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Importance of Constitutive Activity and Arrestin-Independent Mechanisms for Intracellular Trafficking of the Ghrelin Receptor

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The ghrelin receptor (GhrelinR) and its related orphan GPR39 each display constitutive signaling, but only GhrelinRs undergo basal internalization. Here we investigate these differences by considering the roles of the C tail receptor domains for constitutive internalization and activity. Furthermore the interaction between phosphorylated receptors and β-arrestin adaptor proteins has been examined. Replacement of the FLAG-tagged GhrelinR C tail with the equivalent GPR39 domain (GhR-39 chimera) preserved G_q signaling. However in contrast to the GhrelinR, GhR-39 receptors exhibited no basal and substantially decreased agonist-induced internalization in transiently transfected HEK293 cells. Internalized GhrelinR and GhR-39 were predominantly localized to recycling compartments, identified with transferrin and the monomeric G proteins Rab5 and Rab11. Both the inverse agonist [D-Arg^1, D-Phe^5, D-Trp^{7,9}, Leu^{11}] substance P and a naturally occurring mutant GhrelinR (A204E) with eliminated constitutive activity inhibited basal GhrelinR internalization. Surprisingly, we found that noninternalizing GPR39 was highly phosphorylated and that basal and agonist-induced phosphorylation of the GhR-39 chimera was elevated compared with GhrelinRs. Moreover, basal GhrelinR endocytosis occurred without significant phosphorylation, and it was not prevented by cotransfection of a dominant-negative β-arrestin1(319–418) fragment or by expression in β-arrestin1/2 double-knockout mouse embryonic fibroblasts. In contrast, agonist-stimulated GhrelinRs recruited the clathrin adaptor green fluorescent protein-tagged β-arrestin2 to endosomes, coincident with increased receptor phosphorylation. Thus, GhrelinR internalization to recycling compartments depends on C-terminal motifs and constitutive activity, but the high levels of GPR39 phosphorylation, and of the GhR-39 chimera, are not sufficient to drive endocytosis. In addition, basal GhrelinR internalization occurs independently of β-arrestins. (*Molecular Endocrinology* 21: 3100–3112, 2007)

GHRELIN IS A 28-amino-acid peptide, acylated by octanoic acid at Ser3, that was first characterized as an endogenous mediator of pituitary GH release (1). Since then it has emerged as the only peripheral hormone to stimulate appetite and body weight, in contrast to the more numerous gastrointestinal satiety peptides (2). Most circulating ghrelin originates from endocrine cells of the oxyntic gland in the stomach fundus (3), and the sharp preprandial rise in serum peptide levels supports a role in meal initiation (4). Equally, administration of exogenous ghrelin increases hunger in man (5) and stimulates food intake in rodents (6), whereas plasma ghrelin concentrations are elevated after fasting (7, 8) and decreased in obese individuals (9). Ghrelin modulates the activity of neuropeptide Y/agouti-related peptide and proopiomelanocortin neuronal circuits in the hypothalamic arcuate nucleus to stimulate appetite, potentially by both direct mechanisms and via vagal pathways (8, 10). The peptide also has additional actions that promote adipogenesis and inhibit thermogenesis and lipolysis, which contribute to the increased body weight measured after long-term administration (6, 8).

The ghrelin receptor (GhrelinR) belongs to the rhodopsin-like seven-transmembrane domain (7TM) receptor family. Cloned before the discovery of the peptide, GhrelinR was initially described as the receptor for a number of synthetic GH secretagogues and is therefore also called GHS R-1a (11). It belongs to a family that includes the orphan GPR39 as well as receptors for the peptides motilin and neurotensin (12). The pairing be-

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Abbreviations: GFP, Green fluorescent protein; GhR-39, FLAG-tagged ghrelin receptor with GPR39 C tail; 39-GhR, FLAG-tagged GPR39 with ghrelin receptor C tail; GhrelinR, ghrelin receptor; IP, inositol phosphate; LAMP-1, lysosome-associated membrane glycoprotein 1; NK1, neurokinin 1; PNRC, perinuclear recycling compartment; Rab11Q, GFP-tagged Rab11Q70L; Rab11S, GFP-tagged Rab11S25N; Rab5Q, GFP-tagged Rab5Q79L; Rab5S, GFP-tagged Rab5S34N; SP-A, [D-Arg^1, D-Phe^5, D-Trp^{7,9}, Leu^{11}] substance P; Tf, transferrin; 7TM, seven transmembrane domain; YFP, yellow fluorescent protein.

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tween GPR39 and a second pre-proghrelin peptide product (termed obestatin) has not been confirmed; rather, Zn^{2+} ions activate this receptor at physiological concentrations (13). Both GhrelinR and GPR39 display constitutive activity in the absence of agonist, resulting from the spontaneous adoption of an activated 7TM receptor conformation (12, 14). Although several 7TM receptors display a low level of constitutive activation, basal GhrelinR signaling is unusually high (approximately 50% of the ghrelin response in measurements of G_q-coupled inositol phosphate (IP) accumulation; 15). Thus, GhrelinR expression on hypothalamic neuropeptide Y/AgRP neurons could significantly contribute to tonic appetite stimulation, even in the absence of ghrelin (16). Moreover, a GhrelinR missense mutation, A204E, that associates with familial short stature selectively impairs constitutive activity while preserving the response to ghrelin (17). GhrelinR inverse agonists that stabilize the inactive receptor conformation, such as [D-Arg^1, D-Phe^5, D-Trp^{7,9}, Leu^{11}] substance P (SP-A) (14, 15, 18), may have the ability to inhibit appetite by preventing agonist-independent receptor signaling and thereby have novel therapeutic potential as antiobesity agents.

In this context, it is important to understand how constitutive GhrelinR signaling, and the effects of agonists and inverse agonists, are influenced by processes that regulate receptor activity. Many 7TM receptors undergo internalization and intracellular trafficking after agonist exposure, often initiated by receptor phosphorylation and subsequent recruitment of β-arrestin proteins. β-Arrestin association desensitizes G protein-mediated signaling but also targets receptors for clathrin-mediated endocytosis. In addition, β-arrestins are signaling scaffolds that can confer spatiotemporal organization to protein kinase cascades (*e.g.* ERK1/2) (19). The sorting pathways followed by internalized 7TM receptors can lead to plasma membrane recycling or degradation (down-regulation) in lysosomes and therefore determine the lifespan of receptor proteins in response to chronic ligand stimulation (20).

We have previously demonstrated that the GhrelinR undergoes both constitutive and agonist-induced receptor internalization, in contrast to GPR39, which fails to endocytose despite similar levels of basal activity (12). Here we show that the molecular determinants of the different GhrelinR and GPR39 trafficking profiles reside within the receptor C-terminal domains and that constitutive activity drives GhrelinR endocytosis, in part by β-arrestin-independent mechanisms.

RESULTS

IP Signaling

We exchanged the C-terminal domains of the FLAG-tagged GhrelinR and GPR39 after the conserved NPXXY motif at the intracellular end of transmembrane domain VII (Fig. 1). Surface expression estimated by ELISA in transiently transfected COS-7 cells showed

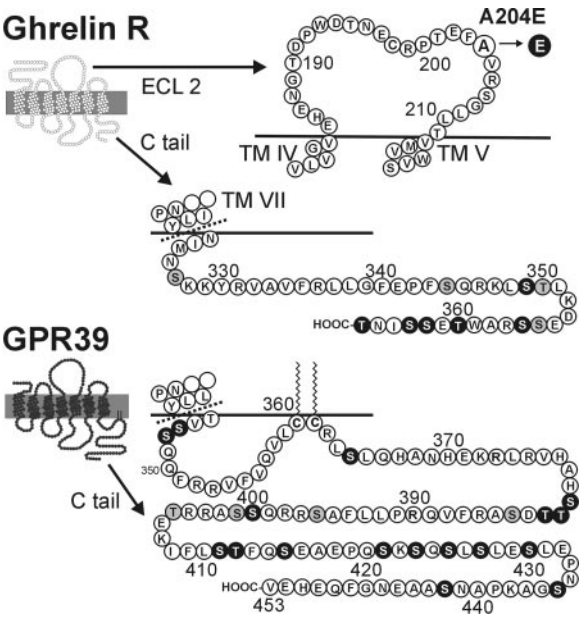

Fig. 1. GhrelinR and GPR39 Amino Acid Sequences

The illustration of the second extracellular loop (ECL 2) of the GhrelinR, between TM IV and V, indicates the position of the A204E mutation. C tail regions after the NPXXY motif of TM VII are shown for both GhrelinR and GPR39, in which potential phosphorylation sites are highlighted (*black text on gray*, protein kinase C consensus; *white text on black*, other Ser/Thr). The two putative Cys palmitoylation sites in GPR39 are also indicated. The position of the C-terminal exchange in the chimeric receptors is shown by the *dotted lines*.

higher expression of the GhrelinR swap mutant with the GPR39 C tail (GhR-39) compared with the GhrelinR, whereas the GPR39 mutant with the GhrelinR C tail (39-GhR) was detected at much lower levels than GPR39 (Fig. 2, A and B). The signaling of each construct via $G\alpha_q$-linked phospholipase C was then compared using an IP accumulation assay. GhrelinR exhibited significant constitutive activity through this pathway [42 $\pm$ 2% of maximal ghrelin-induced stimulation (n =10), as previously reported] (15), and this was elevated further in cells transfected with the GhR-39 construct (56 $\pm$ 3%, n = 10; $P <$ 0.01). IP signaling of both GhrelinR and GhR-39 was also stimulated by ghrelin (EC_{50} values of 0.30 and 0.71 nM, respectively; Fig. 2C). Expression of 39-GhR elevated basal IP accumulation to a lesser extent than GPR39 [11 $\pm$ 8% (n = 4, 39-GhR) *vs.* 27 $\pm$ 3% (n = 8, GPR39) of maximum Zn^{2+}-induced response; $P <$ 0.05]. In both cases, Zn^{2+} (13) increased IP responses further and to a similar maximal level (Zn^{2+} EC_{50} of 40 μM for GPR39 and 69 μM for 39-GhR; Fig. 2D).

GhrelinRs Are Targeted to Recycling Compartments

We examined the intracellular trafficking of native and mutant GhrelinR and GPR39 by immunofluorescence microscopy in transiently transfected HEK293 cells. In

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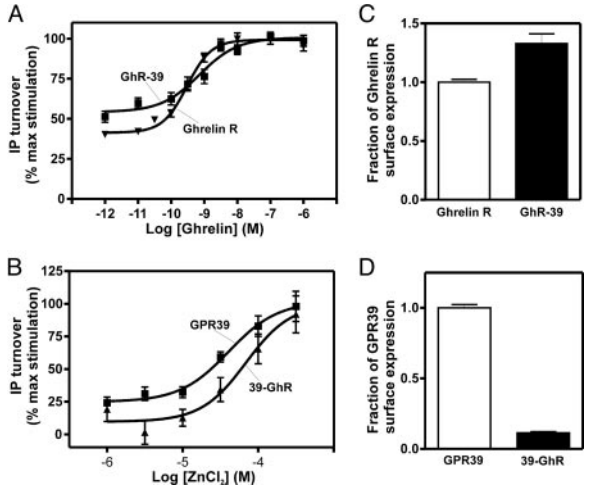

Fig. 2. IP Signaling by GhrelinR and GPR39 Chimeras

A and B, Ghrelin concentration response curves (A) for IP turnover stimulated by GhrelinR (▼) and GhR-39 (■), and Zn^{2+} stimulated IP turnover (B) for GPR39 (■) and 39-GhR (▲); C and D, cell surface expression of GhR-39 as a percentage of Ghrelin R (C), or 39-GhR relative to GPR39 (D), measured by ELISA. Experiments were performed in transiently transfected COS-7 cells, and data are mean ± SE of four to 10 independent triplicate experiments.

these and subsequent experiments (performed at least three times independently), only the plasma membrane receptor population was labeled with M2 anti-FLAG antibody (30 min at 37 C) in living cells before vehicle or ligand treatment, fixation, and detection. However, similar patterns of surface and intracellular receptor immunoreactivity were observed when M2 labeling was carried out on previously fixed cells (supplemental Fig. S1, published as supplemental data on The Endocrine Society's Journals Online web site at http://mend.endojournals.org). As illustrated in Fig. 3, GhrelinRs were constitutively internalized under control conditions, and in marked contrast, GhR-39 was located almost exclusively on the cell surface. Incubation with 100 nM ghrelin increased the proportion of intracellular GhrelinRs and also stimulated GhR-39 internalization less efficiently (Figs. 3 and 4A). However, even after 60 min agonist treatment, the amount of GhR-39 endocytosis (30.0 ± 5.7% of total M2 immunoreactivity, n = 18 cells from six experiments) was significantly lower than for GhrelinR (82.7 ± 2.5%, n = 18 cells from six experiments; $P < 0.001$). Internal GhrelinRs were colocalized with transferrin (Tf, 42–53%), a marker for clathrin-mediated endocytosis and recycling compartments, and not lysosome-associated membrane glycoprotein 1 (LAMP1)-immunoreactive late endosomes and lysosomes. When stimulated with ghrelin, GhR-39 was also targeted to Tf-positive compartments (46.9 ± 7.1% colocalization after 15 min, n = 21 cells from six experiments; Figs. 3 and 4, B and C).

GPR39 immunoreactivity was observed only at the plasma membrane whether in the absence or presence of 100 μM $ZnCl_2$. Many fewer cells transfected with 39-GhR cDNA could be live labeled with M2 antibody, but when observed, positive cells exhibited a mainly intracellular punctuate 39-GhR localization (Fig. 3). The very low intracellular levels of GPR39 and, more surprisingly, the predominantly internal labeling of 39-GhR, were typically not associated (<14%) with either Tf or LAMP1 (Figs. 3 and 4, B and C).

Constitutive Activity Regulates GhrelinR Internalization

As we have previously observed (16) the addition of a GhrelinR inverse agonist, SP-A (100 nM), markedly increased cell surface GhrelinR localization, relative to internal immunoreactivity. SP-A did not alter the plasma membrane distribution of GhR-39 (Figs. 3 and 4A). We also examined the localization of the GhrelinR (A204E) mutant, a natural substitution in the second extracellular loop (Fig. 1) that results in undetectable constitutive activity without impairment of ghrelin-stimulated responses (17). In contrast to native FLAG-tagged GhrelinRs, GhrelinR (A204E) receptors were observed predominantly at the cell surface in both unstimulated and SP-A-treated cells but rapidly internalized after ghrelin treatment (Fig. 5).

GhrelinR Trafficking through Rab5 and Rab11 Compartments

The constitutive endocytosis and recycling of Tf receptors occurs through distinct membrane domains, including their initial internalization to early endosomes, fast recycling directly to the plasma membrane, and slower transport back to the cell surface from recycling endosomes such as the perinuclear recycling compartment (PNRC). This organization depends on the successive presence of three monomeric G proteins (Rab5, Rab4, and Rab11) on this trafficking pathway, which regulate vesicle transport and fusion with specific target compartments (21). We coexpressed green fluorescent protein (GFP)-tagged Rab and FLAG-GhrelinR cDNAs in HEK293 cells to identify whether internal GhrelinRs were located in Rab5a-positive structures (predominantly early endosomes; Fig. 6) or in Rab11a compartments mediating slow recycling (such as the PNRC; Fig. 7). Expression of these tagged G proteins did not significantly alter basal or ghrelin-stimulated GhrelinR internalization, compared with cells transfected only with FLAG-GhrelinR (Figs. 6B and 7B). Equally, the redistribution of GhrelinR immunoreactivity to the cell surface after SP-A treatment was maintained, although its extent was reduced in cells coexpressing GFP-Rab5a (Fig. 6B).

In unstimulated cells, 46% of GhrelinR immunoreactivity was colocalized with GFP-Rab5a, and 61% colocalized with GFP-Rab11a, due to the overlapping nature of Rab5- and Rab11-positive intracellular compartments. Small increases in GFP-Rab5a colocalization and significant decreases in GFP-Rab11a colo-

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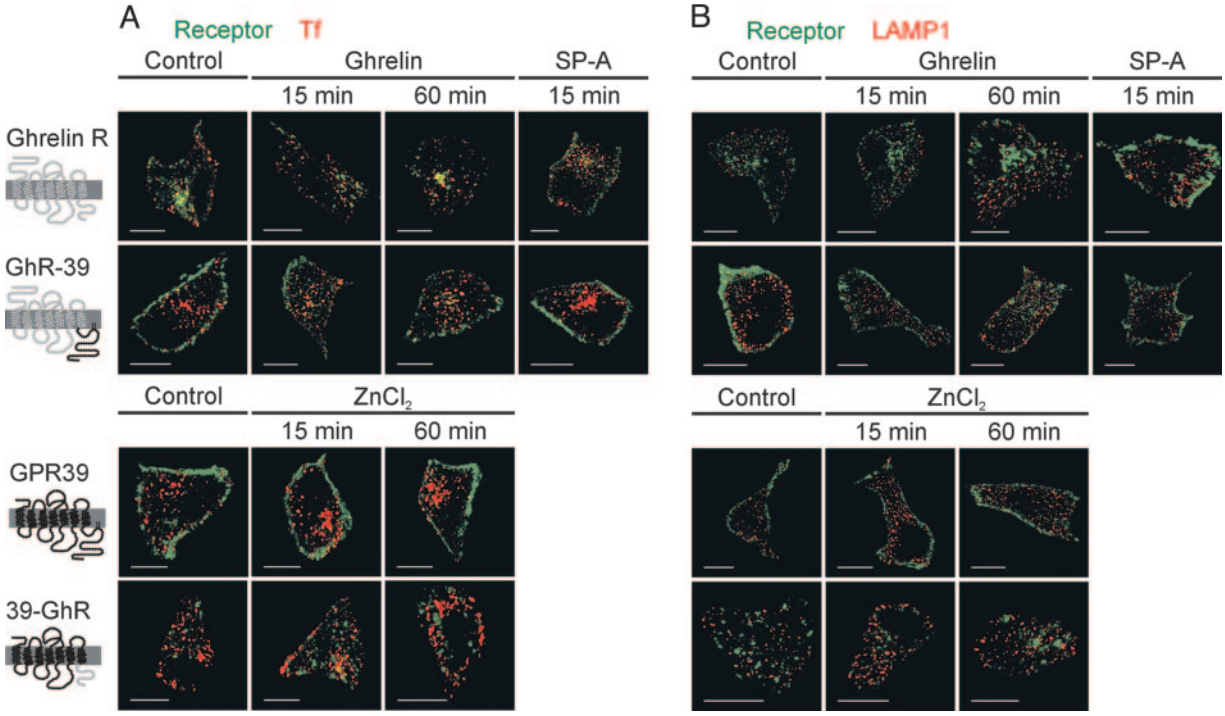

Fig. 3. GhrelinR and GPR39 Trafficking Profiles

HEK293 cells expressing GhrelinR, GPR39 or the C tail chimeras were incubated with M2 antibody for 30 min at 37 C to initially label surface receptors. They were treated with vehicle (control, 15 min), the appropriate agonist (100 nM ghrelin/100 μM $ZnCl_2$, 15 or 60 min), or 100 nM SP-A (15 min). Cells were then fixed and permeabilized, and receptor-bound M2 was detected with Alexa Fluor 488 goat antimouse secondary antibody (*green*). A, Ligand additions also included Texas Red-conjugated Tf (*red*) to highlight the clathrin-dependent endocytic and recycling pathways; B, lysosomal compartments were identified with anti-LAMP1 (*red*) in fixed cells. Example cells illustrate similar results from three experiments. A vertical stack of images (acquired on a Zeiss Axiovert 100 microscope) was deconvoluted and reconstructed in Volocity, to obtain the 3.0-μm z-sections (30 images) shown as a view from above. *Scale bar*, 10 μm.

calization were observed after ghrelin treatment (Figs. 6C and 7C). The proportion of GFP-labeled structures occupied by GhrelinRs in unstimulated cells was 36.7 $\pm$ 4.3% of total Rab5a fluorescence (n = 19 from six experiments) and 26.7 $\pm$ 3.2% of total Rab11a fluorescence (n = 22 from seven experiments).

After ghrelin stimulation, the Ghr-39 chimera also internalized to GFP-Rab5a- or GFP-Rab11a-positive compartments in cotransfected cells (supplemental Figs. S2 and S3). Expression of GFP-Rab5a did not alter the distribution of GPR39 or 39-GhR receptors, and less than 22% of the internal 39-GhR immunoreactivity was colocalized in Rab5 or Rab11 compartments (supplemental Figs. S2 and S3).

Effect of Rab5 and Rab11 Mutants

GhrelinR trafficking was significantly altered by coexpression of GFP-Rab5 variants that either constitutively bind GTP (Rab5Q79L, Rab5Q) or are permanently GDP bound (Rab5S34N, Rab5S). Rab5Q expression resulted in enlarged GFP-positive early endosomes, within which nearly all labeled GhrelinR was localized (Fig. 6). Neither ghrelin nor SP-A incubation changed this distribution. Despite the exclusive presence of GhrelinR in Rab5Q endosomes (97.2 $\pm$ 1.1%, n = 10; unstimulated cells), it was still concentrated into a subpopulation of these compartments (40.3 $\pm$ 6.6% of GFP-labeled puncta; n = 10). Conversely, both basal and agonist-induced GhrelinR endocytosis was significantly inhibited in cells cotransfected with GFP-Rab5S, with FLAG-GhrelinR immunoreactivity concentrated on or close to the plasma membrane (Fig. 6). In transiently transfected COS-7 cells, coexpression of Rab5Q, but not wild-type Rab5, slightly decreased constitutive GhrelinR IP signaling compared with mock cotransfection ($P = 0.02$), and a similar decrease in surface expression was observed. In contrast, Rab5S coexpression had no effect on basal or ghrelin-induced IP accumulation (Fig. 6D).

In cells cotransfected with the GTP-bound Rab11 mutant (Q70L and Rab11Q), the majority of GhrelinR immunoreactivity localized to the cell surface under control or SP-A-treated conditions, and clear ghrelin-stimulated internalization (100 nM, 15 min) was then observed (Fig. 7). Conversely GDP-bound Rab11S25N (Rab11S) cotransfection did not significantly alter the

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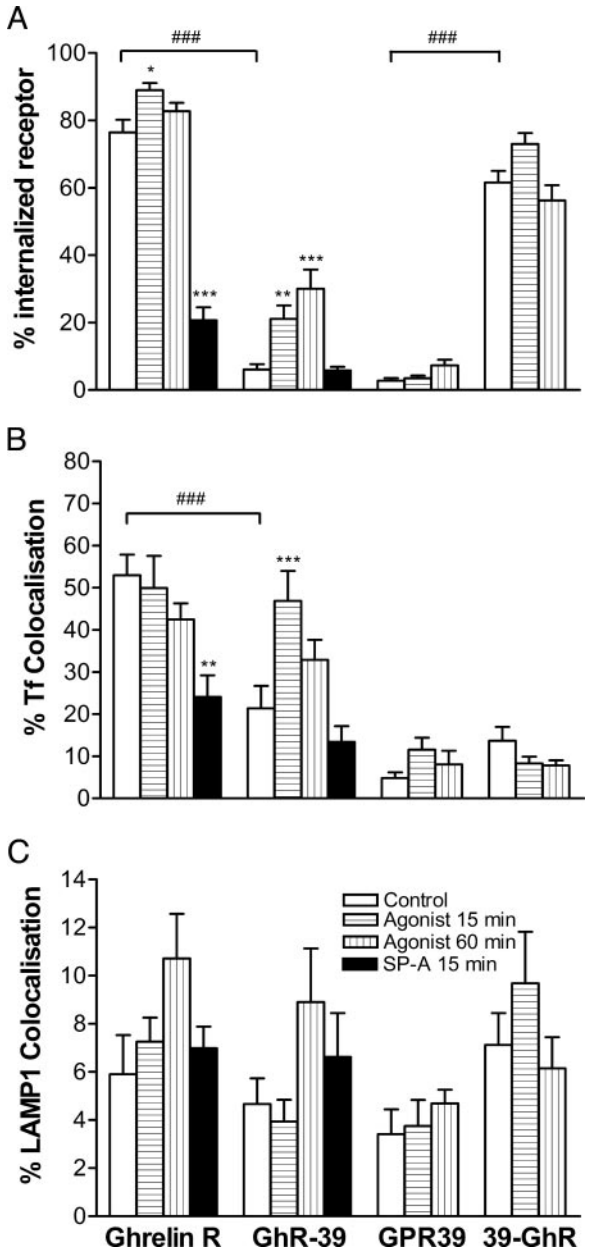

Fig. 4. Colocalization with Tf and LAMP1

Histograms present the pooled data from the experiments illustrated in Fig. 3, as the mean ± SE (at least three cells per data group in three to six experiments; n = 9–20 cells). External and internal receptor immunoreactivity was manually defined for each cell z-section and quantified with the Volocity measurements module. A, Histogram expresses the internal receptor intensity as a percentage of the total receptor immunoreactivity in the presence and absence of agonist (100 nM ghrelin for GhrelinR and GhR-39; 100 μM $ZnCl_2$ for GPR39 and 39-GhR) and SP-A (100 nM, GhrelinR and GhR-39 only); B and C, the percentage of internal receptor immunoreactivity overlapping with Tf-positive (B) or LAMP1 (C) structures is illustrated for each receptor treatment. Note the change of y-axis scale for LAMP1 colocalization. Significant differences are indicated: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ for basal *vs.* agonist or SP-A treatments at the same receptor; ###, $P < 0.001$ for GhrelinR *vs.* GhR-39, or GPR39 *vs.* 39-GhR control data groups.

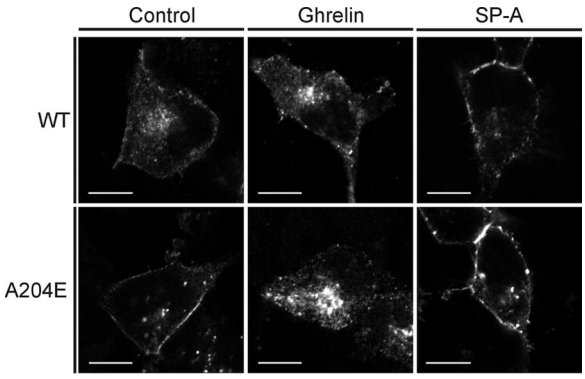

Fig. 5. Basal GhrelinR Internalization Is Prevented by the A204E Mutation

Cells transfected with FLAG-GhrelinR wild type (WT) or FLAG-GhrelinR (A204E) cDNAs were incubated with M2 antibody before 15-min treatments with vehicle (control), 100 nM ghrelin, or 100 nM SP-A. Cells were fixed and permeabilized before secondary detection with a Rhodamine Red X-conjugated secondary antibody. Representative confocal images are illustrated from three independent experiments. *Scale bars*, 10 μm.

intracellular localization of unoccupied or ghrelin-bound GhrelinRs, but this mutant completely inhibited SP-A-bound receptor redistribution to the cell surface.

Phosphorylation of GhrelinR, GPR39, and C Tail Chimeras

We measured receptor phosphorylation to assess its potential contribution to the differences between GhrelinR and GPR39 trafficking profiles. FLAG-tagged receptors were immunoprecipitated from transiently transfected HEK293 cells loaded with $^{32}P_i$, and radiolabeled proteins were detected by gel autoradiography or Western blotting using the M2 antibody (Fig. 8A). Multiple specific species were observed for GhrelinR (34–40, 58–75, and 82–114 kDa), GhR-39 (40–46, 70–91, and 92–160 kDa), and GPR39 (42–50, 60–75, and 78–115 kDa). It is likely that these bands represent the increasing glycosylation state of receptor proteins as they reach maturity and potentially the presence of receptor dimers. In contrast, only two low-molecular-mass bands were observed in immunoprecipitates from 39-GhR-transfected cells (35–38 and 40–43 kDa; data not shown). The levels of GhrelinR phosphorylation were relatively low under basal conditions but were markedly enhanced by 15 min ghrelin treatment (incorporated only into the 58- to 75-kDa band). Surprisingly high levels of basal GPR39 phosphorylation (60- to 75-kDa species) were observed, 4.5 times that for GhrelinR despite immunoprecipitation of equivalent numbers of receptors (Fig. 8). In the example presented in Fig. 8, $^{32}P_i$ incorporation was reduced by Zn^{2+} pretreatment; however, the size of this decrease varied between experiments. Similarly, transfer of the GPR39 C terminus elevated both basal and ghrelin-induced GhR-39 phosphorylation (70- to 91-kDa band) compared with wild-type GhrelinR responses.

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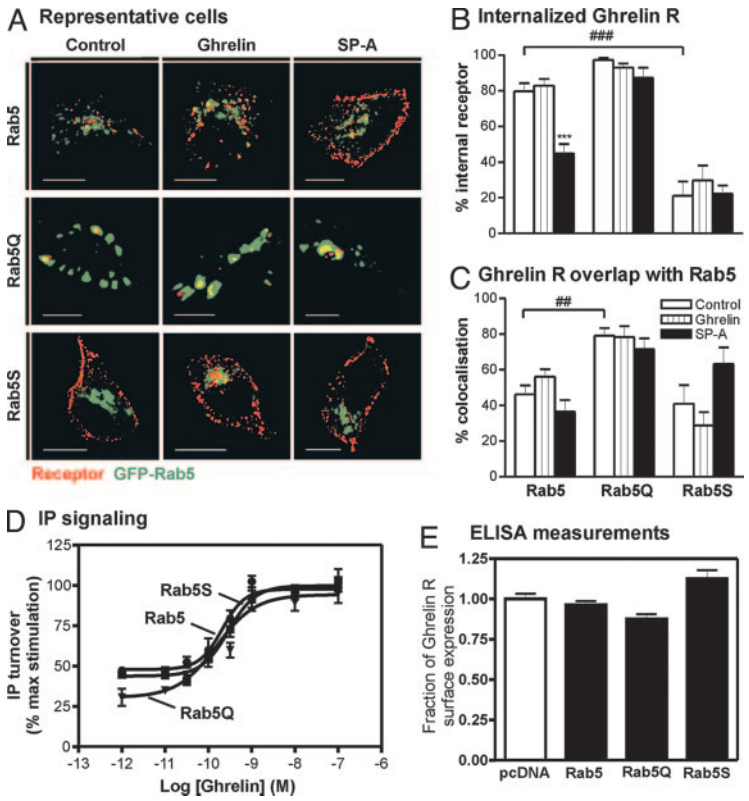

Fig. 6. Effect of Rab5 Coexpression on GhrelinR Trafficking and Signaling

Cells cotransfected with GhrelinR and GFP-Rab5a were live labeled with M2 antibody and treated for 15 min with vehicle (control), 100 nM ghrelin, or 100 nM SP-A. In each case, the distribution of GhrelinR immunoreactivity (*red*) was analyzed in cells expressing wild-type GFP-Rab G proteins or the appropriate GTP-bound (Rab5Q) or GDP-bound (Rab5S) mutants (*green*). Examples of 3.0-μm Volocity z-sections (A; single cells) are presented as a view from above. *Scale bar*, 10 μm. To the *right*, histograms show pooled Rab5, Rab5Q, and Rab5S data groups (mean ± SE, n = 9–19 cells) from three to six experiments. In B, the amount of internal receptor is expressed as a proportion of the total GhrelinR immunoreactivity, comparing the relative extent of receptor internalization after each treatment. In C, the percentage overlap between GFP and GhrelinR immunofluorescence is indicated in each case. Significant differences are indicated: ***, $P < 0.001$ for basal *vs.* agonist or SP-A treatments; ##, $P < 0.01$; ###, $P < 0.001$ comparing control data groups from wild-type and mutant Rab G protein experiments. D, Effect on basal and ghrelin-stimulated IP turnover in COS-7 cells transiently cotransfected with GhrelinR and Rab5 (■), Rab5S (●), or Rab5Q (▲). ELISA measurements (E) indicate the effect on GhrelinR surface expression in these cells of coexpression with empty vector, Rab5, Rab5S, and Rab5Q. Data are mean ± SE of three to five independent experiments performed in triplicate.

Constitutive GhrelinR Internalization Is Not Dependent on β-Arrestins

To investigate whether GhrelinR, GhR-39, and GPR39 associate with β-arrestins, we cotransfected each receptor with N-terminal GFP-tagged β-arrestin2 cDNA into HEK293 cells (Fig. 9). Despite constitutive GhrelinR internalization, GFP-β-arrestin2 recruitment to the GhrelinR was primarily dependent on stimulation by ghrelin (Fig. 9A). A transient redistribution of GFP-β-arrestin2 to intracellular compartments containing GhrelinR was observed after ghrelin treatment, which was most pronounced after 5 min stimulation. In contrast, we observed less recruitment of GFP-β-arrestin2 from the cytoplasm to GhR-39 receptor-containing compartments after ghrelin stimulation, although some colocalized puncta were identified after 5 min agonist treatment (Fig. 9B). GPR39 did not associate with GFP-β-arrestin2 under basal or Zn^{2+}-stimulated conditions (Fig. 9C). We next examined whether GhrelinR internalization persisted under conditions in which the ability of β-arrestins to act as clathrin adaptors was compromised. Coexpression of the yellow fluorescent protein (YFP)-tagged C-terminal domain of β-arrestin1 (319–418), a dominant-negative inhibitor of β-arrestin-dependent sequestration, reduced substance P-induced endocytosis of the neurokinin 1 (NK1) receptor (data not shown), as previously observed (22). However, both basal and ghrelin-stimulated GhrelinR endocytosis was maintained in the presence of β-arrestin1 (319–418)-YFP (Fig. 10A). Furthermore, constitutive GhrelinR internalization was observed in transfected mouse embryonic fibroblasts lacking both β-arrestin1 and β-arrestin2 (23) compared with plasma membrane localization of GPR39. In contrast, NK1 receptor internalization in response to substance P was abolished in these cells (Fig. 10B), as expected for the reported β-arrestin-dependent mechanism (22).

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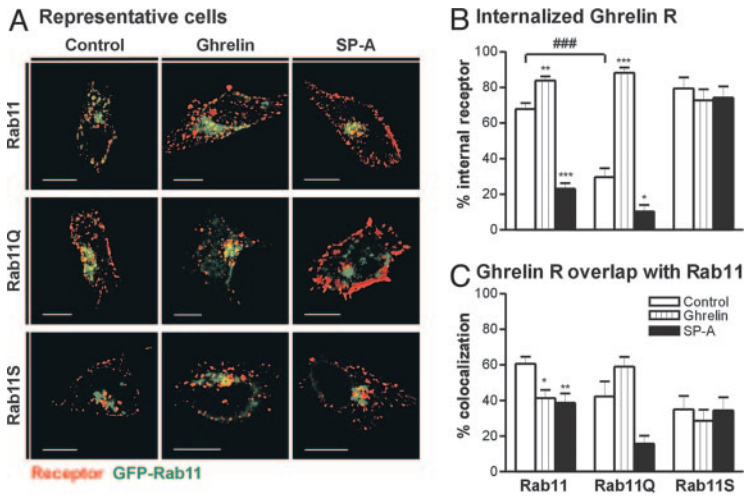

Fig. 7. GhrelinRs Are Targeted to Rab11 Compartments

Example Volocity sections (A) show FLAG-GhrelinR (*red*) and GFP-Rab11a (*green*) distribution in cotransfected cells left untreated (control) or in the presence of ghrelin or SP-A (each 100 nM, 15 min). As in Fig. 6, the effect of wild-type Rab11 expression is compared with the constitutively active (Rab11Q) and dominant-negative (Rab11S) mutant. Pooled internalization and colocalization data groups are presented in histograms B and C, respectively (mean ± 1 SE, n = 9–22 cells from three to seven experiments). Significant differences are indicated: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ for basal *vs.* agonist or SP-A treatments; #, $P < 0.05$; ###, $P < 0.001$ comparing control data groups from wild-type and mutant Rab G protein experiments.

DISCUSSION

The GhrelinR 7TM receptor family includes three members that display high agonist-independent activity (GhrelinR, GPR39, and neurotensin NT2), and both the GhrelinR and NT2 receptor are constitutively internalized as a consequence (12, 24). Importantly, this has been observed not only in heterologous expression systems but also for endogenous GhrelinRs detected by specific antibodies in both central and gastrointestinal neurons (25, 26), endothelial cells (27), and astrocytoma cells (28). Surprisingly, we found that in contrast to the GhrelinR, GPR39 did not undergo internalization, even after a mutation in TMVI that further enhanced its agonist-independent signaling (12). The purpose of this study was to probe the molecular basis for the differences in GhrelinR and GPR39 internalization and to define the mechanisms involved in ligand-independent and ligand-coupled GhrelinR trafficking.

GhrelinR Internalization Is Regulated by Its C Terminus

By exchanging the C tails on the ghrelin and GPR39 receptors, we reversed their trafficking profiles, demonstrating the critical importance of this domain. The GhR-39 receptor was located at the plasma membrane in unstimulated cells in contrast to the predominantly internal GhrelinRs. Ghrelin stimulated a low level of GhR-39 endocytosis, but the percentage of internalized receptors after agonist treatment was still significantly less than for GhrelinRs. The converse chimera (39-GhR) was not effectively expressed at the cell surface after synthesis, suggesting that its export from the endoplasmic reticulum may be prevented by changes in the proximal C tail (a known binding site for 7TM receptor chaperones) (29). However surface 39-GhRs detected by live antibody labeling did undergo constitutive internalization, unlike GPR39. The altered patterns of trafficking resulted in elevated (GhR-39) or reduced (39-GhR) cell surface expression of the chimeras compared with the parent receptors. These changes in receptor expression levels are a likely the cause of the altered chimera responses in the IP accumulation assay, including increased constitutive activity of GhR-39 receptors relative to GhrelinR and decreased G_q coupled signaling of the 39-GhR construct. Thus, the GhrelinR and GPR39 C termini are regulatory domains that define patterns of basal and agonist-induced endocytosis, findings that are supported by several previous studies of 7TM receptors (19).

GhrelinR Recycling Proceeds through Rab5 and Rab11 Domains

We showed that both unoccupied and ghrelin-stimulated GhrelinRs followed intracellular trafficking pathways leading to their cell surface recycling (Tf labeled), rather than to lysosomes and associated proteolysis (LAMP1 associated; Fig. 11). Coexpression of GFP-tagged Rab5 and Rab11 G proteins identified successive domains within the Tf-positive compartments, including early endosomes (predominantly Rab5), and recycling from the PNRC (Rab11) (21). Internal GhrelinRs extensively colocalized with Rab5 and Rab11, and their trafficking was also dependent on the

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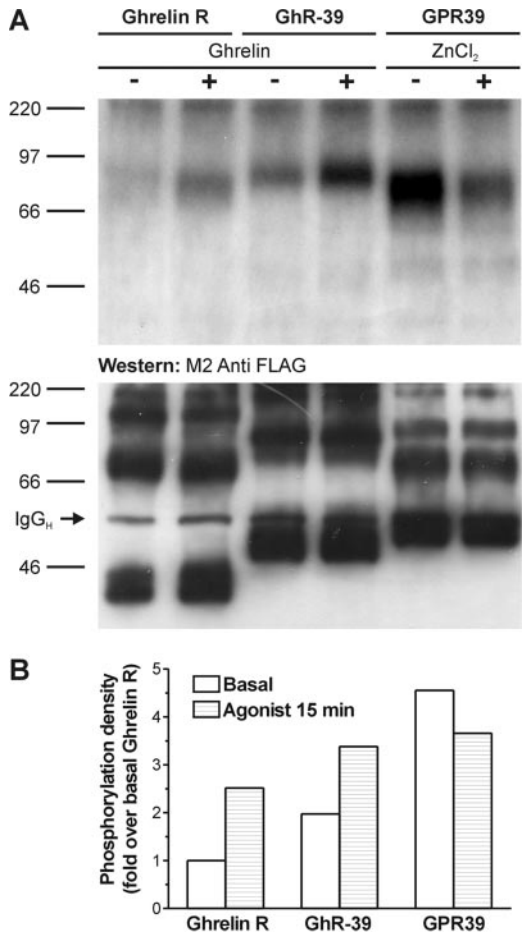

Fig. 8. GhrelinR and GPR39 Phosphorylation

HEK293 cells expressing GhrelinR, GhR-39, or GPR39 were loaded with 50 μCi $^{32}P_i$ for 1 h before treatment with vehicle or agonist (100 nM ghrelin or 100 μM $ZnCl_2$, 15 min). FLAG-tagged proteins were isolated from solubilized cell extracts using M2-agarose, and the washed immunoprecipitates were resolved on 10% SDS-PAGE gels. A representative autoradiograph (95% of immunoprecipitated sample) and M2 Western blot (5%) from a single experiment are illustrated in A, demonstrating $^{32}P_i$ incorporation in mature GhrelinR, GhR-39, and GPR39 proteins. B shows the mean density of phosphorylated bands in the presence or absence of agonist expressed relative to the basal GhrelinR response and quantified from the autoradiographs of two similar experiments.

activity of both G proteins (Fig. 11). The inhibition of GhrelinR endocytosis by the GDP-bound Rab5 mutant (S34N) has been observed for NK1, protease-activated receptor 2, β_2-adrenergic, and angiotensin II 1a receptors (30, 31). Interestingly expression of the GTP-bound Rab5Q79L concentrated live-labeled GhrelinRs into only a subpopulation of enlarged GFP-positive endosomes, consistent with the proposed heterogeneity of Rab5 domains as signaling and trafficking compartments (32, 33).

GhrelinR recycling was enhanced in cells overexpressing either Rab11 or GTP-bound Rab11Q70L, which in turn revealed clear ghrelin-stimulated receptor internalization. Thus, rates of constitutive and agonist-induced GhrelinR endocytosis and recycling will be influenced by the relative expression of Rab5 and Rab11 in different cell types. This provides one mechanism for the reported absence of constitutive GhrelinR endocytosis in CHO cells, although in this study the C-terminal GFP tag might also alter sequestration rates (34).

Perhaps surprisingly, C tail exchange did not alter the sorting of internalized GhR-39 receptors, which were also predominantly recycled after ghrelin stimulation. Most 7TM receptor sorting signals have been identified in the C terminus, but both GhrelinR and GPR39 C tails lack known consensus motifs, and *in vitro*, the GhrelinR C tail does not bind scaffold proteins associated with receptor recycling ezrin/radixin/moesin-binding phosphoprotein 50 and *N*-ethylmaleimide-sensitive factor) or degradation (sorting nexin 1 and G protein-coupled receptor-associated sorting protein) (35). Furthermore, the GhrelinR tail is shorter than the average 7TM receptor tail consisting of only 30 amino acids after helix VIII. The continued recycling of the GhR-39 chimera may be due to as yet uncharacterized signals or could occur by default in the absence of specific lysosomal targeting sequences.

Constitutive Activity Required, But Not Sufficient, for GhrelinR Endocytosis

Two lines of evidence indicated that a spontaneously active conformation of the GhrelinR initiated its internalization. First, the A204E GhrelinR mutant, in which constitutive but not agonist-stimulated signaling is abolished (17), exhibited low levels of basal endocytosis, whereas ghrelin-induced internalization was preserved. Second, the inverse agonist SP-A resulted in GhrelinR redistribution to the plasma membrane, in the same way that constitutive CB1 cannabinoid receptor trafficking is inhibited by its inverse agonist AM281 (36). SP-A effects were blocked both by Rab5Q79L and Rab11S25N, confirming that SP-A inhibits the reinternalization of spontaneously active, recycled receptors (sensitive to both Rab5 and Rab11 mutants), rather than enhancing the delivery of newly synthesized proteins (sensitive only to Rab11-dependent transport from the *trans* Golgi network to the surface; Fig. 11). For some 7TM receptors, constitutive activity can be completely dissociated from effects on basal internalization by mutagenesis (*e.g.* C5a and US28) (37, 38). In contrast, our findings demonstrate that constitutive activity is a key requirement for GhrelinR endocytosis but that it is not a sufficient stimulus. The presence of additional regulatory elements in the C-terminal domain is clearly necessary because the absence of these in GPR39 and the GhR-39 chimera prevents constitutive internalization despite agonist-independent signaling.

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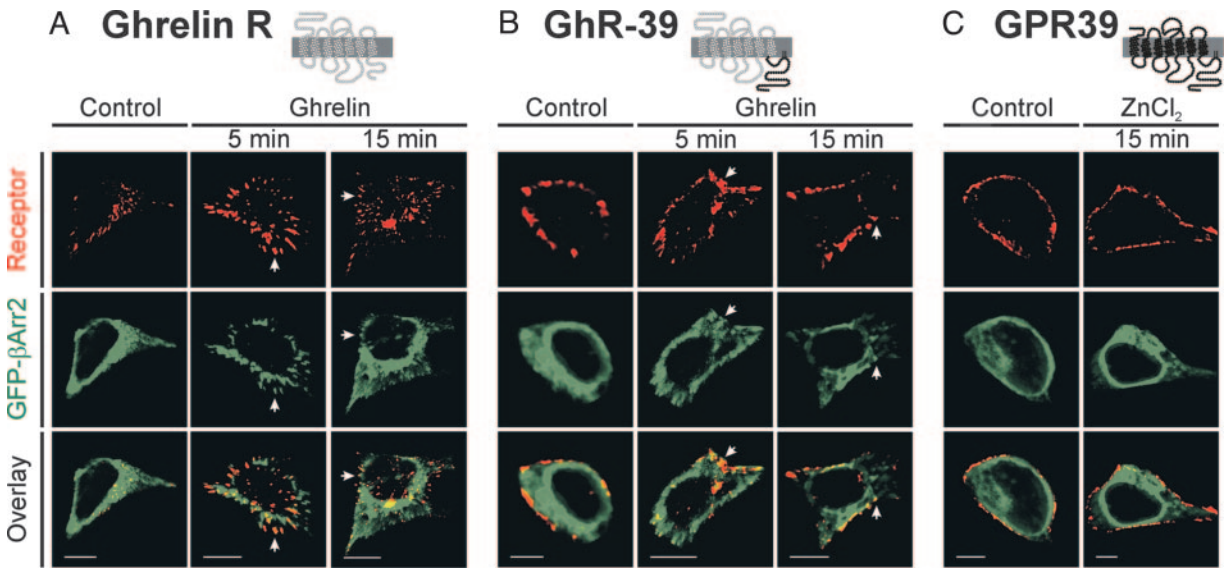

Fig. 9. Recruitment of GFP-β-Arrestin2

HEK293 cells were cotransfected with GFP-β-arrestin2 and GhrelinR (A), GhR-39 (B), or GPR39 (C) cDNAs. Twenty-four hours after transfection, M2-labeled cells were left unstimulated (control, 15 min vehicle) or treated with agonists (100 nM ghrelin or 100 μM $ZnCl_2$) for the times indicated before fixation (4% paraformaldehyde for 15 min). Receptor-bound M2 was detected with an Alexa Fluor 594-conjugated secondary antibody. Representative 3.0-μm z-sections in the nuclear plane illustrate similar results from three independent experiments. In each case, receptor (*red*) and GFP-β-arrestin2 (*green*) channels are shown together with the overlaid composite. *Arrows* indicate example sites of recruitment of GFP-β-arrestin2 by GhrelinR and GhR-39. *Scale bar*, 10 μm.

Agonist-Independent GhrelinR G_q Signaling Involves Interactions at the Plasma Membrane

Given that $G_q\alpha$ proteins are distributed both on the plasma membrane and in intracellular compartments (39), and that an endosomal 7TM receptor G protein signaling pathway has recently been identified (40), we considered whether GhrelinRs might also couple to G_q proteins while constitutively internalized. However, we observed that trapping GhrelinRs for longer in Rab5Q79L endosomes selectively inhibited constitutive GhrelinR IP signaling, in contrast to the elevated basal G_q signaling of the GhR-39 chimera located only at the cell surface. Thus, although GhrelinRs continually cycle between the surface and endosomes, the changes in constitutive IP accumulation when this trafficking is perturbed indicate that GhrelinR G_q signaling occurs predominantly at the plasma membrane, consistent with the cell surface recruitment of $G_q\alpha$ after 7TM receptor stimulation (39).

β-Arrestin-Independent Mechanisms Contribute to GhrelinR Internalization

β-Arrestin isoforms are the most widely recognized adaptor proteins that target 7TM receptors for clathrin-mediated endocytosis (19), but only ghrelin stimulation was able to strongly increase GhrelinR phosphorylation and GFP-β-arrestin2 endosomal recruitment. Moreover, constitutive GhrelinR endocytosis persisted in the presence of a dominant-negative β-arrestin construct that competes for clathrin interaction and in cells that lack both β-arrestin1 and -2 isoforms. Thus, the GhrelinR joins a select number of 7TM receptors that are capable of arrestin-independent internalization, perhaps through dileucine- or tyrosine-based motifs in the GhrelinR C tail (YXXXV; Fig. 1) acting as binding sites for a second clathrin adaptor AP-2 (41–44). A key requirement of this mechanism would be that AP-2 interaction requires an active GhrelinR conformation, as observed for the α1b-adrenoceptor (41). Agonist-stimulated β-arrestin recruitment is thus at least partly redundant for initiating GhrelinR internalization, but it may have more significance for ERK1/2 signaling mediated through β-arrestin interaction (19). The absence of basal β-arrestin2 binding is a plausible explanation for the selective lack of GhrelinR constitutive activity through this protein kinase cascade (12).

GPR39 Phosphorylation Is Not Sufficient for β-Arrestin2 Recruitment

We were surprised to find that GPR39 was heavily phosphorylated under basal conditions, especially because this phosphorylation did not result in β-arrestin2 recruitment or internalization. Equally, transfer of the GPR39 C terminus to the GhrelinR also enhanced constitutive and ghrelin-induced phosphorylation but inhibited GFP-β-arrestin2 interaction. One origin of the differences in phosphorylation lies in the differential trafficking of the receptors, which influences the location from which they are immunoprecipitated. Thus, GhrelinRs would be isolated principally from endosomal compartments, sites of 7TM receptor dephosphorylation (31), whereas immunoprecipitated GPR39 and GhR-39 proteins would originate

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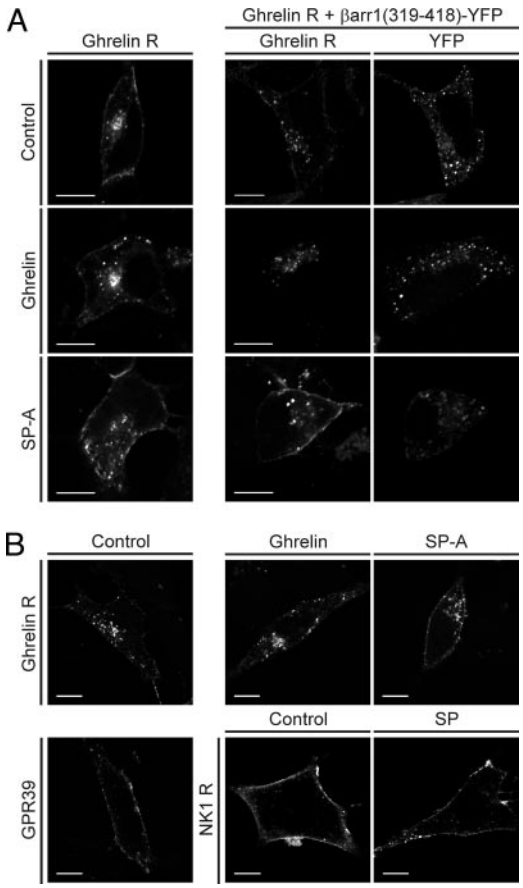

Fig. 10. Constitutive GhrelinR Internalization Occurs Independently of β-Arrestins

HEK293 cells (A) or embryonic fibroblasts from β-arrestin1/2 double-knockout mice (B) were transiently transfected with FLAG-tagged receptor cDNAs 24 h before live labeling with M2 and detection with a Rhodamine Red X-conjugated secondary antibody. In A, representative confocal images compare the distribution of GhrelinR immunoreactivity in cells transfected with the receptor cDNA alone (*left panel*) or with cotransfection of a β-arrestin1 (319–418)-YFP construct (*two right panels*), confirming presence of both FLAG immunoreactivity and YFP fluorescence in the same cell. In B, β-arrestin1/2 knockout fibroblasts expressing FLAG GhrelinR (representative of four experiments) are compared with those transfected with FLAG-GPR39 (n = 3) or the FLAG-NK1 receptor with or without its agonist substance P (SP, n = 2). Ligand treatments (100 nM ghrelin, 100 nM SP-A, and 10 nM substance P) were for 15 min in each case. *Scale bar*, 10 μm.

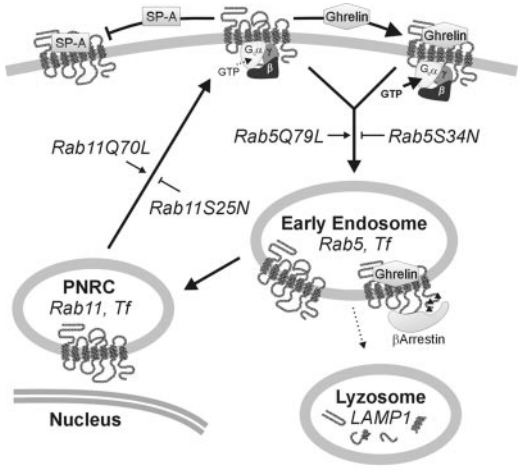

Fig. 11. Internalization and Recycling of the GhrelinR

GhrelinRs internalize under basal or agonist-stimulated conditions and recycle to the surface via Rab5 domains (including early endosomes) and Rab11 compartments (including the PNRC) that are also identified by Tf. Little colocalization exists with LAMP1-positive late endosomes and lysosomes. Individual stages of GhrelinR trafficking can be enhanced by GTP-bound (Q to L) or inhibited by GDP-bound (S to N) Rab mutants as shown. Only ghrelin-stimulated GhrelinRs show significant phosphorylation and endosomal β-arrestin recruitment. By inhibiting constitutive activity, the inverse agonist SP-A inhibits basal GhrelinR internalization and receptors accumulate at the plasma membrane.

from the plasma membrane where kinase activity predominates. However our results also demonstrate that it is the context of particular phosphorylated residues (*e.g.* a Ser/Thr cluster) (45) that is critical for β-arrestin recruitment and not phosphorylation *per se*. Indeed, it is the absence of such a specific motif in GPR39 that limits β-arrestin interaction, because GPR39 can efficiently bind a phosphorylation-independent β-arrestin2 containing a mutated anion sensor (13).

In summary, we have shown that constitutive GhrelinR internalization is regulated both by its agonist-independent activity and C-terminal domain and involves a contribution from β-arrestin-independent mechanisms. GhrelinR endocytosis is inhibited by inverse agonists, or a naturally occurring mutation, which prevent constitutive activity. Future studies will address the importance of these findings for the new roles of constitutive 7TM receptor trafficking in providing a quiescent intracellular pool of receptors protected from desensitization and in aiding their delivery to the appropriate domains of polarized cells such as neurons (25, 46).

MATERIALS AND METHODS

Materials

cDNAs encoding N-terminal GFP-tagged human Rab5a and Rab11a fusion proteins (in vectors pEGFP-C2 or -C3; Clontech, Palo Alto, CA) were kindly provided by Prof. M. Zerial (Max Planck Institute of Molecular Cell Biology and Genetics, Dresden, Germany). N-terminal GFP^2-tagged human β-arrestin2 cDNA was provided by Lene Martini (47) and β-arrestin1 (319–418) by Rasmus Jørgenson (both 7TM; Pharma, Copenhagen, Denmark). The β-arrestin1/2 double-knockout mouse embryonic fibroblasts (23) were a kind gift of Prof. R. J. Lefkowitz (Duke University, Durham, NC). The following antibodies and fluorophores were purchased from commercial sources: M2 anti-FLAG mouse monoclonal IgG (stored in single-use aqueous aliquots at −20 C) and M2 agarose conjugate (Sigma-Aldrich, Poole, UK), anti-LAMP1 rabbit polyclonal IgG (Santa Cruz Biotechnology, Santa Cruz, CA), Alexa Fluor 488 and 594 secondary antibodies and Texas Red-Tf

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(Invitrogen, Paisley, UK), and horseradish peroxidase (HRP)-conjugated goat antimouse IgG (GE Biosciences, Little Chalfont, UK). The following cell culture reagents were also purchased: DMEM (GIBCO Invitrogen, Paisley, UK), fetal bovine serum of Australian origin (Invitrogen), trypsin (Lorne Laboratories, Reading, UK), and antibiotics (MP Biomedicals, Oxford, UK). Ghrelin and SP-A were purchased from Bachem (Bubendorf, Switzerland), and stock solutions were frozen in small aliquots at −20 C. Other reagents were from Sigma-Aldrich or VWR International (Poole, UK).

Receptor Mutagenesis

These studies used GhrelinR and GPR39 cDNAs cloned into pTag2 (Stratagene, La Jolla, CA) as previously described (12) in which each receptor was tagged at the N terminus with the FLAG epitope (DYKDDDDK). C termini were exchanged by PCR using the overlap expression method. The PCR primers were designed to make the exchange just after the conserved Tyr in NPxxY motif. The PCR products were digested with appropriate restriction endonucleases, purified, and cloned into pcDNA3. All PCR experiments were performed using *pfu* polymerase (Stratagene) according to the instructions of the manufacturer. All mutations were verified by restriction endonuclease mapping and subsequent DNA sequence analysis using an ABI 310 automated sequencer.

Cell Culture and Transfection

Cells were maintained in DMEM containing supplements for HEK293 or embryonic fibroblasts (10% fetal bovine serum, 100 μg/ml kanamycin, and 1.2 μg/ml amphotericin B) or COS-7 (10% fetal bovine serum, 2 mM glutamine, 0.01 mg/ml gentamicin). They were incubated at 37 C in a humidified atmosphere of 95% O_2/5% CO_2 and passaged when confluent by trypsinization (0.25% wt/vol in versene). HEK293 and fibroblast transfections were then performed using Lipofectamine 2000 (Invitrogen) in the absence of antibiotics and in accordance with the manufacturer's instructions. COS-7 cells were transiently transfected by calcium phosphate coprecipitation with chloroquine addition.

Phosphatidylinositol Turnover

One day after transfection, COS-7 cells were incubated for 24 h with 5 μCi [^{3}H]myoinositol (GE Biosciences) in 1 ml supplemented DMEM. Cells were washed twice in buffer [20 mM HEPES (pH 7.4), supplemented with 140 mM NaCl, 5 mM KCl, 1 mM $MgSO_4$, 1 mM $CaCl_2$, 10 mM glucose, and 0.05% (wt/vol) fetal bovine serum] and then incubated in 0.5 ml buffer including 10 mM LiCl at 37 C for 30 min. After agonist stimulation for 45 min at 37 C, cells were extracted with 10 mM formic acid followed by incubation on ice for 30 min. The resulting supernatant was purified on Bio-Rad (Hercules, CA) AG 1-X8 anion-exchange resin to isolate the negatively charged IPs. Determinations were made in duplicate.

Cell Surface Expression (ELISA) Measurement

Cells were transfected and seeded in parallel with those used for IP accumulation assay. After two washes, cells were fixed and incubated in blocking solution (PBS, 0.2% BSA) for 30 min at room temperature. Cells were kept at room temperature for all subsequent steps. Cells were incubated with M2 antibody in 1:300 dilution and after three washes were incubated with antimouse HRP-conjugated (Amersham Biosciences, Piscataway, NJ) antibody in a 1:4000 dilution. After extensive washing, the immunoreactivity was revealed by the addition of HRP substrate according to the manufacturer's instructions.

Immunofluorescence Microscopy

HEK293 cells or mouse embryonic fibroblasts were grown to 30–50% confluence on poly-L-lysine-coated 13-mm diameter glass coverslips and transiently transfected with receptor cDNAs alone or in combination with GFP-tagged Rab or β-arrestin cDNAs. After 24–48 h, the medium was exchanged for serum-free DMEM including 1% BSA (1 h at 37 C), and the cells were then incubated for 30 min at 37C in DMEM/1% BSA including M2 anti-FLAG antibody (2.5 μg/ml, 30 μl per coverslip). Ligand treatments (in 10 μl medium) were applied for the time and concentrations indicated, including Tf-Texas Red (1:250 dilution) for the final 15 min in some experiments. Cells were then washed in PBS, fixed for 15 min at 21 C in paraformaldehyde in PBS (3%, or 4% for β-arrestin translocation measurements), and permeabilized with 0.075% Triton X-100 for 5 min at 21 C. In assays that localized lysosomes, samples were subsequently incubated with PBS/1% BSA (1 h), followed by the same medium including anti-LAMP1 (1:500 dilution, 90 min). M2 and LAMP1 binding were detected by an appropriate secondary antibody (1:1000 dilution) conjugated to Alexa Fluor 488, Rhodamine Red X, or Alexa Fluor 594. Cells were postfixed (3% paraformaldehyde), and nuclear DNA was stained with 4′,6-diamidino-2-phenylindole before coverslips were mounted in Mowiol 4-88 (Calbiochem, Nottingham, UK).

Confocal images (Figs. 5 and 10) were obtained on a Zeiss LSM 510M laser scanning microscope with a Plan-Apochromat ×63 NA 1.4 oil objective, using a HeNe 543-nm laser and LP 560-nm filter to excite and collect fluorescence from the Rhodamine Red X secondary antibody. For quantitation, wide-field cell images were captured digitally on a Zeiss Axiovert 100 microscope (×63 oil objective from Carl Zeiss GmbH, Jena, Germany; excitation and emission filter sets from Omega Optical, Brattleboro, VT) using Openlab 4.0 (Improvision, Coventry, UK) with a piezo focus drive to obtain 50–80 image stacks in 0.1-μm z-axis steps. Out-of-focus light was removed by deconvolution in Volocity 3.5 (Improvision), applying a theoretical point spread function for each wavelength. The central 30 deconvoluted images, through the nuclear plane, were reconstructed to produce the 3.0-μm z-section used for further analysis. The internal receptor immunoreactivity (expressed as a percentage of the total), and the percentage internal receptor intensity colocalized with each marker, were calculated with the Volocity measurements module, manually subdividing plasma membrane and cytoplasmic immunoreactivity. At least three cells were analyzed for each experiment, and the pooled data were then compared by one-way ANOVA with Student-Newman-Keuls *post hoc* test (Prism 3.0; GraphPad, San Diego, CA). Statistical significance was indicated by $P < 0.05$.

Receptor Phosphorylation

HEK293 cells were transfected with FLAG-tagged receptor cDNAs in six-well plates and were loaded 24 h later (at 70–80% confluence) with 50 μCi $H_3{}^{32}PO_4$ (GE Biosciences; 10 mCi/ml stock) in phosphate-free Krebs buffer (50 min at 37 C). They were then incubated for 15 min with vehicle (10 μl buffer), ghrelin (100 nM), or $ZnCl_2$ (100 μM) as appropriate. Receptors were solubilized in RIPA buffer containing phosphatase inhibitors (50 mM Tris, 100 mM NaCl, 10 mM NaF, 10 mM $Na_4P_2O_7$, 5 mM EDTA, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS, 0.1 mM phenylmethylsulfonyl fluoride, 200 μM activated Na_3VO_4, and 10 μg/ml leupeptin and aprotinin, pH 8.0), rotating the samples end over end for 90 min at 4 C. Extracts were clarified by centrifugation and equalized for protein content (BCA protein assay; Pierce, Cheshire, UK) before overnight immunoprecipitation at 4 C with directly conjugated M2 agarose. Immunoprecipitates were washed twice with RIPA and once each with wash buffers [50 mM Tris, 5 mM EDTA, 0.1% Nonidet P-40, and

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0.05% sodium deoxycholate (pH 8.0), at 4 C] successively containing 500 mM NaCl and then no salt. Samples eluted in Laemmli buffer were resolved by SDS-PAGE (10% Bio-Rad Tris-HCl ready gels), and the dried gel was exposed to preflashed GE Hyperfilm MP for 24–48 h at −70 C to detect ^{32}P-labeled proteins. Five percent of each sample was resolved on a separate gel and transferred to polyvinyl difluoride membrane. Equivalent receptor loading was then verified by Western blots with M2 anti-FLAG (50 ng/ml, overnight at 4C), detected by a secondary goat antimouse HRP conjugate and enhanced chemiluminescence (GE Biosciences). The relative density of bands on scanned Western blots or autoradiographs was determined by Scion Image (version 4.0; Scion Corp., Frederick, MD).

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