Activation of EGF receptor endocytosis and ERK1/2 signaling by BPGAP1 requires direct interaction with EEN/endophilin II and a functional RhoGAP domain

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Summary

Rho GTPases are important regulators for cell dynamics. They are activated by guanine nucleotide exchange factors and inactivated by GTPase-activating proteins (GAPs). We recently identified a novel RhoGAP, BPGAP1, that uses the BNIP-2 and Cdc42GAP homology (BCH) domain, RhoGAP domain and proline-rich region to regulate cell morphology and migration. To further explore its roles in intracellular signaling, we employed protein precipitations and matrix-assisted laser desorption/ionization mass-spectrometry and identified EEN/endophilin II as a novel partner of BPGAP1. EEN is a member of the endocytic endophilin family but its function in regulating endocytosis remains unclear. Pull-down and co-immunoprecipitation studies with deletion mutants confirmed that EEN interacted directly with BPGAP1 via its Src homology 3 (SH3) domain binding to the proline-rich region 182-PPPRPPLP-189 of BPGAP1, with prolines 184 and 186 being indispensable for this interaction. Overexpression of EEN or BPGAP1 alone induced EGF-stimulated receptor endocytosis and ERK1/2 phosphorylation. These processes were further enhanced when EEN was present together with the wildtype but not with the non-interactive proline mutant of BPGAP1. However, EEN lacking the SH3 domain served as a dominant negative mutant that completely inhibited these effects. Furthermore, BPGAP1 with a catalytically inactive GAP domain also blocked the effect of EEN and/or BPGAP1 in EGF receptor endocytosis and concomitantly reduced their level of augmentation for ERK1/2 phosphorylation. Our findings reveal a concomitant activation of endocytosis and ERK signaling by BPGAP1 via the coupling of its proline-rich region, which targets EEN and its functional GAP domain. BPGAP1 could therefore provide an important link between cytoskeletal network, endocytic trafficking and Ras/MAPK signaling.

Key words: BPGAP1, Endophilin, RhoGAP, Endocytosis, EGF, ERK

Introduction

The Rho family GTPases have emerged as key signal transducers that regulate a wide variety of cellular events including cell growth, division, motility, survival and apoptosis (Bishop and Hall, 2000; Etienne-Manneville and Hall, 2002). Like all other signaling networks, the execution of Rho GTPase pathways is characterized by the assembly and disassembly of multiple complexes, and they are activated by certain classes of guanine nucleotide exchange factors and inactivated by GTPase-activating proteins (GAPs). These two regulators provide an important interface that allows the duration and amplitude of the cellular activity to be fine-tuned, thus ensuring that the biological activities of Rho GTPases are well orchestrated at the molecular level.

Many different proteins have RhoGAP activity, including those with multiple domains or motifs and differential substrate specificities (Ridley et al., 1993; Moon and Zheng, 2003; Bernards and Settleman, 2004). To date, there is no specific GAP for a single GTPase – instead a GAP may recognise more than one GTPases, and a single GTPase can be a target of multiple GAPs. Furthermore, the in vitro substrate profile can vary when compared with the in vivo results (Ridley et al., 1993) and the substrate recognition can be modulated by lipids (Ligeti et al., 2004). Although GAPs usually function to inactivate their cognate GTPase substrates, some are believed to function as effectors that mediate downstream signaling – for example, the RasGAP neurofibromatosis 1 (Yunoue et al., 2003) and TcGAP (Chiang et al., 2003). By contrast, some enhance the effect of the protein they interact with, such as the RhoGAP domain in the regulatory subunits of phosphoinositide 3-kinase, p85 (Zheng et al., 1994). Furthermore, GAPs can be subjects of signaling crosstalk by providing multiple signaling modules linked to other signaling pathways such as tyrosine kinase, phosphoinositides and serine/threonine kinases. All these point to the complex nature of GAP and small GTPase regulation.

We recently cloned and characterized a novel RhoGAP, BPGAP1 (Shang et al., 2003), that harbors three distinctive protein domains: the BNIP-2 and Cdc42GAP homology (BCH) domain that we first described (Low et al., 1999; Low et al., 2000a; Low et al., 2000b), a proline-rich region (PRR) and a functional GAP domain. BPGAP1 induces the formation

of pseudopodia, which requires both BCH and GAP domains, whereas its interaction with cortactin via its PRR facilitates cortactin translocation to the cell periphery for enhanced cell migration (Lua and Low, 2004). To explore the molecular mechanisms underlying other cellular function(s) elicited by BPGAP1 through its multiple protein domains, we continued using protein precipitations and matrix-assisted laser desorption/ionization-time of flight mass spectrometry to reveal novel partner(s) of BPGAP1. Here, we report the identification of the protein encoded by extra eleven-nineteenth (EEN) leukemia fusion gene as a novel partner of BPGAP1. EEN [also known as the human homolog of rat endophilin II; SH3-containing GRB2-like protein 1 (SH3GL1); SH3P8] (Ringstad et al., 1997; So et al., 1997), belongs to a family of the Src homology 3 (SH3) domain-containing endocytic proteins that might be involved in normal cellular processes such as endocytosis (Ringstad et al., 1997; So et al., 2000a) and cell cycle (Cheung et al., 2004), as well as malignant cellular processes including chromosomal translocation in human leukemia (So et al., 1997; So et al., 2000b), and is implicated in the pathogenesis of Huntington's disease (Zechner et al., 1998). Although most of the studies are focused on endophilin I (also known as SH3-containing GRB2-like protein 2, SH3GL2), the biochemical and cellular functions of endophilin II remain unclear. In this study, various protein interaction assays confirmed that EEN interacted directly with BPGAP1 in a manner that required its SH3 domain binding to the PRR of BPGAP1. Overexpression of EEN or BPGAP1 alone enhanced epidermal growth factor (EGF)-stimulated receptor endocytosis and extracellular signal-regulated kinase (ERK)1/2 phosphorylation. When present together, EEN and BPGAP1 augmented endocytosis of EGF receptor further and increased the level and duration of phosphorylation of ERK1/2. In addition, the N-terminal part of EEN, which lacked the SH3 domain, acted as a dominant negative mutant that could completely inhibit BPGAP1 effects in both processes, indicative of the strong requirement for an intact EEN function for such coupling. In comparison, introducing BPGAP1 with an inactive GAP domain devoid of the catalytic 'arginine-finger' motif also blocked EEN and/or BPGAP1's effect in EGF receptor endocytosis with a reduction in their level of augmentation for ERK1/2 phosphorylation. These results provide the support for a RhoGAP that functionally interacts with an endocytic protein to promote EGF-receptor-linked ERK signaling. Specifically, our findings reveal a concomitant regulation of endocytosis and ERK signaling by BPGAP1 via the coupling of its PRR, which targets EEN, with its functional GAP domain, while exerting additional activation on ERK through a yet unidentified mechanism. The significance of such novel integration of BPGAP1 in the context of endocytic trafficking and cell signaling is discussed.

Materials and Methods

Identification of BPGAP1 interacting partners

Cells were lysed in lysis buffer containing 50 mM HEPES, pH 7.4, 150 mM sodium chloride, 1.5 mM magnesium chloride, 5 mM EDTA, 10% (v/v) glycerol, 1% (v/v) Triton X-100, a mixture of protease inhibitors (Roche Molecular Biochemicals), 5 mM sodium orthovanadate and 25 mM glycerol phosphate (Sigma). The GST-BPGAP1 proteins, coupled to glutathione beads, were incubated with pre-cleared cell lysates. The bound proteins were resolved by SDS-PAGE and were visualized by silver-staining (Bio-Rad). The unique bands were excised and digested with trypsin (Shevchenko et al., 1996). Mass spectra were acquired with a matrix-assisted laser desorption ionization-time of flight (MALDI-TOF) mass spectrometer (Voyager STR BioSpectrometry work station; Applied System) operating in the delayed-extraction reflectron mode. Peptide mass fingerprints of the tryptic peptides from MALDI-TOF MS data were used to search the National Center for Biotechnology Information (NCBI) protein database with the programs MS-Fit (http://prospector.ucsf.edu/ucsfhtml4.0/msfit.htm) and Mascotsearch engine (http://www.matrixscience.com).

Plasmids construction

To obtain EEN/endophilin II cDNA (GenBank AAB86800) (So et al., 1997), 5 μg of total RNA isolated from HeLa cells (RNeasy; Qiagen) was subjected to first-strand cDNA synthesis with AMV Reverse Transciptase (Promega) primed with oligo(dT) (Operon) and amplified by high fidelity, long-template DyNAzyme (Finnzymes) using specific primers. Various domains were generated from the full-length template using specific PCR primers with *Bam*HI (forward) and *Xho*I (reverse) sites for cloning into Flag epitope-tagged pXJ40 vector (kindly provided by E. Manser, Institute of Molecular and Cell Biology, Singapore), pGEX-4T1 vector (Amersham Pharmacia Biotech), pCMV-Tag vector (Stratagene), pET-32a vector (Novagen) or with *Xba*I site (reverse) into maltose-binding protein (MBP)-tagged pMAL-c2X (kindly provided by T. S. Sim, National University of Singapore). All BPGAP1 constructs used were as described previously (Shang et al., 2003; Lua and Low, 2004). Clones were verified after sequencing entirely in both directions and propagated in *Escherichia coli* strain DH5α, BL21 and XL1-blue. All plasmids were purified using Qiagen miniprep or midiprep kit for subsequent use in transfection experiments. Reagents used were of analytical grade, and standard protocols for molecular manipulations and media preparation were as described (Sambrook and Russell, 2001).

Cell culture and transfection

293T cells were grown in RPMI 1640 medium supplemented with 10% (v/v) fetal bovine serum, 2 mM L-glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin (all from Hyclone), and maintained at 37°C in a 5% CO_2 atmosphere, whereas HeLa cells were grown in Dulbecco's modified Eagle's medium (high glucose). The 293T cells and HeLa cells were transfected using Fugene 6 (Roche Molecular Biochemicals) according to the manufacturer's instructions.

Production of EEN antibodies

GST fusion protein for the N-terminal (aa 1-332) of human EEN protein (1 mg) was eluted from Sepharose beads by boiling in 1% (w/v) SDS. The supernatant was dialyzed overnight at 4°C in phosphate-buffered saline, and mixed with complete adjuvant (Sigma) until miscelles were formed and injected subcutaneously into female New Zealand White rabbits. Every 2 weeks, two more boosters were administered in incomplete adjuvant followed by another injection 1 month later. Ten days after the final injection, sera were collected for purification. The sera were first incubated with GST proteins immobilized on a polyvinylidene difluoride membrane to remove anti-GST antibodies. The supernatant, containing mostly EEN antibodies, was then incubated with His-EEN immobilized on another polyvinylidene difluoride membrane to capture EEN antibodies. The bound antibodies were eluted by incubating in glycine (pH 2) and vortexed several times, and the solution was neutralized in 1.5 M Tris (pH 8).

Precipitation/pull-down, direct binding studies and western blot analyses

Control cells or cells transfected with expression plasmids were lysed in 1 ml of lysis buffer as described above. Lysates were directly analyzed, either as whole-cell lysates (25 μg) or aliquots (500 μg) used in affinity precipitation/pull-down experiments with various GST fusion proteins (10 μg), or M2 anti-Flag agarose beads (Sigma) as previously described (Low et al., 1999). For in vitro direct-binding studies, immobilized GST fusion proteins were incubated with either 100 ng thrombin-cleaved GST-fusion proteins or purified MBP-fusion proteins in 250 μl of lysis buffer at 4°C for 1 hour. Samples were run in SDS-PAGE gels followed by western blotting and signals detected using the *ECL* system (Amersham Pharmacia Biotech). Antibodies used were anti-Flag (monoclonal and polyclonal, both from Sigma), polyclonal anti-HA (Zymed), monoclonal anti-MBP (New England), polyclonal anti-Myc (Santa Cruz), polyclonal anti-EGF receptor (Upstate Biotechnology) and anti-ERK1/2 (Transduction Laboratories). To assay for the phosphorylation status of EGF receptor and ERK1/2, antibodies specific to their phosphorylated forms were used – monoclonal anti-phospho EGF receptor (Cell Signaling Technology) and monoclonal anti-dualphospho threonine tyrosine for ERK1/2 (Sigma). The control and transfected cells were starved for 18 hours in serum-free medium prior to treatment without or with 100 ng/ml EGF (Sigma) for 2, 5, 10 and 20 min. Cells were lysed and lysates were analyzed by western blots.

Immunofluorescence

HeLa cells seeded on sterilized glass cover slips were transfected with various epitope-tagged expression plasmids for BPGAP1 and/or EEN for 24 hours. Cells were then made quiescent for 2 hours by serum removal, followed by treatment with 30 μM monensin (Biomol) for 15 minutes to inhibit recycling prior to treatment with EGF (100 ng/ml) for 10 minutes. They were washed with cold PBS and fixed with 3% paraformaldehyde for 20 minutes at 4°C. Fixed cells were washed twice with PBS, twice with PBS containing 50 mM NH_4Cl, and twice again with PBS, followed by permeabilization with 0.2% Triton X-100 (BioRad) (room temperature, 5 minutes) and incubation at room temperature, for 1 hour with 20 μl (0.2 μg) of monoclonal anti-EGF receptor (Transduction Laboratories), polyclonal (chicken) anti-HA (Immunology Consultants Laboratory), polyclonal (rabbit) anti-Flag or polyclonal (rabbit) anti-Myc [in 2% (v/v) fetal bovine serum, 2% (w/v) bovine serum albumin in PBS]. Samples were washed three times (2 minutes) in 0.2% Triton X-100 containing PBS before incubation with the appropriate fluorophore-conjugated secondary antibodies such as rhodamine-conjugated goat anti-chicken IgY (Abcam), fluorescein isothiocyanate-conjugated goat anti-mouse IgG (Chemicon), Alexa Fluor 594-conjugated donkey anti-rabbit IgG (Molecular Probes) or Pacific Blue-conjugated goat anti-rabbit IgG (Molecular Probes). Filamentous actin was identified by staining with rhodamine-phalloidin (Sigma). After the final wash (five times in 0.2% Triton X-100 containing PBS and twice in PBS), coverslips were mounted with FluorSave™ (Calbiochem) and examined by confocal fluorescence microscopy (Olympus FV500). All images were captured with a ×60 objective lens and presented by Microsoft PowerPoint software (Microsoft 2002).

EGF receptor endocytosis assay

The quantitative extent of endocytosis was measured by following the uptake of dye-conjugated EGF that was bound to the receptor during stimulation, similar to the procedures previously described (Shearwin-Whyatt et al., 2004). To increase detection sensitivity, the signals for the EGF uptake were further amplified using anti-fluorescein/Oregon Green antibody (Molecular Probes) and cells that took up the EGF were analyzed according to the specific fluorescence emitted by fluorophore-conjugated antibodies against Flag-tagged or HA-tagged proteins expressed in the particular populations of the cells. Essentially, transfected or control 293T cells were serum-starved for 2 hours, followed by treatment with 30 μM monensin (Biomol) for 15 minutes at 37°C to inhibit recycling. Treated cells were then incubated with 100 ng/ml Oregon-Green-labeled EGF (Molecular Probes) for 1 hour at 4°C. The cells were washed with serum-free medium three times to remove unbound EGF, fresh starvation medium was added at 4°C before warming to 37°C to allow the uptake of EGF at specific time points (10, 20 and 30 minutes). Then, cells were immediately chilled on ice to stop the reaction and acid-stripped in ice-cold 0.2 M acetic acid (pH 2.5; containing 0.5 M NaCl) for 5 minutes. This procedure selectively strips bound EGF from the cell surface and leaves intracellular receptor-ligand complexes intact. After acid-stripping, cells were dislodged, washed three times with ice-cold PBS and fixed in 1% paraformaldehyde, followed by permeabilization with 0.2% saponin (Sigma) (room temperature, 15 minutes). Cells were then incubated at room temperature for 1 hour with 100 μl PBS [with 2% (v/v) fetal bovine serum, 2% (w/v) bovine serum albumin, 0.1% saponin plus 1 μg each of polyclonal anti-Flag and monoclonal anti-fluorsecein/Oregon-Green (for single-transfected cells) or polyclonal anti-HA and monoclonal anti-fluorsecein/Oregon-Green (for double-transfected cells)]. To determine the transfection efficiency in double-transfected cells expressing Flag-tagged and HA-tagged proteins, a portion of the sample was incubated with monoclonal anti-Flag and polyclonal anti-HA. All samples were washed three times in PBS before and after incubation with Alexa Fluor 488-conjugated donkey anti-mouse IgG and/or Alexa Fluor 594-conjugated donkey anti-rabbit IgG (Molecular Probes). Stained cells were suspended in PBS containing 1% paraformaldehye and subjected to fluorescence-activated cell sorter analysis (Cyan LX, Dakocytomation). The average intensity of amplified EGF signals (measuring the extent of EGF uptake) within the population that expressed either one or both tagged proteins was computed after subtraction of their respective background binding at zero time points. The experiments were repeated at least three times, and 50,000 events were collected three times per sample and analyzed using Summit V3.3 software (Dakocytomation). This protocol has established a linear uptake of EGF for the control cells up to 30 minutes, typically with less than 10% variation among the test samples within a specific given time. For clarity purposes, error bars were omitted from multiple points in the figures. Please refer to the text and legend for details. Statistical comparisons were performed using analysis of variance (StatsDirect). P values of <0.01 indicate a significant difference compared with the reference control as described in the text.

Results

EEN/endophilin II as a novel BPGAP1-interacting partner

To explore the molecular mechanisms underlying the signaling network that could be elicited by BPGAP1 through its multiple protein domains, we employed protein precipitations and matrix-assisted laser desorption/ionization-time of flight (MALDI-TOF) mass spectrometry to reveal novel partner(s) of BPGAP1. Several putative partners have been identified, including cortactin, which we recently described (Lua and Low, 2004). In a similar experiment using the kidney epithelial 293T cells, two unique bands were also consistently observed, one with an apparent molecular mass of 50 kDa, the identity of which remains unknown, and the other at 46 kDa, which is the subject of the current study (Fig. 1A, arrow). This band was excised from the gel and digested in-gel with trypsin. Following this, the generated peptide mixture was analyzed as

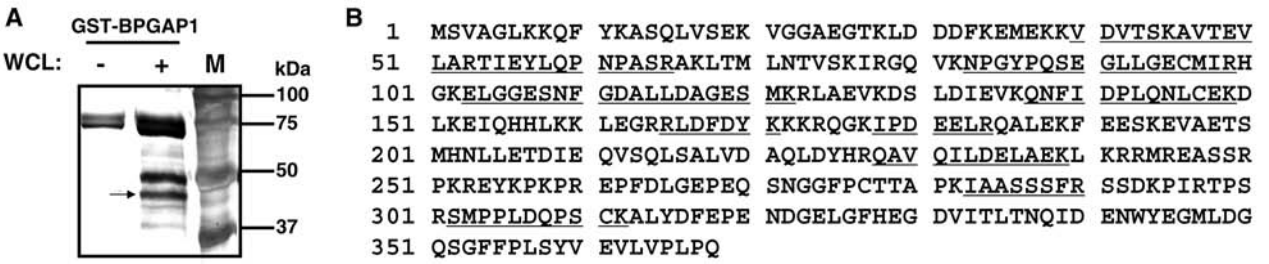


Fig. 1. Identification of BPGAP1-interacting partner, EEN/endophilin II via protein precipitation and MALDI-TOF. (A) Full-length BPGAP1 expressed as GST-recombinant coupled to glutathione beads were incubated with either lysis buffer (–) or 293T cell lysates (+) that had been pre-cleared with GST-beads to remove non-specific binding. Bound proteins were resolved by SDS-PAGE and revealed by silver-staining. *M*, molecular weight marker in kilodalton (kDa). A unique band at 46 kDa (indicated by an arrow) was subjected to trypsin digestion followed by MALDI-TOF analyses as described in Materials and Methods. (B) Protein sequence of EEN with sequence coverage of 34% (underlined) over the protein.

described in Materials and Methods. Based on their mass spectra, 9 fragments could be clearly identified as parts of the protein encoded by the extra eleven-nineteenth leukemia fusion gene (*EEN*) (Fig. 1B) (Ringstad et al., 1997; So et al., 1997), a new member of the SH3 domain-containing endocytic protein family. EEN was also identified in similar experiments using the cervical HeLa and breast cancer MCF cells (data not shown).

BPGAP1 interacts with EEN via its proline-rich region

To confirm that EEN was indeed a bona fide partner of BPGAP1 and to map their interaction sites, we sought to first employ GST pull-down protein binding assays to examine the interaction of the endogenous EEN with various BPGAP1 domains. Different fragments of BPGAP1 were prepared as bacterially expressed GST recombinants, harboring either the BCH domain at the N-terminus but no PRR (NNP), BCH domain plus PRR (NP), or PRR plus GAP domain at the C-terminus (PC) (Fig. 2A). Cell lysates prepared from 293T cells maintained in normal culture condition were subjected to pull-down assays using the purified recombinants, analyzed by SDS-PAGE and probed with antibody raised against EEN, as described in Materials and Methods. Fig. 2B shows that the full-length BPGAP1, NP and PC fragments interacted strongly with endogenous EEN, as indicated by marked enrichment when compared with the whole-cell lysates. The antibody specifically detected EEN at 46 kDa, the expected mobility as verified using the overexpressed Flag-tagged EEN in the cells. The NNP fragment devoid of PRR, however, did not reveal any binding to the endogenous EEN. Similar results were observed when another epithelial A431 cell was used or when EEN was overexpressed in 293T cells (data not shown).

Prolines 184 and 186 of BPGAP1 are essential for its direct interaction with EEN

Within the BPGAP1 PRR (176-PPPTKTPPPRPPLP-189), a putative EEN-binding motif PPPRP positioned from aa 182-186 matches the consensus proline-rich binding region, PPXRP, previously identified in other proteins that interact with the SH3 domain of EEN and other endophilins (Ringstad et al., 2001). To verify whether this stretch of amino acids was important in mediating its interaction with the EEN, several mutants were generated as GST fusion proteins for pull-down assays using lysates isolated from 293T cells, which express endogenous EEN (Fig. 3). The mutants included internal deletion mutants that removed either all (P1) or part (P2 and P3) of the PRR, and point mutants, where prolines at 184 and 186 were substituted with alanines (PP) such that all potential PXXP core motifs for SH3 binding had been removed. As a negative control, proline 422 was substituted with alanine (P422A) (Fig. 3A). As shown in Fig. 3B, deletion of the entire PRR (P1 mutant) or the KTPPPRPPLP sequence in the P2 mutant abolished the binding between BPGAP1 and EEN,

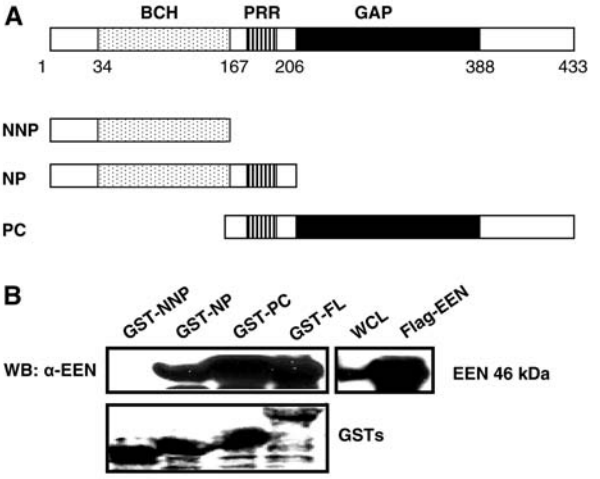


Fig. 2. BPGAP1 interacts with EEN via its PRR. (A) BPGAP1 constructs used for identifying functional interactive domain for EEN. The NNP domain (aa 1-166), NP domain (aa 1-206), PC domain (aa 167-433) and full-length BPGAP1 were expressed as GST-recombinants in *E. coli* and affinity-purified with glutathione-sepharose beads. NNP, N-terminus with BNIP-2 and Cdc42GAP homology domain (BCH); NP, N-terminus with BCH domain and PRR; PC, C-terminus with GTPase-activating protein domain (GAP) and PRR. (B) BPGAP1 GST-recombinants were prepared and used for pull-down assays with 293T cell lysates as described in Materials and Methods. Blots were stripped and stained with amido black to reveal loading of GST-recombinants. Bound endogenous proteins were detected by EEN antibody. Lysates expressing Flag-EEN are used as positive controls and reference for the specificity of the EEN antibody used in detecting the endogenous EEN.

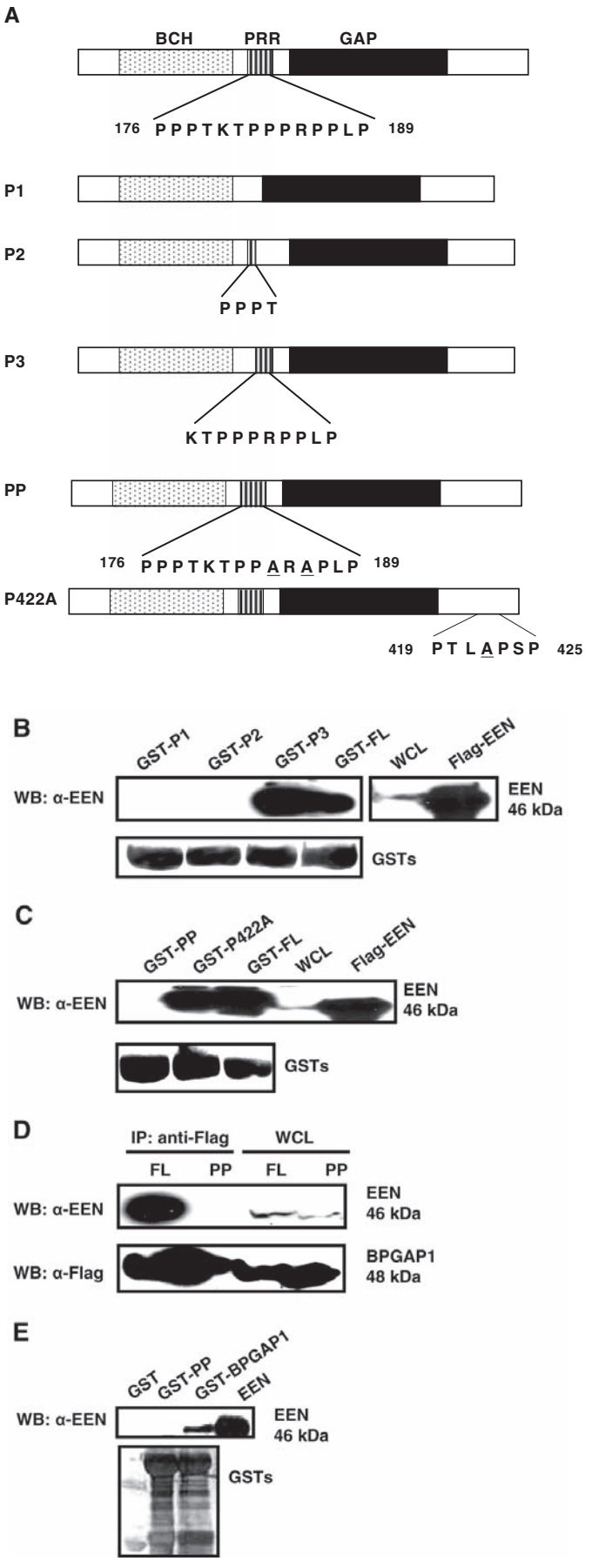


Fig. 3. The specific PXXP core motif within the PRR of BPGAP1 is indispensable for targeting the SH3 domain of EEN. (A) The three deletion mutants and two site-directed point mutants of BPGAP1. P1, devoid of the entire PRR; P2, retaining sequence PPPT; P3, retaining sequence KTPPPRPPLP; PP, substitution of the proline residues at 184 and 186 with alanines (underlined); P422A, substitution of proline residue at 422 with alanine (underlined). GST-recombinants of these deletion constructs (B) and site-directed mutants (C) were prepared as sepharose beads and used for pull-down assays using 293T cell lysates expressing endogenous EEN. Beads from the pull-down experiments were washed and processed for western analysis using EEN antibody. The antibody specifically detected endogenous EEN at 46 kDa, the expected mobility as verified using the overexpressed Flag-tagged EEN in the cells. (D) Cells were transfected with either the wildtype FL or PP mutant of Flag-tagged BPGAP1 and immunoprecipitated with anti-Flag M2 beads, washed and analyzed for bound endogenous EEN using EEN antibody. (E) Purified EEN from thrombin-cleaved GST recombinant was incubated with sepharose beads conjugated with GST fusions of full-length wildtype, the PP mutant of BPGAP1, or GST control, and bound targets revealed by western blot analyses using EEN antibody. WCL, whole cell lysates. FL, full-length.

which suggests that this stretch of sequence is essential for their interaction. Consistent with this was the observation that the P3 mutant, which still retained the sequence KTPPPRPPLP, could indeed pull-down endogenous EEN. Furthermore, Fig. 3C shows that proline residues at 184 and 186 but not 422 were important in mediating the interaction with EEN.

To ascertain that the wildtype BPGAP1 but not the PP mutant could indeed interact with endogenous EEN when expressed inside intact cells, they were separately tagged with Flag-epitope, expressed in 293T cells and immunoprecipitated with anti-Flag beads followed by western blot analyses with anti-EEN, as described in Materials and Methods. Unlike the wildtype BPGAP1, the Flag-tagged PP mutant did not interact with endogenous EEN (Fig. 3D). To further confirm that their interaction was truly mediated by direct binding and not due to the association with other protein complexes inside the cells, purified full-length EEN was prepared and shown to interact with the wildtype GST-BPGAP1 but not with the GST-PP mutant in a direct binding assay (Fig. 3E). Taken together, these results confirm that prolines 184 and 186 within the PRR of BPGAP1 are indispensable for directly targeting BPGAP1 to EEN.

EEN binds BPGAP1 via its SH3 domain

We set out to determine whether the SH3 domain of EEN could serve as the functional interactive domain for the PRR of BPGAP1. Two deletion constructs of EEN were made as GST fusion proteins, as described in Fig. 4A. The NT fragment comprises the BAR (BIN/amphiphysin/Rvsp) domain but lacking the SH3 domain, whereas the CT fragment harbors only the SH3 domain at the C-terminus. Full-length BPGAP1 was expressed as a Flag-tagged protein in 293T cells and subjected to pull-down experiments with GST-recombinants of either the full-length, NT or CT fragments of EEN. As shown in Fig. 4B, the SH3-containing CT fragment of EEN interacted with BPGAP1. However, the NT fragment devoid of the SH3 domain failed to pull-down BPGAP1 from the lysates.

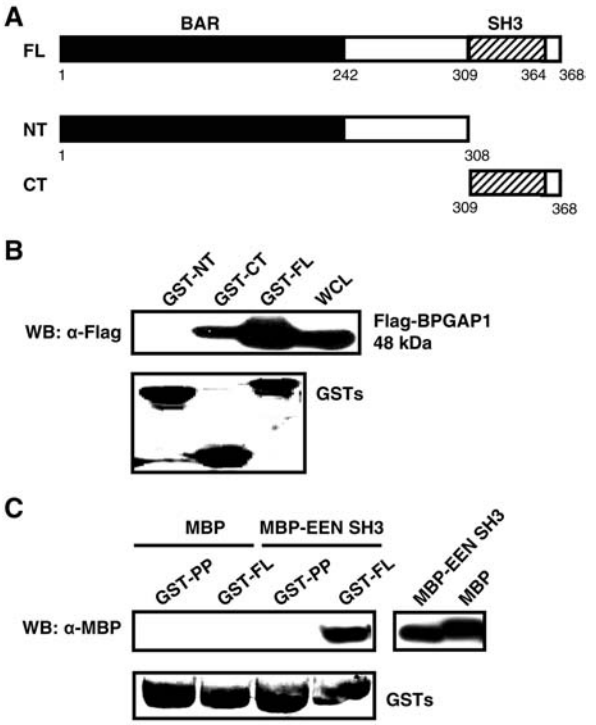


Fig. 4. EEN binds to BPGAP1 via its SH3 domain. (A) EEN domains used in binding studies. NT, N-terminal (aa 1-308) and CT, C-terminal (aa 309-368). NT contains a BIN/amphiphysin/Rvsp (BAR) domain and a coiled-coil region; CT contains an SH3 domain. (B) GST-recombinants of various constructs of EEN were prepared as sepharose beads and used for pull-down assays on lysates prepared from 293T cells that expressed Flag-tagged full-length BPGAP1. Beads from the pull-down experiments were washed and processed for western analyses using the Flag antibody. The blot was stripped and stained with amido black to reveal loading of GST-recombinants. (C) Purified EEN SH3 fusion of maltose binding protein, MBP-EEN SH3 or the MBP control were incubated with sepharose beads conjugated to purified GST recombinant proteins of the full-length wildtype, the PP mutant of BPGAP1, or GST control, and bound targets revealed by western blot analyses using MBP antibody. Purified MBP and MBP-EEN SH3 were analyzed with MBP antibody and revealed intact targets used in the direct binding assays. The lower apparent molecular mass for MBP-EEN SH3 compared with the MBP alone was due to the removal of internal *lacZ* coding sequence upon cloning of the target insert. The blot was stripped and stained by amido black to verify loading of equal amounts of GSTs.

Consistently, the Flag-tagged NNP fragment of BPGAP1, which lacked the PRR, did not interact with any of the EEN GST-constructs, whereas Flag-tagged NP or PC (both contain the PRR) interacted with both the full-length or the SH3-containing CT fragment of EEN, but not with the NT fragment (data not shown). Furthermore, the purified SH3 domain of EEN in the form of a fusion protein with maltose-binding protein was prepared and shown to bind to the wildtype GST-BPGAP1 but not with the GST-PP mutant (Fig. 4C). Taken together, the result strongly suggested that the SH3 domain alone was indeed sufficient to mediate its direct interaction with BPGAP1. To further verify the binding specificity, the N-terminal SH3 domain of Grb2 and one of the SH3 domain of Nck adaptor proteins were used in the similar experiments. None of these two SH3 domains could recognize the PRR of BPGAP1 (data not shown), further strengthening the notion that EEN is a bone fide partner of BPGAP1 and together they might be involved in distinct functions in cell signaling. No other SH3-containing proteins were readily identified from the subproteomics of BPGAP1 pull-down, probably because of the low abundance of such putative target(s).

BPGAP1 enhances EGF receptor endocytosis via its interaction with EEN and its functional GAP domain

EEN is a member of the endophilin family originally known to interact with several endocytic proteins, including dynamin II, synaptojanin and amphiphysin (de Heuvel et al., 1997; Ringstad et al., 1997; So et al., 2000b; Micheva et al., 1997). However, the exact role(s) of EEN in this and other cellular processes linked to endocytosis has yet to be demonstrated. Since endocytosis is one of the primary mechanisms by which growth factors and their receptors are internalized for initiating or terminating signaling (Le Roy and Wrana, 2005), we wanted to examine the functional consequences of the interaction between EEN and BPGAP1 within the context of EGF signaling. Three important cellular and physiological read-outs were monitored using cells that were optimally responsive to EGF while being amenable for such biological assays: (1) the intracellular disposition of BPGAP1 and EEN and their effects on EGF-stimulated EGF receptor internalization, observable by confocal immunofluorescence microscopy; (2) the rates of internalization for EGF still bound to their receptor tyrosine kinases in the cells that had expressed specific constructs of BPGAP1 and/or EEN, quantified using fluorescence-activated cell sorter assays; and (3) their impacts on the canonical downstream signaling leading to the activation of extracellular signal-regulated kinases 1/2 (ERK1/2), which are commonly measurable by their phosphorylation status in western blot analyses.

To study EGF receptor internalization, human cervical epithelial HeLa cells were chosen and the efficacy of the assay validated. Cells were made quiescent and left untreated or treated with EGF for 10 minutes and the internalization of labeled EGF receptor was monitored in the presence of monensin to prevent recycling, as described in Materials and Methods. As seen in Fig. 5A, only cells stimulated with EGF exhibited profound disposition of the labeled EGF receptor in vesicles. Similar results were also observed for stimulated cells transfected with vector control alone (data not shown). As reported by others (Jullien-Flores et al., 2000; Wang et al., 1999; Murthy et al., 1986), the apparent EGF receptor signals at the perinuclear region had mostly remained unchanged.

To examine the effects of BPGAP1 and/or EEN on EGF receptor internalization, HeLa cells were transfected with various expression plasmids for the wildtype or mutant BPGAP1 and/or EEN, either separately (Fig. 5B) or in different combinations as indicated (Fig. 5C). These constructs, as depicted earlier for binding studies, were tagged with the HA or Flag epitope, thus allowing cells specifically expressing one or more constructs to be traced with specific primary antibodies, followed by secondary antibodies pre-conjugated with designated fluorophores. The extent of the internalization for the EGF receptor in the untransfected cells

or cells expressing these constructs were then monitored after EGF stimulation using confocal immunofluorescence microscopy. In all images, cells expressing the protein(s) in question were presented with adjacent untransfected cells as the 'internal control' and results were representative for more than 90% of the total cells viewed. As seen in Fig. 5B, cells expressing Flag-tagged wildtype BPGAP1 exhibited multiple protrusions with the protein mainly localized as punctate structures in the cytoplasm and also along the cell periphery (Shang et al., 2003; Lua and Low, 2004). However, when compared with control cells, cells overexpressing BPGAP1 exhibited more EGF receptor internalization. By contrast, the PP mutant did not induce any significant increase in the EGF receptor internalization despite showing a similar disposition to the wildtype counterpart, implying the importance of this motif in conferring enhanced EGF receptor internalization. Endocytosis is a dynamic process that involves rapid remodeling of the actin cytoskeleton, and which is regulated by Rho small GTPases (Qualmann and Mellor, 2003). We next examined whether the RhoGAP activity of BPGAP1 was also necessary for its effect on EGF receptor internalization. To this end, a catalytically inactive mutant of BPGAP1, where its catalytic arginine finger motif Arg232 had been mutated to an inert alanine (R232A) (Shang et al., 2003), was introduced into the cells and shown to strikingly reduce the basal EGF receptor internalization. This inhibition was probably due to sequestration of the Rho GTPases from participating in such process. Taken together, these results indicate that the functional GAP domain of BPGAP1, although itself not sufficient to drive endocytosis (as seen for the PP mutant, which still retained the GAP domain), still remained an integral entity for its adjacent PRR to elicit enhanced endocytosis. However, cells over-expressing EEN alone were more cuboidal in shape and the proteins were localized mainly on the periphery and tubular network, with a smaller proportion being localized to the nucleus. More significantly, these cells showed a more profound degree of internalization of the EGF receptor when compared with untransfected cells (Fig. 5B). However, once the SH3 domain of EEN was removed, the truncated mutant (NT fragment) showed a greater tendency to be localized to the nucleus, although a significant proportion of them were still found to be membrane-associated and present in the tubular network. Intriguingly, all cells expressing this mutant exhibited complete inhibition in the EGF receptor internalization (Fig. 5B), indicative of its dominant-negative role in such process.

When both the wildtype of BPGAP1 and EEN were coexpressed in the same cell, they were found to be colocalized in most parts of the cell, especially along the periphery. They also led to more profound stimulation of EGF receptor internalization when compared with cells transfected with only BPGAP1 (Fig. 5C, first row). However, when EEN was present together with the non-interactive PP mutant, such augmentation in the stimulatory effect was no longer apparent, indicating that the combined BPGAP1-EEN effect for the enhanced internalization of EGF receptor was probably due to their constructive interaction rather than a mere additive effect operating from separate pathways (Fig. 5C, second row). Furthermore, it could be seen that some EEN were still colocalized with the PP mutant in the periphery, whereas most of them were now located in the nucleus. It seemed plausible that EEN proteins colocalized on the cell periphery could be targeted independently of BPGAP1 or could still associate to the BPGAP1 complex through an indirect accessory protein, even though they had lost their direct interaction. Consequently, functional interaction with the wildtype BPGAP1 could help retain some of the EEN in the cytosol for their subsequent regulation of endocytosis. Likewise, it was found that when NT mutants were coexpressed with the wildtype BPGAP1, the majority of the NT remained in the nucleus, while some were found to be associated with BPGAP1 on the membrane and tubular network, probably because of the autonomous targeting or indirect binding to BPGAP1, as described above. In this case, no internalization of EGF receptor was observed (Fig. 5C, third row). These results were therefore consistent with the dominant negative role of NT in the process of endocytosis, and further supported the notion that BPGAP1 enhanced EGF receptor internalization through its functional interaction with EEN.

In comparison, if EEN was present together with the interactive but catalytically inactive BPGAP1 mutant, R232A, very few EEN remained in the nucleus while they both showed colocalization in most parts of the cell, mainly on the cell periphery. However, similarly to cells expressing only the GAP mutant (see Fig. 5B, third row), cells that harbored both R232A and EEN showed less EGF receptor internalization compared with control cells, indicative of an absolute requirement for the functional RhoGAP activity by BPGAP1 to facilitate the process of endocytosis catalyzed by EEN (Fig. 5C, fourth row). Finally, to demonstrate further that the lack of augmentation by EEN with the non-interactive PP mutant (as described above) was not simply due to the global distortion of the endocytic machinery as a result of overexpressed PP, cells were cotransfected with Myc-tagged EEN together with HA-tagged PP and functional Flag-tagged BPGAP1. The assay was optimized to express the wildtype BPGAP1 in all the cells that expressed PP mutant, as validated by a parallel assay (data not shown). As seen in Fig. 5C (last row), re-introduction of wildtype BPGAP1 could help augment the enhanced endocytosis by EEN even in the background of the PP mutant, which indicates that constructive BPGAP1-EEN interaction alone was sufficient to bring about the combined effect.

To obtain a more quantitative measure of the effects of BPGAP1 and EEN on endocytosis and to provide separate evidence of their regulation of endocytosis upon EGF stimulation, we used the human kidney epithelial cells 293T, expressing one or more of the constructs, and the rates for the internalization of the EGF ligand still bound to the intracellular pools of the receptors were measured. Unlike HeLa cells, which are useful for confocal fluorescence imaging, the 293T cells are generally more amenable for high-efficient multiple transfections that are crucial for such quantitative measurements based on fluorescence-activated cell sorter assays.

Cells were transfected with the vector control or various expression plasmids coding for the wildtype or mutants forms of BPGAP1 or/and EEN, either separately or in combination, and assayed for their effects on EGF-bound EGF receptor endocytosis. The mean intensity of EGF uptake per cell was quantified after analyses of specific populations of cells that had expressed the tagged proteins and had taken up the labeled EGF using the fluorescence-based technique as described in

Materials and Methods. This protocol had established a linear uptake of EGF for the control cells for up to 30 minutes, typically with less than 10% variation among the test samples within a given time. For clarity purposes, error bars were omitted in the figures.

After EGF stimulation, control cells elicited a slow linear uptake of EGF over 30 minutes. Cells over-expressing either EEN or BPGAP1 alone enhanced the initial uptake after 10 minutes by sevenfold and tenfold, respectively ($P<0.01$); thereafter they reached a plateau (Fig. 6A). However, when both proteins were coexpressed, the initial rate during the first 10 minutes remained about the same magnitude as that induced by either of the single transfectants, but it continued linearly at a much greater rate (18-fold) than the control. The augmented level reached the plateau after 20 minutes. For the maximal uptake at 20 minutes, the degree of enhanced EGF uptake by BPGAP1 present together with EEN was significantly more than the combined effects from those stimulated separately by BPGAP1 or EEN ($P<0.01$). By contrast, overexpression of the NT fragment of EEN appeared to stimulate the uptake only for the first 10 minutes, but declined rapidly to the level below the control. This pattern of decline was also apparent when NT and BPGAP1 were coexpressed in the same cells, indicative of a

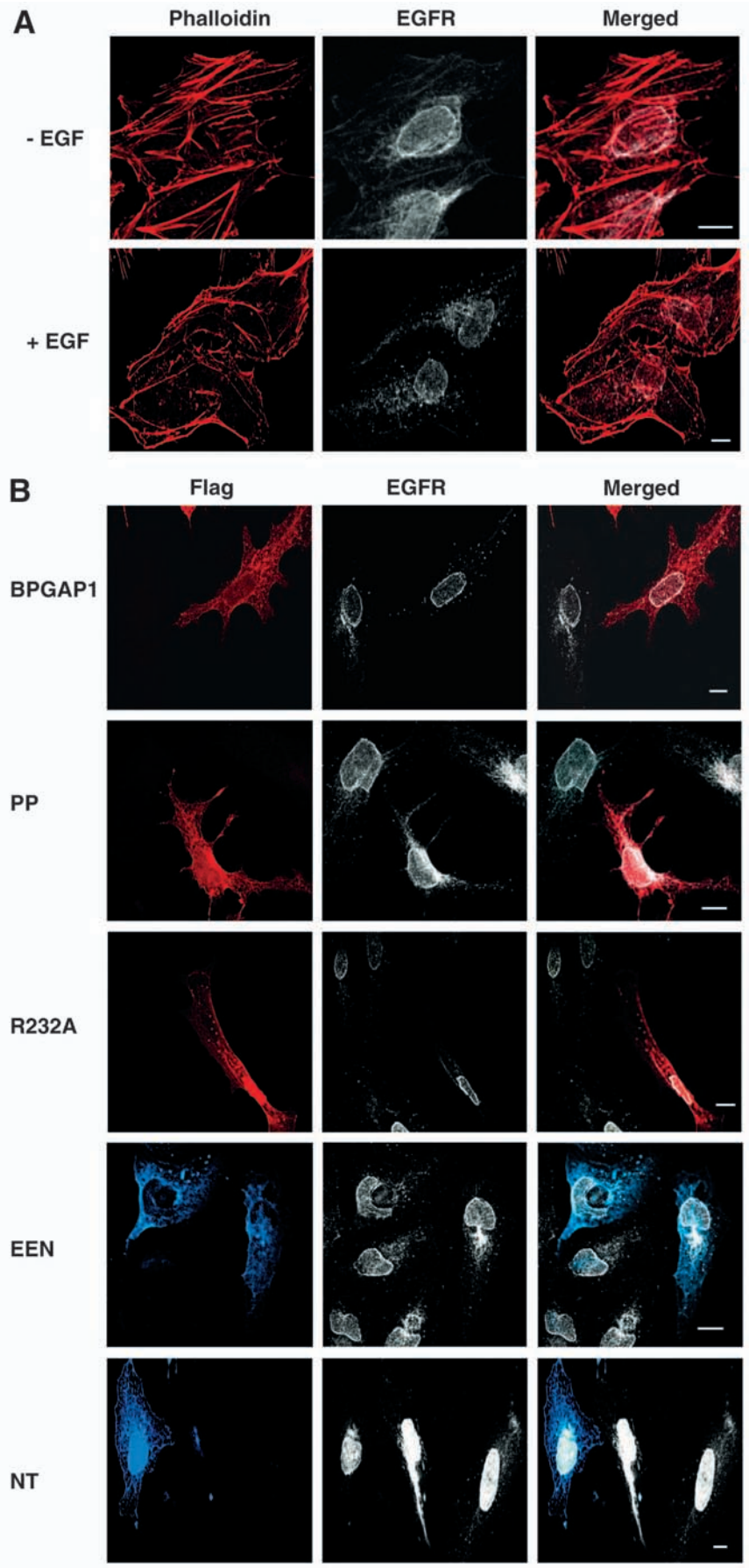


Fig. 5. Effects of BPGAP1 and EEN on EGF receptor endocytosis. HeLa cells were untransfected (A) or transfected with different epitope-tagged expression plasmids coding for wildtype BPGAP1, PP, R232A and/or wildtype EEN and NT, either alone (B) or in combination (C) as indicated. Cells were then made quiescent by serum-removal for 2 hours, followed by treatment with 100 ng/ml EGF for 10 minutes in the presence of monensin. Cells were permeabilized, stained and visualized under confocal fluorescent microcopy as described in Materials and Methods. Untransfected cells in A were labeled with rhodamine-phalloidin to mark the shape of cells and the intracellular vesicles (A-C) indicate uptake of EGF receptor (grey). Cells expressing different constructs of BPGAP1 (red) and EEN (blue) were detected by appropriate anti-Flag, anti-HA or anti-Myc antibodies followed by fluorophore-conjugated secondary antibodies. Images from cells transfected with multiple constructs were merged (Merged[1]) to show their colocalization (purple). The second merged images (Merged[2]) illustrate the endocytosed EGF receptor in transfected cells. For the triple transfectants (asterisk), the transfection was optimized to express the wildtype BPGAP1 in all the cells that expressed PP mutant, validated by parallel experiments (data not shown). The intensities of images were enhanced to capture changes in the cell peripheries, including their cell protrusions. Bars, 10 μm.

dominant negative effect of NT on the otherwise stimulatory effect by BPGAP1. The significance of this will be described below (Fig. 6B). By comparison, the PP mutant of BPGAP1 did not affect the rate of endocytosis to any great extents. As such, EEN and PP together elicited an endocytosis rate profile similar to that incurred by EEN alone. However, overexpression of the GAP mutant (R232A), either alone or together with EEN, led to lower levels of EGF uptake than in

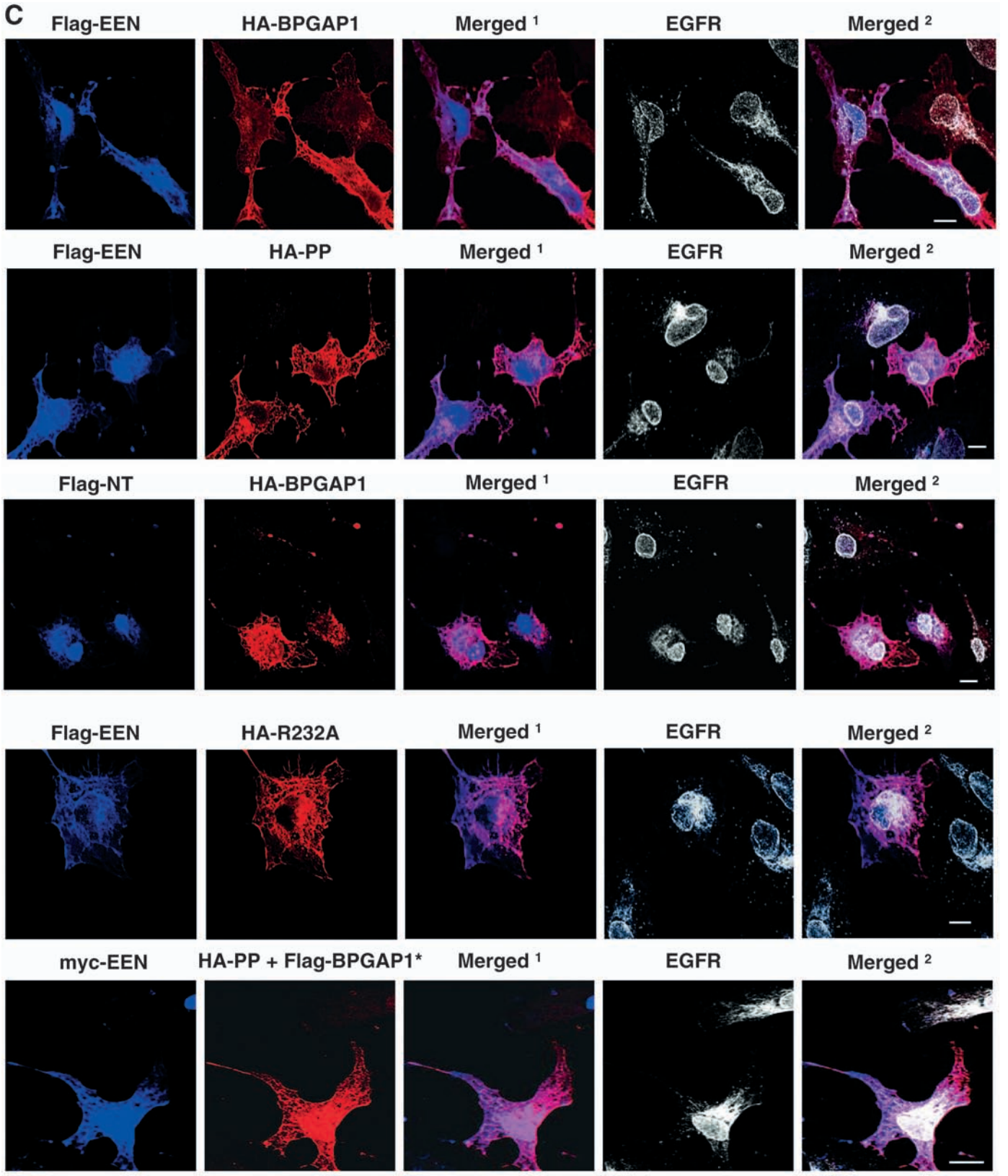


Fig. 5C. See previous page for legend.

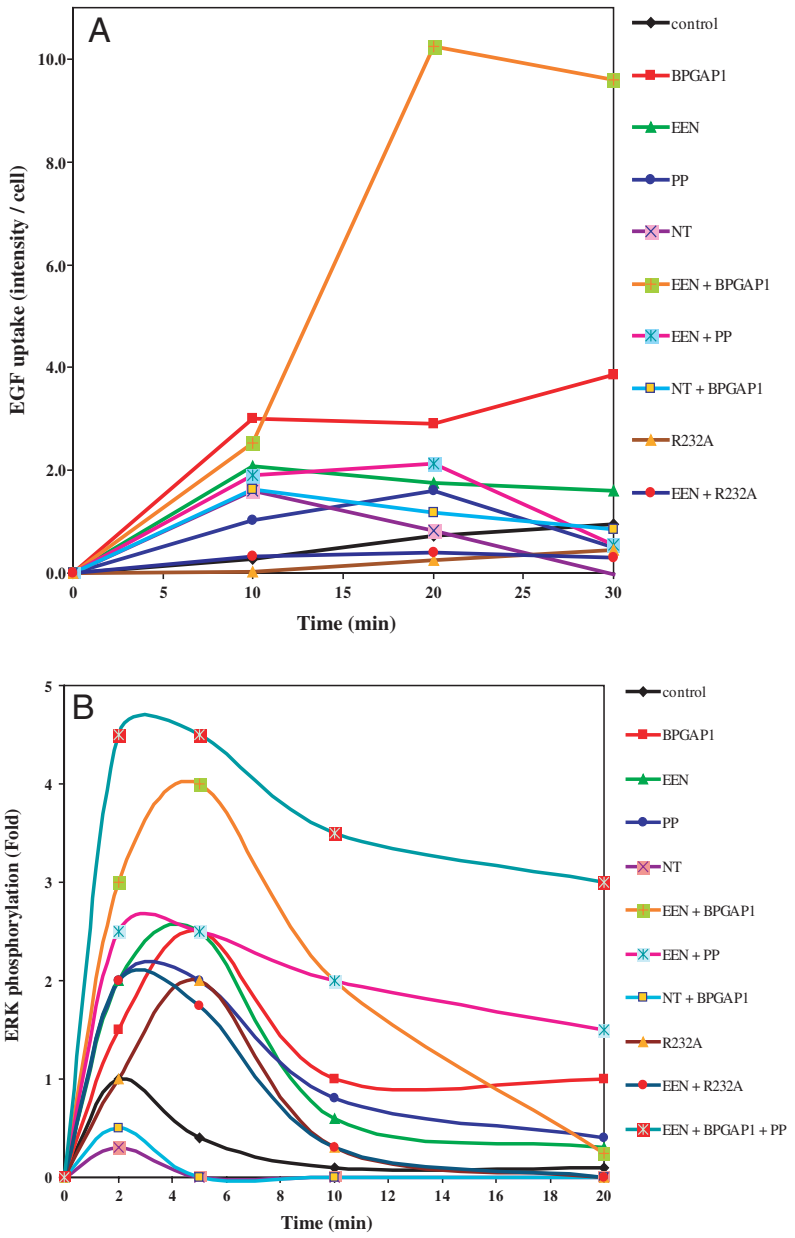
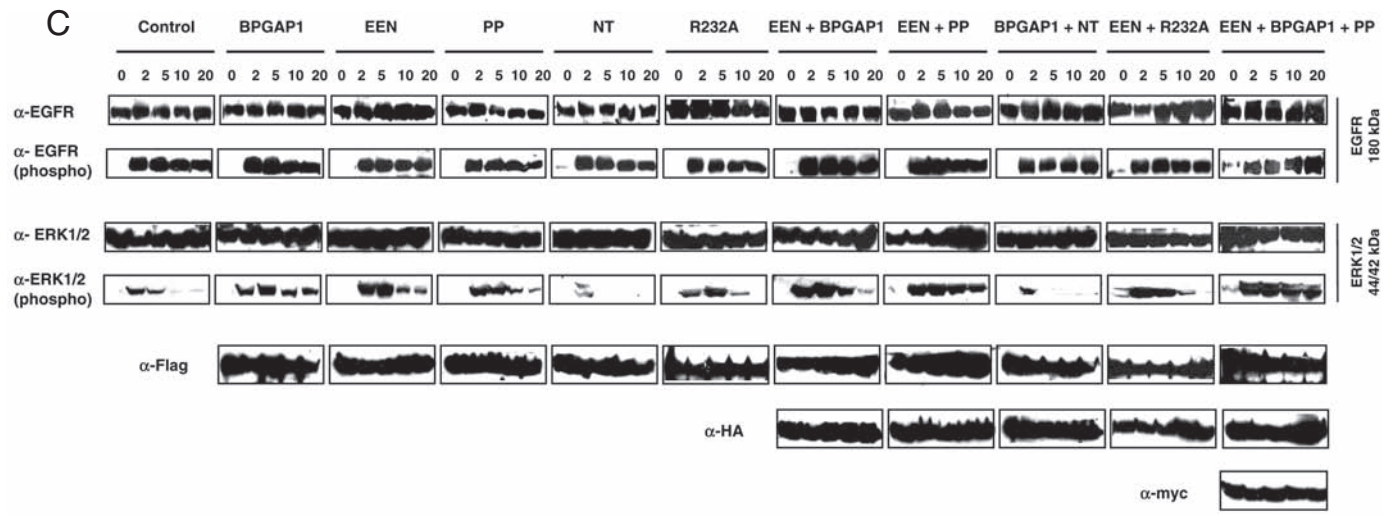


Fig. 6. BPGAP1 and EEN stimulate EGF receptor endocytosis and ERK1/2 phosphorylation. (A) 293T cells were transfected with vector control or expression plasmids coding for the tagged wildtype or mutant proteins, either alone or in combinations as indicated. They were made quiescent by serum depletion for 2 hours followed by stimulation with 100 ng/ml Oregon-Green-labeled EGF for the times indicated and processed for EGF uptake in the presence of monensin as described in the Materials and Methods. The total amounts of EGF-bound EGF receptor being internalized within certain populations of cells that had expressed specific tagged proteins were measured by fluorescence-based detection and calculated as mean intensity per cell, as described in Materials and Methods. Data are means of two to three independent experiments, each counted in triplicate. Variations for each specific time point and sample were typically less than 10% and the error bars are omitted for clarity. The control uptake exhibits a linear uptake rate up to 30 minutes. (B) 293T cells were transfected with vector control or expression plasmids coding for the tagged wildtype or mutant proteins, either alone or in combination as indicated. They were made quiescent by serum depletion for 18 hours followed by the stimulation with 100 ng/ml EGF for the periods indicated. Activation of the EGF receptor and ERK1/2 were analyzed by western blotting of equal amount of whole-cell lysates using anti-phospho EGF receptor and anti-phospho ERK1/2, respectively. To show equal loading of whole-cell lysates and expression of the appropriate tagged proteins, the blots were analyzed with anti-EGF receptor (EGFR), anti-ERK1/2 or anti-Flag (for EEN and NT in single or double transfectants, and for BPGAP1, PP and R232A in single transfectants), or anti-HA (for BPGAP1, PP and R232A in double-transfectants). For triple transfectants, anti-Myc (for EEN), anti-HA (for PP) and anti-Flag (for BPGAP1) were used. The ERK phosphorylation signals were expressed as number of fold over the maximal level seen by the control cells after 2 minutes. These results are representative of three independent sets of assays, each time with the control-stimulated cell lysates prepared concurrently and analyzed alongside the test constructs. This serves as an internal control for gel variations and also for normalizing the film exposures. One of the representative set is shown as Fig. 6C.



control cells. This supports the apparent dominant negative role of BPGAP1 GAP mutant on endocytosis as described earlier for the confocal immunofluorescence assays (Fig. 5).

Taken together, all these results strongly support the notion that EEN and BPGAP1 are imperative modulators that are involved in the similar regulatory pathway for EGF-stimulated EGF receptor endocytosis. Specifically, their interaction is instrumental for the augmentation of this process which also requires a functional RhoGAP activity from BPGAP1 inside the cells.

BPGAP1 enhances ERK1/2 phosphorylation partly via interaction with EEN and its functional GAP domain

To correlate the significance of the enhanced EGF-stimulated EGF receptor endocytosis to its intracellular signaling, the same combinations of expression plasmids were transfected into 293T cells. Transfected cells were made quiescent before being challenged with an optimal dose of EGF (100 ng/ml) for various times up to 20 minutes, lysed and analyzed for the status of phosphorylation for ERK1/2, a marker for the activation of the Ras/mitogen-activated protein kinase (MAPK) pathway. Profiles for the ERK1/2 phosphorylation and endocytosis rates were then compared. To provide a semi-quantitative measure of the strength and durability for the ERK signaling capacity elicited by different proteins, the signals were compared with the maximal level seen in the control cells, which were analyzed concurrently with each test sample on the same western blots. EGF stimulated ERK1/2 phosphorylation maximally in the control cells by 2 minutes but it rapidly returned to the basal level after 10 minutes. Overexpression of EEN and BPGAP1 alone enhanced the maximal levels at 5 minutes by 2.5-fold, respectively and sustained the activation further after 10 minutes (Fig. 6B,C). Similarly to their augmentation in endocytosis, co-expression of EEN and BPGAP1 stimulated the phosphorylation further by fourfold, sustained by twofold after 10 minutes, before it returned to almost the basal level after 20 minutes. By contrast, the NT mutant reduced the maximal activation level for the control cells by more than 50% and, consequently, inhibited any stimulatory effects otherwise brought about by BPGAP1 alone. These results are therefore in accordance with the dominant negative effect that NT exerts on BPGAP1-induced endocytosis (Fig. 5, Fig. 6A). Interestingly, in contrast to its lack of stimulatory effect on endocytosis, overexpressed PP mutant unexpectedly induced activation of ERK1/2 phosphorylation, albeit at different kinetics compared with the wildtype BPGAP1. Consequently, EEN and PP together did not increase their maximal level further, but only helped to sustain the duration of ERK1/2 phosphorylation by 1.8-fold even after 20 minutes.

To ensure that the lack of augmentation by EEN with the non-interactive PP mutant and the apparent stimulatory effect by PP mutant were not simply due to the global distortion of ERK signaling resulting from the overexpression of PP, cells were cotransfected with Myc-tagged EEN together with HA-tagged PP and the functional Flag-tagged BPGAP1, and their triple-combined effects on ERK1/2 phosphorylation were examined. As for the rescue experiments performed for endocytosis in HeLa cells (Fig. 5), transfection in 293T cells was optimized to maximize the coexpression of the wildtype

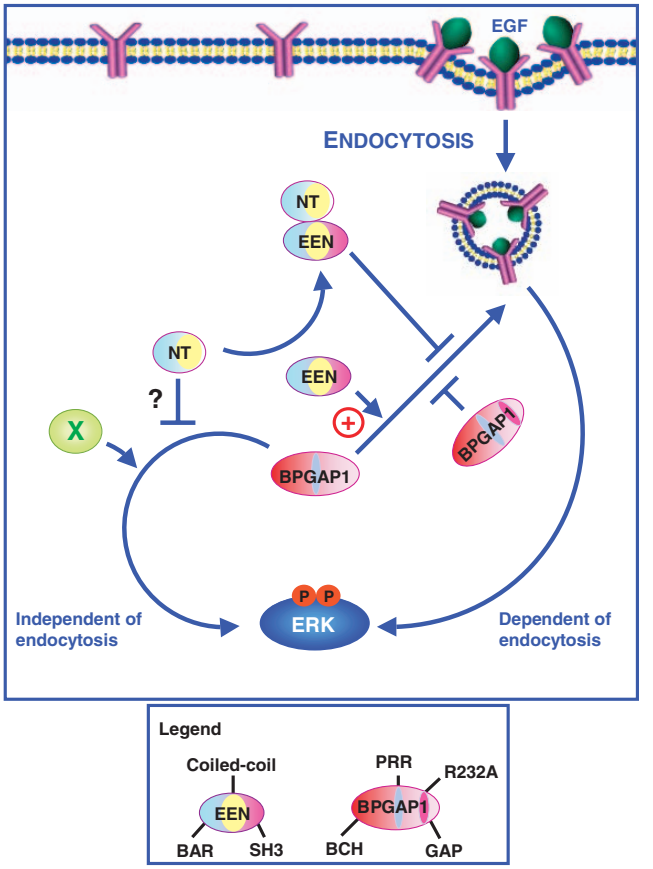


Fig. 7. A model for the stimulatory effects by BPGAP1 and EEN on EGF-stimulated EGF receptor endocytosis and ERK1/2 phosphorylation. Stimulation of cells with EGF triggers the internalization of the EGF-bound EGF receptor in the form of vesicles, leading to the phosphorylation of ERK1/2. This process is enhanced by BPGAP1 or EEN alone and augmented through their interaction via their PRR and SH3 domain, respectively. However, the presence of NT mutant of EEN, which lacks the SH3 domain, completely prevents the effects of BPGAP1 in both endocytosis and ERK1/2 phosphorylation, thus acting as a dominant negative mutant in this pathway. This could involve displacing and sequestering functionally active EEN or other endophilins from BPGAP1, by forming homodimer or heterodimer complexes via their coiled-coil regions. Similarly, the GAP mutant R232A devoid of the catalytic arginine finger motif also reduces basal and EEN-stimulated EGF receptor endocytosis, suggesting that functional RhoGAP activity is required to promote endocytosis and ERK1/2 phosphorylation. Furthermore, despite failing to augment the EEN effects further and its inability to enhance endocytosis, the PP mutant (devoid of the interaction with EEN) or the GAP mutant (R232A) can also stimulate ERK1/2 phosphorylation, which suggests a distinct regulatory pathway independent of BPGAP1's stimulatory role in endocytosis. This could involve a different protein X possibly via its interaction with the BCH domain, but not linked to its direct interaction with EEN. However, this pathway still functions downstream of the control that requires an intact EEN function, as the NT mutant completely abolishes all stimulatory effect by BPGAP1, perhaps through a feedback or crosstalk from the endocytosis-dependent mechanism. BAR, BIN/amphiphysin/Rvsp domain; BCH, BNIP-2 and Cdc42GAP homology domain; GAP, GTPase-activating protein domain; PRR, proline-rich region; R232A, GAP mutant devoid of the catalytic arginine finger motif; NT, EEN mutant without the SH3 domain; ERK, extracellular signal-regulated kinase. Plus sign in circle denotes stimulatory effect while T-bars denote inhibitory effect. P, phosphorylation.

BPGAP1 with the PP mutant. Significantly, re-introduction of the wildtype BPGAP1 could help restore and augment the enhanced ERK1/2 activation by EEN even in the background of the PP mutant. Interestingly, the total level of activation now reached a robust maximum of 4.5-fold between 2 and 5 minutes, with the enhanced levels at all sub-optimal time points exhibiting additive effects from 'EEN with BPGAP1' (Fig. 6B, orange line) and the 'EEN with PP' (Fig. 6B, pink line). These results therefore strongly support the notion that (1) functional BPGAP1-EEN interaction could bring about the constructive and enhanced effect on ERK1/2 phosphorylation, similarly to their effects on EGF receptor endocytosis, and (2) BPGAP1 could also activate ERK1/2 through an additional mechanism not linked to its direct interaction with EEN, but is clearly still downstream of the pathway regulated by a functionally intact EEN protein, as shown by the dominant negative effect of NT (see Fig. 7 for model).

Since the earlier endocytosis results indicated that the BPGAP1 inactive GAP mutant, R232A, also served as a dominant negative mutant on EGF receptor endocytosis (Fig. 5, Fig. 6A), it would provide an additional tool to further dissect the roles of BPGAP1 in ERK1/2 regulation. As seen in Fig. 6B,C, overexpression of R232A alone did not reduce the basal level of ERK1/2 activation by EGF. Instead, it enhanced the level with an early time-course profile similar to that induced by the wildtype BPGAP1 but with lower magnitude of activation. Notably, unlike the wildtype BPGAP1 or PP, R232A alone did not cause any prolonged activation after 20 minutes. Furthermore, when present together with EEN, R232A could not augment the level of activation induced by EEN despite its ability to interact with the latter. Instead, it shifted and reduced the maximal level of activation and duration of EEN effects, notably the decay of the activated signal, to the basal level after 20 minutes. This result indicates that R232A inhibited the EEN effect in a manner similar to its dominant negative role during endocytosis, while still activating ERK1/2 phosphorylation, independently of the coupling between GAP function and its interaction with EEN.

As a control for equal loading of lysates and also to rule out the possibility of perturbation of the expression levels of ERK1/2, same blots of anti-phopsho ERK1/2 were stripped and the levels of these two proteins across the samples were shown to be constant. Furthermore, in all experiments, the phosphorylation of the EGF receptor was not being significantly affected by any of the overexpressed proteins, adding to the specificity of the stimulatory or inhibitory effects seen by the wildtype or mutant proteins in both the endocytosis and ERK1/2 phosphorylation.

Taken together, all these results are consistent with the notion that functional BPGAP1-EEN interaction is instrumental in enhancing EGF receptor endocytosis leading to the phosphorylation of ERK1/2 while eliciting its additional stimulatory effect via a yet unknown pathway independently of the roles of PRR and GAP domain in BPGAP1. How the additional mechanism operates and whether these two mechanisms are linked remain the subject of our on-going studies. BPGAP1 and EEN thus represent two novel determinants that could integrate signaling crosstalk between RhoGAP and endophilin-mediated endocytosis for the regulation of Ras/MAPK signaling pathway.

Discussion

We recently showed that BPGAP1 induces cell morphological changes (Shang et al., 2003) and facilitates cell motility through its interaction with cortactin (Lua and Low, 2004). In the present study, we have further identified EEN/endophilin II as an important interacting partner for BPGAP1 and together they lead to enhanced EGF receptor endocytosis and ERK1/2 phosphorylation. This interacting complex provides an important link between RhoGAP and endophilins in regulating endocytic machinery for intracellular signaling.

BPGAP1 and EEN act in concert to promote endocytosis: the absolute requirements for its proline-rich region for targeting EEN and the functional GAP domain

The endophilin family of proteins consists of two subfamilies – endophilin A and B. Endophilin A consists of three members, AI (SH3GL2; SH3P4), AII (EEN; SH3GL1; SH3P8) and AIII (SH3GL3; SH3P13) (Ringstad et al., 1997; Giachino et al., 1997), whereas the recently identified endophilin B subfamily comprises BI (SH3GLB1) and BII (SH3GLB2) (Pierrat et al., 2001; Modregger et al., 2003). All endophilin isoforms share similar structural features: an N-terminal BAR (BIN/amphiphysin/Rvsp) domain, followed by a coiled-coil region, and a C-terminal SH3 domain. They interact with a common set of cellular proteins and are implicated in different cellular processes (Reutens and Begley, 2002). Endophilin I (SH3GL2) (Simpson et al., 1999) and endophilin III (SH3GL3) (Sugiura et al., 2004) have been shown to stimulate and inhibit endocytosis, respectively, while work on the biochemical characterization and cellular roles of endophilin II (EEN) are just starting to unfold. Functionally, the BAR domain of endophilin I has an acyltransferase activity (Schmidt et al., 1999) that generates lysophosphatidic acid required for the invagination of the plasma membrane during endocytosis (Takei et al., 1998; Farsad et al., 2001).

In the current study, we have demonstrated that EEN exerts its stimulatory effect on EGF-stimulated EGF receptor endocytosis in epithelial cells by coupling to BPGAP1 via its SH3 domain. Furthermore, introduction of the NT mutant of EEN, which lacks the SH3 domain, reduced basal and BPGAP1-activated endocytosis as well as ERK1/2 phosphorylation. We speculate that the NT mutant, which still retained the coiled-coil region normally used for homo- or heterodimerisation (Ringstad et al., 2001), could have sequestered and displaced endogenous EEN or other endophilins from functionally interacting with BPGAP1 (Fig. 7). These results also highlight the potential roles of BPGAP1 in regulating EEN function with other partners that also associate with its SH3 domain. In this regard, the SH3 domain of EEN has been shown to interact with several target proteins containing PRRs (Ringstad et al., 1997; So et al., 2000; Yam et al., 2004), including dynamin, a large GTPase that has been implicated in the scission of clathrin-coated vesicles during receptor-mediated endocytosis (Damke et al., 1994). It is possible that BPGAP1-EEN and EEN-dynamin complexes are functionally linked or they might compete with each other for EEN. Indeed, BPGAP1 binding to EEN does not interfere with the EEN-dynamin complex when expressed inside the cells

(B.L.L. and B.C.L., unpublished). Given the fact that dynamin, EEN and BPGAP1 are all positive regulators of endocytosis, they are likely to act in concert, rather than in competition, to bring about enhanced endocytosis. Possible links between BPGAP1-EEN with EEN-dynamin are now being investigated.

BPGAP1 and EEN as novel determinants for extracellular signal-regulated kinase signaling

Following EGF activation, receptor tyrosine kinase recruit Sos to the plasma membrane, where it converts Ras to its GTP-bound form, which then interacts with its effector Raf-1, recruiting Raf-1 to the plasma membrane. Raf-1, a mitogen-activated protein kinase kinase kinase, phosphorylates and activates the MAP kinase kinase, MEK1, which in turn phosphorylates and activates the MAP kinase, ERK1/2. This well-characterized Ras/MAPK pathway can be stimulated by a number of activated receptors and a functional endocytic trafficking event is essential for activation of ERK1/2 (Vieira et al., 1996; Le Roy and Wrana, 2005). To date, the role of receptor endocytosis in the activation of the MAP kinase cascade has received much attention, although its precise mode of action still remains to be elucidated. In addition, there have been substantial disputes in regard to whether receptor internalization occurs to activate or attenuate, or shows no involvement in the MAP kinase signaling (Vieira et al., 1996; Leof, 2000; Ceresa and Schmid, 2000; Le Roy and Wrana, 2005). Here, we observed that interaction of BPGAP1 with EEN enhances endocytosis and leads to ERK1/2 phosphorylation. Using the BPGAP1 mutant that lacks the binding motif for EEN (PP), the EEN mutant that lacks the SH3 domain that binds BPGAP1 (NT), as well as the BPGAP1 mutant that is catalytically inactive towards RhoA (R232A) we have uncovered several important aspects of ERK1/2 regulation by BPGAP1 (see model in Fig. 7): (1) BPGAP1 exerts its stimulatory effect via its functional GAP domain collaborating with its PRR, which interacts with EEN to initiate EGF receptor internalization, leading to subsequent activation of ERK1/2. This is supported by the observation that the dominant negative NT mutant of EEN completely abolished BPGAP1-induced activation of endocytosis as well as ERK1/2 phosphorylation, whereas the R232A mutant greatly inhibited the effect of EEN on endocytosis and failed to augment further the effect of EEN on ERK activation. (2) BPGAP1 can also induce ERK via a separate mechanism, independently of its direct binding to EEN and the functional GAP domain. However, it is still downstream of the pathway regulated by a functionally intact EEN protein as NT could completely inhibit the BPGAP1 effect. We speculate that this activation is likely to be independent of BPGAP1's direct effect on endocytosis (as it is insensitive to inhibition of endocytosis by the GAP mutant) and the NT effect might indicate a feedback loop or crosstalk regulation from the first endocytic-dependent mechanism. In this regard, our ongoing studies show that the N-terminus of BPGAP1, comprising mostly the BCH domain, could indeed activate ERK1/2 phosphorylation (B.L.L. and B.C.L., unpublished), the molecular mechanism of which is now being investigated.

In this study, BPGAP1 appears to help retain EEN in the cytosol, as well as colocalizing with EEN in the periphery. Recently, Yam et al. demonstrated that overexpression of EEN in the murine fibroblast cell line R6 inhibits Ras-mediated transactivation and transformation through an EEN-binding protein (EBP) (Yam et al., 2004). In addition, EEN might act by shuttling between the nucleus, cytosol and membranes, or distinct pools of EEN could exist for their different functions under different conditions (Cheung et al., 2004). It will be interesting to find out whether EEN-EBP and EEN-BPGAP1 represent two mutually exclusive entities or whether BPGAP1 and EBP could compete for EEN and determine the final outcome of the Ras/MAPK signaling. EEN is part of a new family of SH3-domain-containing proteins, collectively known to be involved in both normal cellular processes, such as cell proliferation and differentiation, as well as malignant cellular processes as a result of chromosomal translocation in leukaemia or pathogenesis of Huntington's disease. It remains to be seen how BPGAP1 and EEN can regulate such a diversity of dynamic cellular processes.

BPGAP1: a potential link between the actin cytoskeleton, endocytic trafficking and cell signaling

The Rho subfamily of small GTPases has been known to play an important role in regulating the actin cytoskeleton (Hall, 1998) and more recently in endocytic trafficking (Qualmann and Mellor, 2003). However, precise involvement of specific GTPase(s) and/or their regulators, as well as the signaling mechanism linking GTPases, actin dynamics and endocytosis remain to be explored. Expression of constitutively active mutants of either Rac or RhoA block the internalization of transferrin receptor (Lamaze et al., 1996), whereas expression of constitutively active mutants of RhoD (Murphy et al., 1996; Murphy et al., 2001) or RhoB (Gampel et al., 1999) impedes early endocytic motility and internalization of EGF receptor into lysosomes, respectively. Similarly, an effector of Ral small GTPase known as RLIP76/RalBP1 interacts with AP2 complex during receptor endocytosis, whereby activated RalB interferes with endocytosis of both transferrin receptor and EGF receptor (Jullien-Flores et al., 2000). RalBP1 itself harbors a RhoGAP homology domain and exhibits GAP activity towards Rac1 and Cdc42 but not RhoA. Deletion of this GAP domain at its N-terminus, while still retaining its Ral-binding domain, inhibits EGF- and also insulin-dependent endocytosis, whereas the region containing the GAP domain inhibits only insulin-dependent endocytosis (Nakashima et al., 1999). These two processes, however, are not affected by the full-length RalBP1, suggesting that RalBP1 mutants could have disrupted the endogenous complex of RalBP1 necessary for endocytosis (Nakashima et al., 1999). In the absence of the catalytically inactive point mutant, the precise role of GAP function in RalBP1 and its physiological role in EGF signaling still remain unknown. Here, we show that BPGAP1, being a RhoGAP for RhoA but not Rac1 and Cdc42 in vivo (Shang et al., 2003), stimulates EGF receptor internalization leading to ERK1/2 phosphorylation. These two processes require the functional GAP domain of BPGAP1 to collaborate with its adjacent PRR as the direct binding site for EEN. Our results also shed light on the requirement for an inactivation of RhoA for the stimulation of EGF receptor endocytosis and the Ras/MAPK pathway.

We have recently shown that BPGAP1 also interacts with cortactin, the cortical actin-associated protein to elicit actin

cytoskeleton rearrangement for cell motility (Lua and Low, 2004). CD2-associated protein has been shown to couple cortactin to endophilin I, thus providing a potential link between EGF receptor endocytosis and the actin cytoskeleton (Lynch et al., 2003). In addition, cortactin is found to interact with dynamin (McNiven et al., 2000), which is also a target for EEN (Ringstad et al., 1997; So et al., 2000b). Such a cortactin-dynamin interaction requires actin polymerization (Zhu et al., 2005). Therefore, it remains an exciting prospect to see how BPGAP1, cortactin, EEN and dynamin converge to orchestrate the coupling of the cytoskeletal network to endocytic trafficking and cell signaling.

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