence (ECL) system (Amersham Biosciences).

Cell transfections and immunoprecipitations
HeLa cells were transfected with the indicated plasmids using Lipofectamine 2000 reagent (Invitrogen) according to the manufacturer's instructions. Immunoprecipitations were carried out 24 hours post-transfection as described previously (Chin et al., 2001) using whole cell lysates with anti-HA, anti-GFP or anti-GRIF1 antibodies. Immunocomplexes were then analyzed by SDS-PAGE and immunoblotting.

Small interfering RNAs (siRNAs) transfection
Two siRNAs (Dharmacon, Lafayette, CO) were generated against the following rat GRIF1 mRNA sequences: GRIF1 siRNA-1 (5′-GGAACCAUGUCUUAUCUG-AUU-3′) and GRIF1 siRNA-2 (5′-GAACGGAAGCUCCAGAUUCU-3′). In addition, a control siRNA with no known mammalian homology (siCONTROL Non-Targeting siRNA #1; Dharmacon) and untransfected cells were used as negative controls. PC12 cells were transfected with the indicated siRNA (100 nM) using the *Trans*IT siQUEST (Mirus, Houston, TX) reagent according to the

manufacturer's instructions. The cells were transfected a second time with the siRNA 48 hours later. Experiments were performed 48 hours after the final siRNA treatment.

Immunofluorescence microscopy

HeLa and PC12 cells were grown on poly-L-lysine-coated glass coverslips. PC12 cells were differentiated with NGF (50 ng/ml) for 48-72 hours (Li et al., 2002). Cells were fixed in 4% paraformaldehyde and processed for indirect immunofluorescence microscopy as described previously (Li et al., 2002). The distribution of GRIF1 was compared with the staining patterns of various marker proteins using a Zeiss LSM 510 confocal microscope. Images were processed using Adobe Photoshop 7.0 (Adobe Systems, Inc.).

EGFR degradation assays

At 24 hours after transfection for overexpression in HeLa cells or 48 hours after 2 consecutive siRNA treatments in PC12 cells, cells were serum starved for 1 hour and then incubated in the presence or absence of 100 ng/ml EGF for 1 hour at 37°C. Cells were then lysed in 1% SDS. An equal amount of protein from each lysate was subjected to SDS-PAGE and immunoblotting. The intensity of the EGFR band was quantified using NIH Image/Scion software (Chin et al., 2001; Li et al., 2002).

Endocytic trafficking assays

For measurement of TR-transferrin or TR-EGF endocytosis, pEGFP-GRIF1-transfected HeLa cells were incubated in serum-free medium for 1 hour, and then treated with 100 µg/ml TR-transferrin at 37°C for 30 minutes, or with 3 µg/ml TR-EGF in the presence of 0.1% bovine serum albumin (BSA) at 37°C for 10 minutes. The cells were then processed for immunofluorescence microscopy (Li et al., 2002). For measurement of EGF trafficking after internalization, cells were washed three times with HeLa medium to remove extracellular TR-EGF and incubated for an additional 3 hours at 37°C, and then processed for immunofluorescence microscopy (Li et al., 2002).

^{125}I-EGF internalization and degradation assays

For measurement of ^{125}I-EGF internalization, cells were serum starved for 2 hours, then incubated on ice with ~20 ng/ml ^{125}I-EGF (MP Biochemicals, Solon, OH) in binding buffer (1% BSA in serum-free DMEM). Cells were then washed with cold binding buffer and either lysed immediately to measure the initially bound ^{125}I-EGF or transferred to 37°C for 10 minutes. After washing cells with acid wash (0.5 M NaCl, 0.2 M acetic acid, pH 2.8) on ice, the internalized ^{125}I-EGF was measured as described previously (Longva et al., 2002; Valiathan and Resh, 2004) and expressed as a percentage of the initially bound ^{125}I-EGF. For measurement of ^{125}I-EGF degradation after internalization, cells were chased in serum-free DMEM containing 1.5 µg/ml EGF and 1% BSA at 37°C for 60 minutes. Degraded ^{125}I-EGF was measured as described previously (Longva et al., 2002; Valiathan and Resh, 2004) and expressed as a percentage of the initially internalized ^{125}I-EGF.

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