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Differential PI 3-kinase dependence of early and late phases of recycling of the internalized AT_1 angiotensin receptor

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Agonist-induced endocytosis and processing of the G protein–coupled AT_1 angiotensin II (Ang II) receptor (AT_1R) was studied in HEK 293 cells expressing green fluorescent protein (GFP)– or hemagglutinin epitope–tagged forms of the receptor. After stimulation with Ang II, the receptor and its ligand colocalized with Rab5–GFP and Rab4–GFP in early endosomes, and subsequently with Rab11–GFP in pericentriolar recycling endosomes. Inhibition of phosphatidylinositol (PI) 3-kinase by wortmannin (WT) or LY294002 caused the formation of large endosomal vesicles of heterogeneous Rab composition, containing the ligand–receptor complex in their limiting membranes and in small associated vesicular structures. In contrast to Alexa®–transferrin, which was mainly found in small vesicles associated with the outside of large vesicles in WT-treated cells, rhodamine–Ang II was also segregated into small internal vesicles. In cells labeled with ^{125}I-Ang II, WT treatment did not impair the rate of receptor endocytosis, but significantly reduced the initial phase of receptor recycling without affecting its slow component. Similarly, WT inhibited the early, but not the slow, component of the recovery of AT_1R at the cell surface after termination of Ang II stimulation. These data indicate that internalized AT_1 receptors are processed via vesicles that resemble multivesicular bodies, and recycle to the cell surface by a rapid PI 3-kinase–dependent recycling route, as well as by a slower pathway that is less sensitive to PI 3-kinase inhibitors.

Introduction

The most important physiological actions of the octapeptide hormone angiotensin II (Ang II)* are mediated by the type 1 Ang II receptor (AT_1R). The AT_1R is a member of the superfamily of G protein–coupled receptors (GPCRs), and promotes inositol phosphate and calcium signaling via $G_{q/11}$ (De Gasparo et al., 2000). Ang II also causes rapid phosphorylation, desensitization, and endocytosis of the AT_1R, with subsequent intracellular processing and recycling to the cell membrane (Thomas, 1999; Hunyady et al., 2000; Ferguson, 2001). Previous studies have suggested that the fates of the internalized hormone and the receptor differ in that the ligand accumulates in lysosomes while the receptor recycles to the cell surface (Hein et al., 1997).

Agonist-induced endocytosis of most GPCRs occurs primarily via clathrin-coated vesicles (Ferguson, 2001). Previous studies suggested that internalization of the AT_1R differs from that of β-receptors in being dynamin- and β-arrestin–independent (Zhang et al., 1996), but at physiological hormone concentrations, endocytosis of the AT_1R occurs predominantly via a β-arrestin– and dynamin-dependent mechanism (Anborgh et al., 2000; Gáborik et al., 2001; Kohout et al., 2001; Qian et al., 2001). The functions of β-arrestins, β2-adaptin, and dynamin have all been found to involve binding to inositol lipids or inositol phosphates (Gaidarov et al., 1999), and phosphoinositides have been implicated at several steps in the endocytotic process (Achiriloaie et al., 1999; Bottomley et al., 1999; Gaidarov and Keen, 1999). Studies with muscarinic and β-adrenergic receptors indicated that phosphorylation of lipids by phosphatidylinositol (PI) 4-kinase(s) is required for the internal-

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*Abbreviations used in this paper: Ang II, angiotensin II; AT_1R, AT_1 Ang II receptor; EEA1, early endosomal antigen 1; EGF, epidermal growth factor; GPCR, G protein–coupled receptor; GFP, green fluorescent protein; HA, hemagglutinin; PI, phosphatidylinositol; PI(3)P, PI 3-phosphate; Rhod, rhodamine; TfR, transferrin receptor; WT, wortmannin.

Key words: angiotensin II receptor recycling; endosomal sorting; G protein–coupled receptor; PI 3-kinase; Rab proteins

ization of GPCRs (Sorensen et al., 1998, 1999). However, recent studies have suggested that GPCR kinase–mediated translocation of a PI 3-kinase to membranes may mediate this effect (Naga Prasad et al., 2001).

Although considerable progress has been made in defining the mechanisms involved in intracellular processing of internalized nutrient and growth factor receptors (Mellman, 1996), less information is available about the trafficking and fate of internalized GPCRs. Studies on numerous GPCRs have shown that most internalized receptors recycle to the cell surface and utilize a variety of trafficking pathways during this process. For example, the β_2-adrenergic receptor undergoes relatively rapid recycling to the surface via a route that requires a conformational change induced by acidification in the endosomes and subsequent dephosphorylation (Lefkowitz, 1998). Recent studies have demonstrated that GPCRs are also found in perinuclear recycling endosomes during their intracellular trafficking steps (Innamorati et al., 2001; Kreuzer et al., 2001; Schmidlin et al., 2001; Signoret et al., 2000).

Studies on the yeast mating factor receptor suggest that degradation of the internalized receptor via ubiquitination involves sorting into a lysosomal compartment that is also a part of the vacuolar sorting pathway from the Golgi apparatus (Katzmann et al., 2001; Simonsen et al., 2001). Both the sorting of molecules into this compartment and their exit therefrom depend on the function of Vps34p, the sole PI 3-kinase of yeast (Schu et al., 1993; Simonsen et al., 2001). The formation of PI 3-phosphate (PI[3]P) by Vps34p is required for membrane targeting of the early endosomal autoantigen 1 (EEA1), which promotes Rab5-mediated homotypic fusion of early endosomes (Simonsen et al., 2001). Moreover, further phosphorylation of PI(3)P by a 5-kinase, Fab1p, is necessary for the formation and processing of multivesicular bodies (MVBs) (Odorizzi et al., 1998). The functions of inositide kinases and phosphoinositides are also required for the endosomal processing of internalized growth factor and transferrin receptors (TfRs) (Corvera and Czech, 1998; Simonsen et al., 2001). However, little is known about the specific mechanisms by which phosphoinositides determine the endosomal processing of GPCRs in mammalian cells.

The aim of the present study was to characterize the intracellular compartments through which the internalized AT_1R is processed, and to investigate the role of PI 3-kinases in the pathway(s) involved in AT_1R trafficking. Our results indicate that PI 3-kinase inhibitors selectively impair a step in the processing of internalized AT_1R by which the receptor rapidly recycles to the membrane from vesicles that resemble MVBs. We also demonstrate the presence of a slower process that occurs via recycling endosomes and is still operative in the presence of PI 3-kinase inhibitors. Based on these findings, we propose a model of the postendocytic trafficking of the AT_1R that involves at least two major pathways of GPCR recycling.

Results

Properties of the AT_1R–GFP chimera

Green fluorescent protein (GFP) fusion proteins have been used to study the intracellular trafficking of several GPCRs,

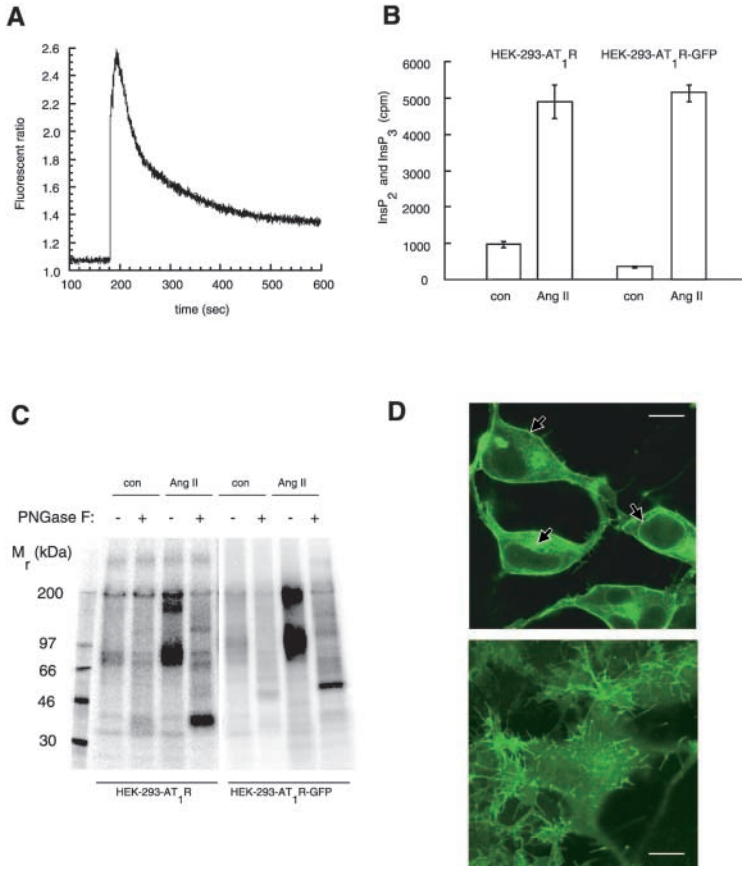

Figure 1. **Functional properties of the GFP-tagged AT_1R expressed in HEK 293 cells.** (A) Cytoplasmic Ca^{2+} signal after addition of Ang II to Fura-2–loaded cells. (B) Inositol phosphate production (mean ± SEM; $n = 3$) of cells expressing AT_1R–GFP or a FLAG-tagged AT_1R in response to Ang II (100 nM). (C) Ang II-induced phosphorylation of wild-type and GFP-tagged AT_1R, and their mobility change after deglycosylation by peptide *N*-glycosidase F. (D) Distribution of GFP-tagged AT_1R stably transfected in HEK 293 cells. Confocal images are shown either through a cross section (top) or on the surface (bottom) of the cells. In addition to the plasma membrane, there is a clear signal in intracellular membranes, most notably in the nuclear envelope and the pericentriolar compartment. Bars, 10 μm.

including the AT_1R (Kallal and Benovic, 2000; Chen et al., 2001; Miserey-Lenkei et al., 2001). In the present study, HEK 293 cells were stably transfected with a fusion protein consisting of the AT_1R and GFP. Ang II stimulation evoked a characteristic cytoplasmic $[Ca^{2+}]$ signal and an inositol phosphate response similar to those induced by the wild-type receptor (Fig. 1, A and B), indicating the functional integrity of the tagged receptor. Because phosphorylation of the cytoplasmic tail is a major determinant of the intracellular trafficking of the AT_1R and other GPCRs, the extent to which fusion of a GFP tag to the COOH terminus of the receptor affected its phosphorylation was also investigated. As shown in Fig. 1 C, Ang II caused prominent phosphorylation of the wild-type AT_1R receptor expressed in HEK cells, typically with several diffuse bands over the 70–200 kD mol wt range that reflects the extensive glycosylation of the receptor (Smith et al., 1998). Accordingly, after deglycosylation with peptide *N*-glycosidase F, the receptor was detected as a more compact 40-kD band (Fig. 1 C). Phosphorylation of the AT_1R–GFP fusion protein showed a similar pattern (Fig. 1 C), except that the molecular weights of the intact and deglycosylated AT_1R–GFP receptors were higher due to the addition of GFP.

On confocal microscopy, the green AT_1R was found predominantly in the plasma membrane in resting HEK 293 cells. However, intracellular localization of the fusion protein was also detectable, especially in cells showing higher levels of receptor expression (Fig. 1 D). In many cells, the receptor was present in the nuclear envelope, but intranuclear localization of the receptor was never observed (Fig. 1 D, top). Receptor expression was also very prominent in cell surface extensions (Fig. 1 D, bottom).

Colocalization of fluorescent agonist with the AT_1R during endocytosis

To follow the fate of the receptor and its ligand simultaneously in live cells, rhodamine-labeled fluorescent Ang II (Rhod–Ang II) was used to stimulate cells expressing the AT_1R–GFP chimera. Rhod–Ang II was rapidly bound to cell surface receptors (Fig. 2 A) and caused clustering of the receptors on the plasma membrane within a few minutes (Fig. 2 B). During the subsequent internalization of the hormone–receptor complex, both ligand and receptor were detectable in punctate intracellular structures (Fig. 2 C). Progressive internalization of the receptor, and its colocalization with the ligand, were evident for up to 30 min (Fig. 2 D). At this time, the receptor and its ligand began to appear in deeper compartments adjacent to the nucleus, in addition to the more peripheral vesicles (Fig. 2, D and E). At these later times, Rhod–Ang II was also detectable in small punctate structures that did not contain the receptor (Fig. 2, D and E). Most cells showed extensive accumulation of the AT_1R–GFP chimera in colocalization with its fluorescent ligand in the juxtanuclear compartment after 1 h of incubation with Rhod–Ang II (Fig. 2 E).

Compartments involved in the endosomal processing of the AT_1R–GFP chimera

Fluorescent markers were used to characterize the intracellular compartments that contained Ang II and the AT_1R during their individual trafficking steps. Early endosomes were identified by the use of a GFP-tagged FYVE domain (FYVE–GFP) of the EEA1, and GFP-tagged Rab proteins were used to identify individual recycling compartments. The FYVE domain construct used in the present study (amino acids 1252–1411) contains part of the adjacent Rab5 binding domain of the EEA1 protein and thereby labels early endosomes, due to its simultaneous binding of PI(3)P and Rab5 in this compartment (Gillooly et al., 2000). The GFP constructs were transiently expressed in HEK 293 cells stably transfected with either wild-type or HA-tagged AT_1R. Soon after the addition of Rhod–Ang II, a significant proportion of the internalized ligand showed colocalization with the FYVE–GFP construct in early endosomes (Fig. 3 A, top). No increase was observed in the number of FYVE–GFP-positive vesicles after stimulation with Ang II. Treatment with wortmannin (WT) caused rapid dissociation of the GFP-tagged FYVE domain from its punc-

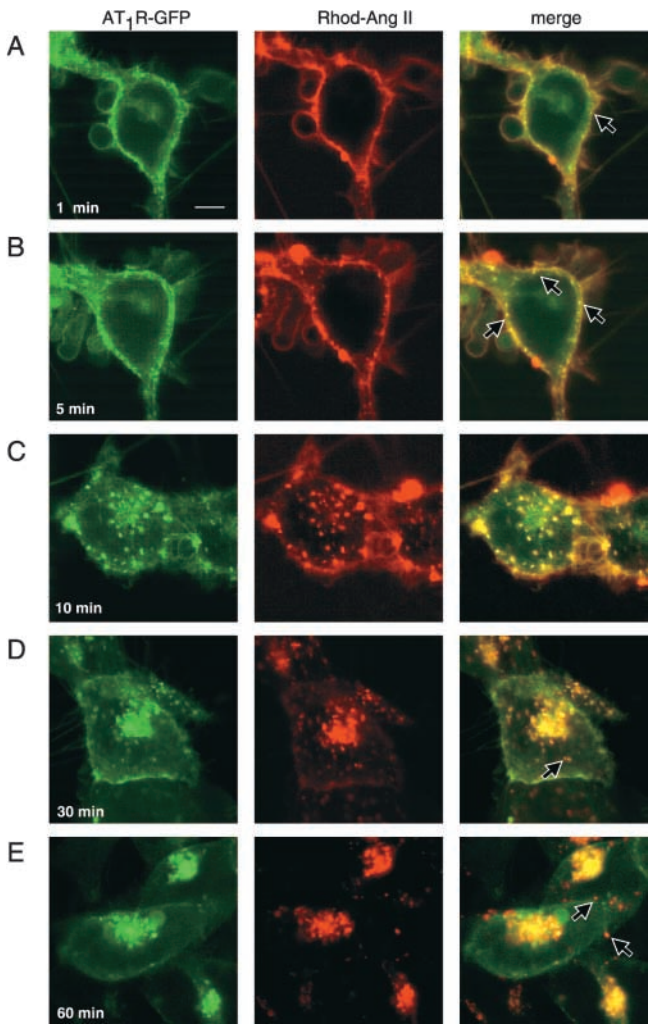

Figure 2. **Internalization of Rhod–Ang II in HEK 293 cells stably expressing the AT_1R–GFP.** Confocal images show the distribution of receptors (green) and the ligand (red) at selected times after stimulation with Rhod–Ang II (50–100 nM) at 37°C. Note the colocalization of receptors and ligand at early times of stimulation (A and B, arrows) and the subsequent appearance of vesicles containing only the ligand after prolonged incubation (D and E, arrows). Bar, 10 μm.

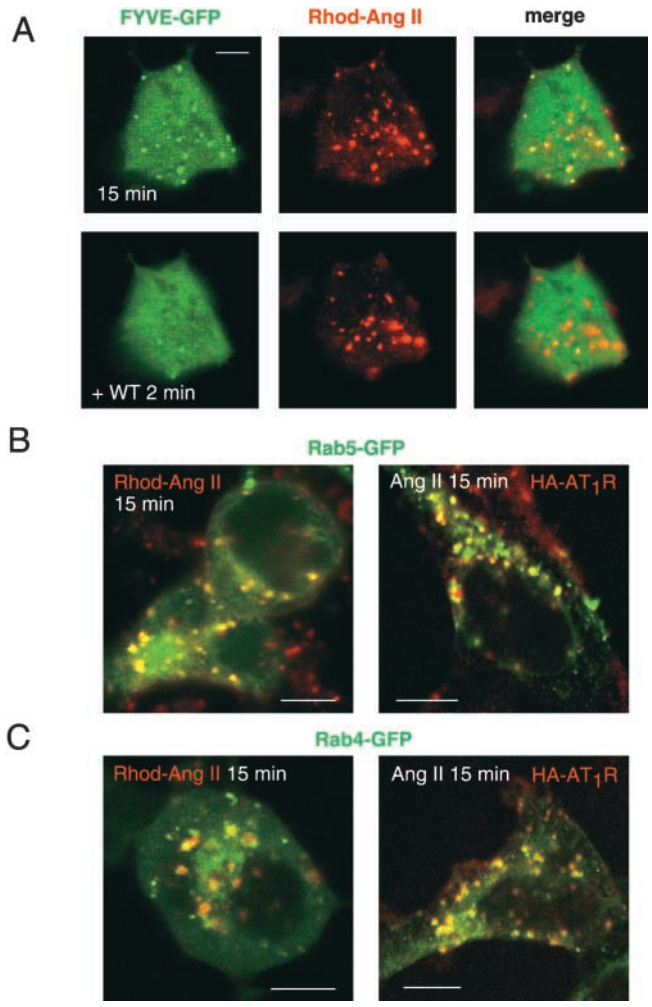

Figure 3. **Localization of AT_1R and selected fluorescent markers after Ang II stimulation in HEK 293 cells.** HEK 293 cells expressing the wild-type or HA-tagged AT_1R were transiently transfected with the GFP-fused FYVE domain of EEA1, or GFP-fused Rab4 and Rab5 proteins. After 24 h, cells were stimulated with Rhod–Ang II (B and C, left) or Ang II (right) for 15 min at 37°C. Appearance of Rhod–Ang II in FYVE–GFP- (A) and Rab5–GFP-positive (B) early endosomes is apparent at this time. Addition of 1 μM WT causes rapid dissociation of FYVE–GFP from the early endosomes (A, bottom). Rhod–Ang II also appears in Rab4–GFP-positive vesicles shortly after stimulation (C). Similar localization of the HA–AT_1R was observed by immunostaining of the receptor in fixed cells (B and C, right). Bars, 10 μm.

tate sites without affecting the association of already internalized Rhod–Ang II with the early endosomes (Fig. 3 A, bottom). Both Rhod–Ang II (in live cells) and HA epitope–tagged AT_1R (in fixed cells) were found in Rab5-positive vesicles after short incubations (Fig. 3 B), consistent with the recently reported role of Rab5 during the initial processing of the internalized AT_1R in early endosomes (Seachrist et al., 2002).

After 15 min, localization of Rhod–Ang II as well as the HA-tagged AT_1R was also observed in vesicles containing Rab4 (Fig. 3 C). However, only after more prolonged incubations (>30 min) was the ligand and receptor found in juxtanuclear vesicles positive for Rab11 (Fig. 4 A). The amount of red fluorescent ligand detected in the latter compartment was greatly reduced in cells expressing dominant-negative Rab11S25N–GFP (Fig. 4 B). The prominent colocalization of AT_1R–GFP with Alexa®594–Tf in the juxtanuclear compartment was also consistent with the passage of internalized AT_1R through pericentriolar recycling endosomes (Trischler et al., 1999; Fig. 4 C).

WT-induced morphological changes in compartments involved in AT_1R trafficking

When HEK 293 cells stably transfected with the AT_1R–GFP chimera were treated with 1 μM WT, stimulation with Rhod–Ang II for 60 min caused prominent accumulation of large vesicles that contained Rhod–Ang II in their lumens, and the receptor (often with its associated ligand) in their limiting membranes (Fig. 5 A). In many of the large vesicles containing Rhod–Ang II, more intense and rapidly moving small red dots were attached to their membranes and often within their lumens (Fig. 5 B). The progressive accumulation of large vesicles was paralleled by a decrease in the small vesicles containing only the ligand without AT_1R–GFP in the cell periphery (Fig. 5, A vs. E), suggesting an impairment of the separation of this compartment from the larger vesicular pool. Because the majority of the small peripheral vesicles containing only the ligand without AT_1R–GFP did not colocalize with the lysosomal marker, Lysotracker (unpublished data), these structures may represent recycling vesicles. Despite the marked alteration in the endosomal processing of the AT_1R in WT-treated cells, colocalization of some Rhod–Ang II and AT_1R in the juxtanuclear compartment was still evident after 60 min of exposure of the cells to the fluorescent agonist (Fig. 5 A). In dose dependence studies, the morphological changes caused by 100 nM WT were almost as marked as those observed with 1 μM WT. However, no significant changes were observed at 10 nM and only slight enlargement was detectable at 30 nM WT. Treatment of cells with 100 μM LY 294002, a less potent PI 3-kinase inhibitor, induced similar changes in morphology (unpublished data).

Characterization of the vesicular pool enlarged in response to WT

To characterize the endosomal compartment that is enlarged in WT-treated cells, GFP-tagged Rab proteins known to be involved in the processing of endosomes were again expressed in HEK 293 cells stably transfected with the HA–AT_1R. After stimulation with Rhod–Ang II in the presence of WT, some of the enlarged vesicles were found to contain each of Rab4–GFP, Rab5–GFP, and Rab11–GFP (Fig. 6 A). Almost all vesicles contained the red fluorescent ligand, often still attached to the receptor, at their limiting membranes. The HA–AT_1R was likewise also found to be associated with enlarged vesicles containing either Rab5–GFP (unpublished data), Rab4–GFP, or Rab11–GFP, and was colocalized with Rab11 in the juxtanuclear recycling compartment (Fig. 6 B). Fluorescent Ang II was also present in large vesicles that contained Rab7–GFP in their limiting membranes (Fig. 6 A). These data demonstrate that WT did not prevent the association of these three Rab proteins with endocytic membranes.

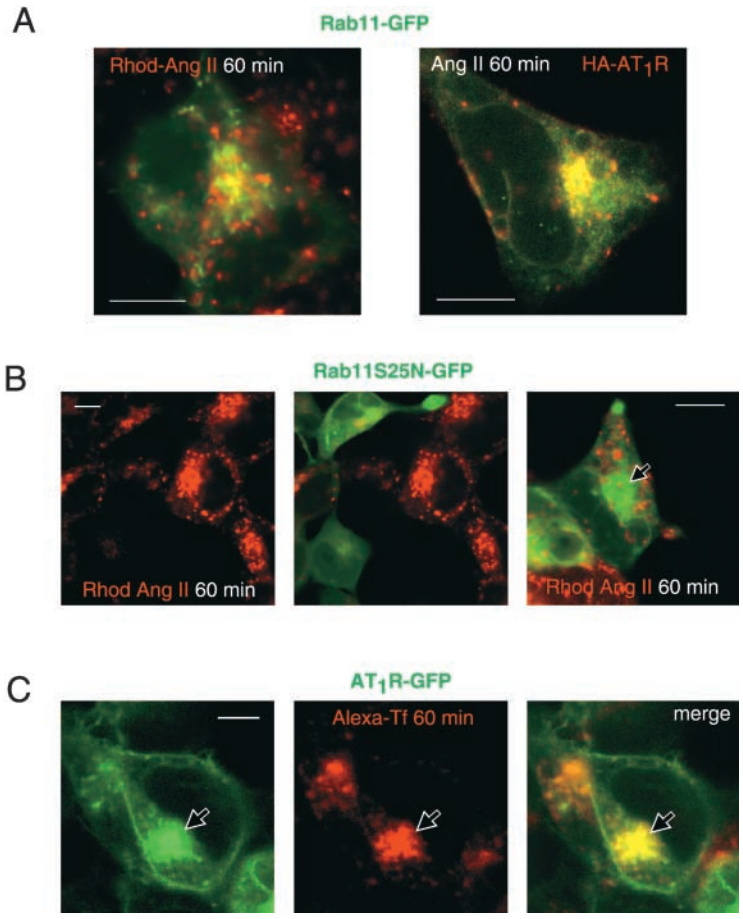

Figure 4. **Localization of the AT_1R and Rhod–Ang II in juxtanuclear recycling endosomes after prolonged Ang II stimulation in HEK 293 cells.** Cells stably expressing AT_1R were transfected with Rab11–GFP (A, left) or Rab11-S25N–GFP (B) or the HA-tagged AT_1R was coexpressed with Rab11–GFP (A, right). After 24 h, cells were stimulated with Rhod–Ang II or Ang II for 1 h at 37°C as indicated. Colocalization of both the ligand and the receptor with Rab11 in juxtanuclear compartments can be observed (A), and the accumulation of the ligand in this compartment is greatly reduced in cells expressing Rab11-S25N–GFP (B). The juxtanuclear area void of Rhod–Ang II is pointed out by an arrow (B, right). (C) Cells expressing the AT_1R–GFP were stimulated with unlabeled Ang II (100 nM) and Alexa®594-conjugated Tf for 60 min. After washing, cells were further incubated for 15 min to chase Tf from the early endosomes. Note the prominent colocalization of AT_1R and Tf in the juxtanuclear compartment (arrows). Bars, 10 μm.

Because large vesicles were also found in WT-treated cells without Ang II stimulation (although without AT_1R–GFP in their wall), we examined the distribution of TfR in WT-treated cells by using Alexa®-labeled Tf in live cells, or by immunostaining the TfR in fixed cells. Fluorescent Tf was present in the membranes of only a minority of the large vesicles of WT-treated cells, often in colocalization either with Rab4, Rab5, or Rab11, but never with Rab7 (Fig. 6 C). Unlike Ang II, Tf was not found within the lumen or inside the small vesicular compartment within the large vesicles. However, it was occasionally present in their limiting membrane and in smaller vesicles often still at-

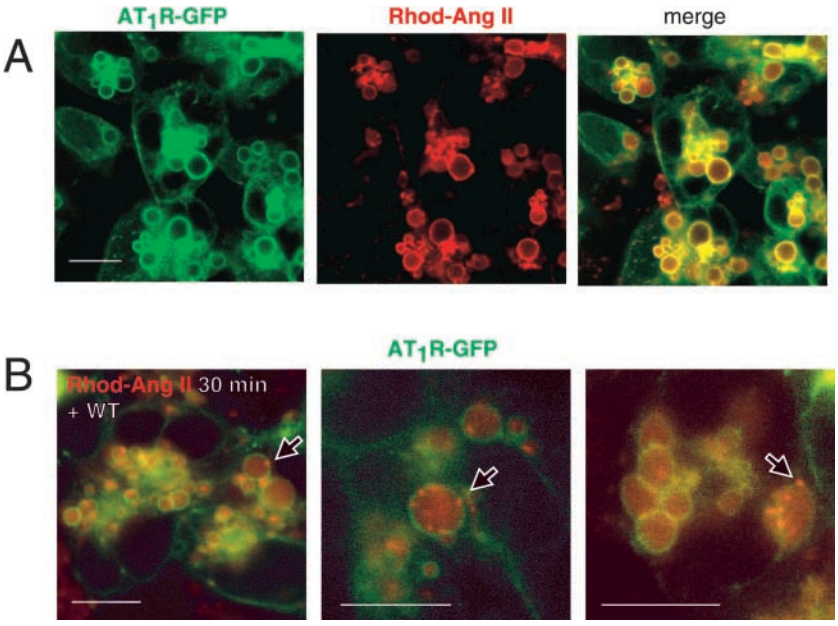

Figure 5. **Effect of WT treatment on the localization of internalized AT_1R.** (A) Cells expressing AT_1R–GFP were stimulated with Rhod–Ang II for 60 min after a 10-min pretreatment with 1 μM WT. Note the significant reduction of receptors at the cell surface and the development of large vesicles containing the receptor still bound with its ligand in their walls. (B) Localization of small vesicles (arrows) containing Rhod–Ang II within the large vesicles that accumulate after stimulation by Ang II for 30 min in the presence of WT. Bars, 10 μm.

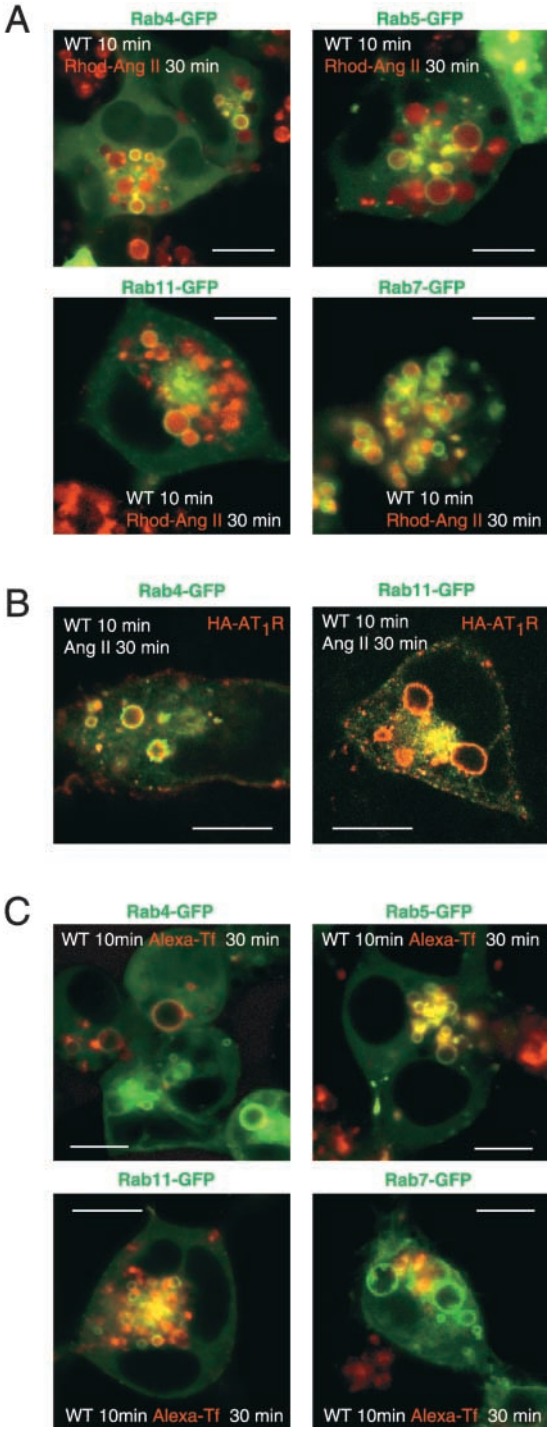

Figure 6. **Localization of Rhod–Ang II, AT_1R, Alexa®–Tf, and individual Rab–GFP proteins in HEK 293 cells after WT treatment.** Cells expressing HA–AT_1R and the respective Rab–GFP constructs were pretreated with 1 μM WT and incubated with Rhod–Ang II (A), Ang II (B), or Alexa®-conjugated Tf (C) for 30 min. After WT treatment, Rhod–Ang II can be found in multiple vesicles, some of which are positive for any of the four Rab–GFP proteins tested (A). The immunostained HA–AT_1R is also found in association with Rab4–GFP-containing large vesicles and with the Rab11-positive juxtanuclear compartment (B). (C) Alexa®–Tf can be found in the limiting membranes (but not in the lumens) of Rab4-, Rab5-, and Rab11-positive vesicles, but not in those positive for Rab7. Bars, 10 μm.

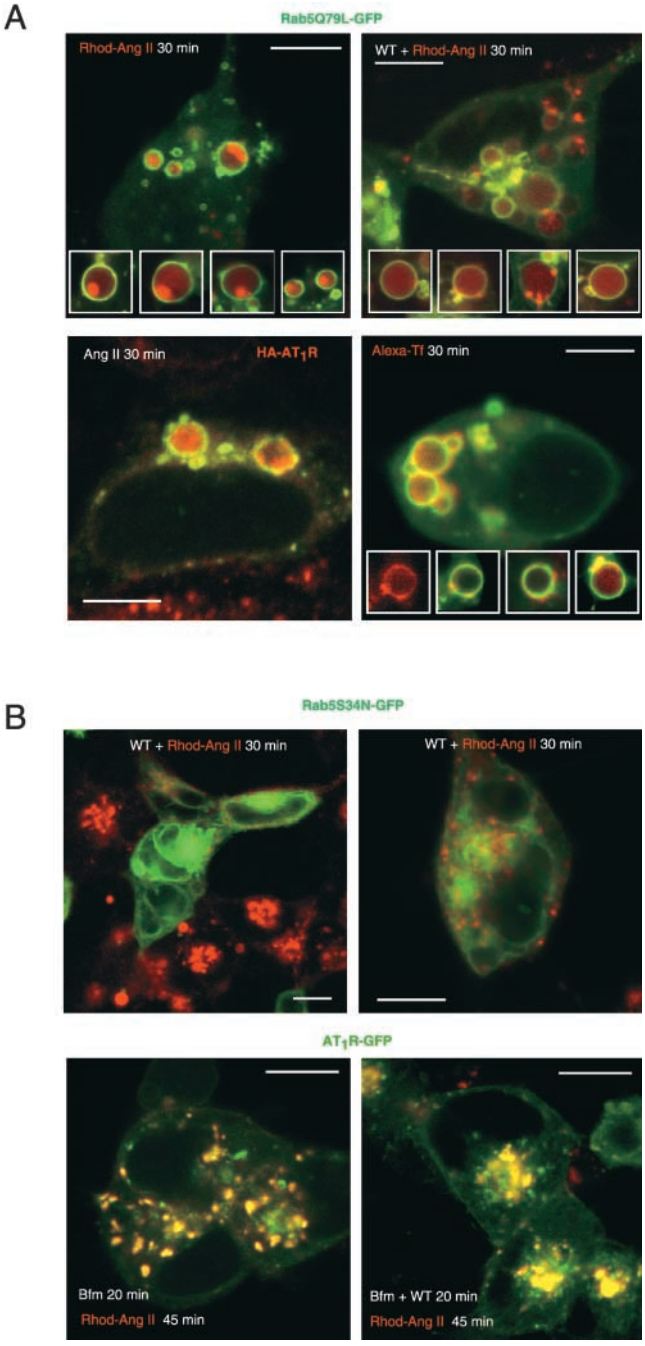

Figure 7. **Localization of Rhod–Ang II, HA–AT_1R, and Alexa®–Tf in HEK 293 cells expressing Rab5Q79L or Rab5S34N.** Cells expressing wild-type or HA–AT_1R and the respective mutant form of Rab5–GFP or only AT_1R–GFP were stimulated with Rhod–Ang II or Ang II for 30 min. In cells expressing Rab5Q79L–GFP, Ang II stimulation leads to the formation of large clusters containing both the ligand (A, top left) and the immunostained HA–AT_1R (A, bottom left) within large vesicles. These clusters are not observed in WT-treated cells (A, top right). Alexa®-Tf is only present in the limiting membranes but not in the lumen of the large vesicles (A, bottom right). Expression of Rab5S34N (B, top) or treatment with 100 nM bafilomycin (Bfm; B, bottom) prevents the formation of large vesicles after WT treatment. Bars, 10 μm.

tached to the larger vesicles (Fig. 6 B). Similar results were obtained after immunostaining with the anti-TfR antibody (unpublished data).

When the GTP-bound mutant version of Rab5 (Rab5Q79L) was expressed in HEK 293 cells, large vesicles similar to those formed after WT treatment in control cells were observed (Fig. 7 A). However, a notable feature of the Rab5Q79L-induced large vesicles, as opposed to those of WT-treated cells, was the presence of large Rhod–Ang II- and AT_1R-containing clusters within their lumens (Fig. 7 A). Such clusters were not observed in cells incubated with Rhod–Ang II after WT treatment and only the large vesicle–associated small red dots characteristic of WT-treated cells were present (Fig. 7 A). In contrast, expression of the GDP-bound mutant, Rab5S34N, prevented the formation of large vesicles after Ang II and WT treatment. The amount of internalized Rhod–AngII was greatly reduced in such cells after 30 min of incubation, although the initial internalization of AT_1R was still observed (Fig. 7 B). Pretreatment of cells with the H^+-ATPase inhibitor bafilomycin (100 nM) completely prevented the separation of AT_1R and its ligand (Fig. 7 C, left) and abolished the formation of the large vesicles in WT-treated cells, leading to the accumulation of the Rhod–Ang II and the AT_1R in the juxtanuclear compartment (Fig. 7 C, right).

WT inhibits rapid, but not slow, recycling of the AT_1R to the cell surface

The inhibitory effect of WT on some, but not all, aspects of AT_1R trafficking, and the association of multiple Rab proteins with the enlarged vesicles, suggested that recycling of the receptor to the cell surface occurs via multiple pathways. To assess the consequences of the WT-induced vesicular changes on the cell surface receptor population, binding studies were performed with ^{125}I-[Sar^{1}Ile^8]Ang II. As expected, incubation of cells with Ang II for 30 min caused a significant decrease in the abundance of AT_1R on the cell surface (Fig. 8 A), as measured by radioligand binding. To analyze the process of receptor recovery and the involvement of PI 3-kinase(s), after 30 min of Ang II treatment, the ligand was removed by thorough washing and the cells were incubated for the indicated times in the presence or absence of WT. Subsequent quantitation of cell surface receptors by radioligand binding revealed that the reduced number of cell surface receptors in control cells showed progressive recovery after removal of the ligand, reaching pretreatment levels by 75 min and continuing to rise for up to 150 min (Fig. 8 A). However, in WT-treated cells, the recovery of sequestered AT_1R at the cell surface was significantly inhibited during the first 30 min after removal of Ang II, but thereafter proceeded at the same rate as in control cells despite the continued presence of WT (Fig. 8 A). The lack of effect of WT on the slow recovery phase was not due to WT degradation, because similar results were obtained when WT was added every 30 min for up to 2 h (unpublished data). These findings suggest that the restoration of AT_1 receptors at the cell surface is mediated by a rapid, WT-sensitive mechanism and by a slower process that still operates after WT treatment. The concentration-dependent actions of two PI 3-kinase inhibitors on the early component of AT_1R recycling are shown in Fig. 8 B. These are in good agreement with the respective potencies of the inhibitors in exerting their morphological effects, indicating the involvement of PI 3-kinase(s) in the process of AT_1R recycling.

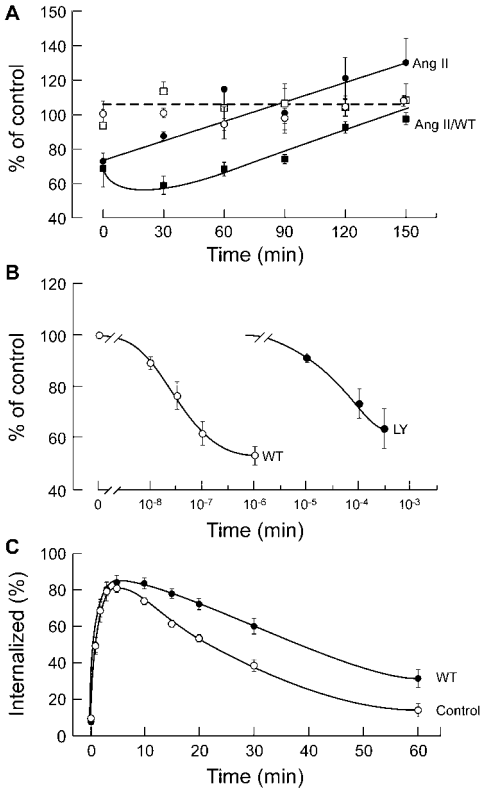

Figure 8. **Changes in cell surface AT_1R abundance as assessed by ^{125}I-[Sar^1,Ileu^8]Ang II.** (A) Cells expressing GFP-tagged AT_1R were stimulated with Ang II (1 μM) for 30 min (labeled as 0 min) and the cells were thoroughly washed before further incubation without the ligand for the indicated times. At selected time points, cells were placed on ice and surface receptors were determined by 12-h binding with the iodinated antagonist ligand at 4°C. When added, WT was present during the last 10 min of the 30-min Ang II treatment. (B) Dose dependence of the inhibitory effect of WT and LY 294002 on the recycling of AT_1R measured at the 30 min time point of the plot shown in panel A. (C) Appearance of acid-resistant (internalized) ^{125}I-Ang II binding as a function of time in a one-cycle internalization at 37°C of cell surface receptors preloaded with the iodinated agonist at 4°C.

The effect of WT on a single cycle of the processing of prebound ^{125}I-Ang II ligand was examined in cells expressing the AT_1R–GFP chimera. Cells were prelabeled with ^{125}I-Ang II on ice to prevent internalization and incubated with or without 1 μM WT for the last 10 min of incubation. After washing at 4°C to remove the unbound ligand, cells were incubated in the absence or presence of WT at 37°C for the indicated times, and the internalized and extracellular fractions of bound ^{125}I-Ang II were determined (see Materials and methods). WT had no effect on the rapid intracellular accumulation of ^{125}I-Ang II during the internalization phase (first 5 min) of the incubation (Fig. 8 C). However, during the subsequent 10 min, the internalized label was cleared more rapidly from control cells than from those treated with

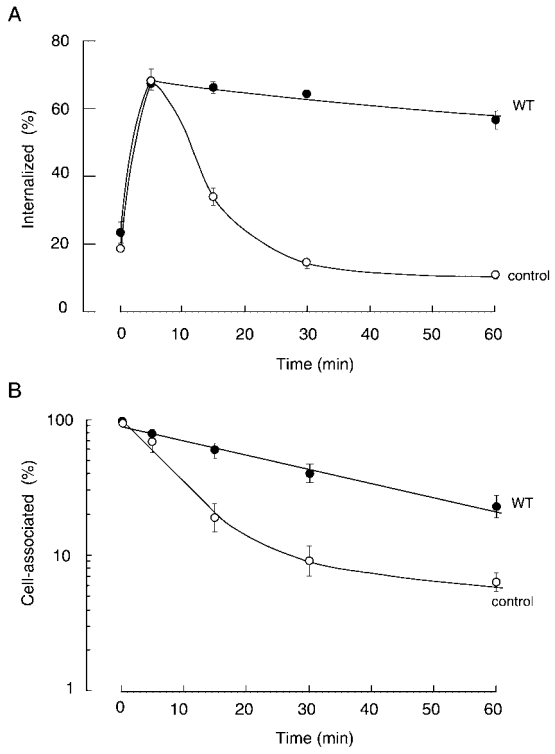

Figure 9. **Recycling of surface-bound or already internalized ^{125}I-Tf in HEK 293 cells.** HEK 293 cells expressing AT_1R–GFP were incubated with ^{125}I-Tf either on ice (A) or at 37°C (B) for 90 and 30 min, respectively. After washing off the unbound ligand, cells were incubated at 37°C for the indicated period of time, and the distribution of the tracer between the medium, cell surface, and cell interior was determined as detailed in the Materials and methods section. Reentry of externalized Tf was prevented by the addition of desferroxamine (100 μM) and unlabeled Tf after 5 min (A) or from 0 min (B).

WT (Fig. 8 C). Furthermore, the intracellular ligand was eventually slowly cleared from the WT-treated cells as well. These data indicate that WT does not affect the process of endocytosis of the AT_1R, but causes selective inhibition of the early recycling process of both the receptor and its ligand.

Finally, the WT sensitivity of the recycling of ^{125}I-Tf was examined by a similar approach to analyze one single cycle of prebound Tf, and in the conventional way by preloading cells with ^{125}I-Tf for 30 min at 37°C and following the exit of internalized Tf in the presence or absence of WT. WT almost completely prevented the recycling of ^{125}I-Tf in the one-cycle experiment (Fig. 9 A), but only partially inhibited the recycling of Tf that was loaded into the cells at 37°C during prolonged incubation (Fig. 9 B).

Discussion

The present study has provided a detailed analysis of AT_1R endocytosis and trafficking, and the effects of PI 3-kinase inhibition on the processing pathways of the internalized ligand–receptor complex. After addition of Ang II, the AT_1R and its ligand formed clusters at the cell surface and rapidly appeared in early endosomes. This was indicated by their colocalization with a GFP-tagged FYVE domain, and with the Rab5 protein as recently described by Seachrist et al. (2002). As predicted, WT treatment caused rapid dissociation of the FYVE domain from this compartment. This might be expected to alter the function of early endosomes because both the heterotypic fusion of endocytic vesicles with endosomes and the homotypic fusion of early endosomes require tethering by the EEA1 protein (Christoforidis et al., 1999). However, the ability of this step to proceed after WT treatment is probably due to the interaction of EEA1 with Rab5 even without PI(3)P, as recently observed during processing of the epidermal growth factor (EGF) receptor (Chen and Wang, 2001b). Accordingly, WT concentrations of up to 10 μM had no effect on agonist-induced endocytosis of either the AT_1R or the TfR. The latter is consistent with previous reports that inhibition of PI 3-kinases did not impair the initial rate of TfR endocytosis (Martys et al., 1996; Spiro et al., 1996). The agonist-dependent recruitment of a PI 3-kinase to the cell membrane has been recently proposed to play a role in β_2-adrenergic receptor sequestration (Naga Prasad et al., 2001). However, other studies have suggested that the inhibitory effect of high WT concentrations on the endocytosis of β_2-adrenergic and muscarinic receptors is caused by inhibition of type III PI 4-kinases rather than PI 3-kinases, with consequent depletion of the plasma membrane PI 4,5-bisphosphate pool (Sorensen et al., 1998, 1999).

The processing of nutrient receptors from early endosomes has been well characterized and is known to involve the small GTP binding proteins Rab4, Rab5, and Rab11. Rab4 is believed to provide rapid recycling of receptors to the surface from Rab5-positive sorting endosomes, whereas Rab11 is believed to be involved in the recycling from the more slowly appearing juxtanuclear compartment often referred to as the recycling endosomes (Mellman, 1996). During maturation of the latter compartment, Rab4 and Rab11 proteins often appear together (Miaczynska and Zerial, 2002). The WT sensitivity of Tf recycling, found in experiments using tracer amounts of ^{125}I-Tf and a "one-cycle" protocol, indicates that a large fraction of internalized TfRs recycle through a WT-sensitive mechanism. Only after longer incubations at 37°C does Tf reach a compartment from which Tf recycling occurs even in the presence of WT. These data are consistent with a model in which Rab4-dependent rapid cycling of TfR from sorting endosomes is more sensitive to PI 3-kinase inhibition than the route of Rab11-dependent recycling endosomes. The Tf receptors found in the latter compartment become loaded with Tf only after prolonged internalization or at high receptor occupancy (Trischler et al., 1999).

Our data on the colocalization of AT_1R with Rab4- and Rab11-containing vesicles indicate that the AT_1R also uses both of these mechanisms for receptor recycling. Processing of internalized AT_1R from early endosomes showed several features that differ from those of the TfR. First, even in one-cycle experiments, only the most rapid fraction of the ^{125}I-Ang II recycling was inhibited by WT and a significant fraction of the ligand was able to leave the cells. Similarly, only the rapid phase of AT_1R recovery to the cell surface after termination of Ang II stimulation was sensitive to WT. These kinetic data were paralleled by morphological differences

that became appreciable only after WT treatment. In the case of the AT_1R, separation of a significant portion of the ligand from its receptor occurs by a WT-sensitive process that causes the appearance of both the receptor and its ligand in small vesicles within the larger WT-induced vesicles. Neither Tf nor its receptor is detected inside such enlarged vesicles, but they were occasionally found in the limiting membranes of the larger vesicles and in tiny vesicles associated with the latter. The vesicular structures observed after Ang II stimulation in the presence of WT were similar to the MVBs, in which inward membrane budding leads to the formation of small internal vesicles (Mellman, 1996). MVBs that are positive for Rab7 are known to process receptor tyrosine kinases, such as the EGF receptor, and appear to be the sorting organelles for proteins destined for lysosomal degradation (Felder et al., 1990; Mellman, 1996). However, kinase-deficient EGF receptors, unlike their wild-type counterpart, do not undergo lysosomal degradation and return to the plasma membrane (Felder et al., 1990). This suggests that receptors can recycle back to the cell surface from this organelle or from its precursor forms. A recent report also demonstrated the presence of the G protein–coupled m4 muscarinic receptor (but not of TfR) inside small vesicles within large endosomes formed in cells expressing Rab5Q79L, a structure considered by the authors to be the MVB (Volpicelli et al., 2001).

Several lines of evidence have indicated that protein sorting at the level of the MVB is dependent on PI 3-kinase products. In *Saccharomyces cerevisiae*, the formation of $PI(3,5)P_2$ from PI(3)P by the Fab1p protein, a PI(3)P 5-kinase, is necessary for the inward invagination of membranes and formation of internal vesicles (Odorizzi et al., 1998). In mammalian cells, injection of an antimammalian Vps34p antibody inhibited internal vesicle formation in multivesicular endosomes (Futter et al., 2001). Other evidence indicates that PI 3-kinases are also involved during the formation of late endosomes and lysosomes (Fernandez-Borja et al., 1999). WT also induced morphological changes similar to those described in the present report during studies on the delivery of lysosomal enzymes mediated by the mannose 6-phosphate receptor (Brown et al., 1995). Our data on the heterogeneity of the large vesicles observed in WT-treated and Ang II–stimulated HEK cells are consistent with multiple defects in the processing of early endosomes as well as the MVB. These include the budding off of external vesicles (used by TfR and perhaps a fraction of the AT_1R) as well as the formation of internal vesicles and the maturation toward late endosomes via MVBs. Inhibition of these processes by WT could contribute to the enlargement of internalized vesicles. Although the WT-sensitive component of AT_1R recycling could represent the same Rab4-dependent pathway used by the TfR, it may also involve a process by which the receptor is retrieved from multivesicular compartments before maturation to late endosomes. It is likely that this route (even if not identical with the pathway of TfR recycling) also utilizes Rab4, because we clearly detected colocalization of Rhod–Ang II and the HA–AT_1R with Rab4–GFP. The G protein–coupled β_2 adrenergic receptor, which is known to use a rapid recycling route, has also been shown to recycle to the cell surface via a Rab4-dependent mechanism (Seachrist et al., 2000).

It is important to note that WT treatment had relatively minor effects on the slow component of AT_1R recycling, and under these conditions both receptor and ligand were found in small juxtanuclear vesicles. This suggests that some small vesicles still can be formed, and that these probably represent the route through which receptor and ligand can recycle after WT treatment. Several observations suggest that this compartment is the Rab11-positive recycling endosome. The AT_1R was found to colocalize with Alexa®–Tf in the pericentriolar area of Ang II–stimulated cells even after WT treatment. Given the relative WT insensitivity of TfR recycling from the Rab11-positive recycling endosomal compartment, it is probable that AT_1R recycling from the enlarged vesicles involves the same route. However, it is also possible that the slow recycling route observed in WT-treated cells is a distorted pathway that shares only some of the characteristics of the pathways occurring in normal cells. The preservation of the slow recovery phase of internalized receptors to the cell surface in WT-treated cells indicates that lysosomal degradation of the AT_1R is not a major route in these cells, at least in the 2-h time frame of stimulation in the present experiments. We also could not detect the AT_1R receptor (only the Rhod–Ang II ligand) in Rab7-positive vesicles in WT-treated cells, consistent with the retrieval of the receptor before late endosomes.

The similarity of the large vesicular phenotype between WT treatment and Rab5Q79L expression, and the inability of added 3-phosphoinositides to reverse the large vesicular phenotype, has suggested that this effect of WT results from inhibition of Rab5 inactivation rather than PI 3-kinase activity (Chen and Wang, 2001a). However, although hydrolysis of Rab5-bound GTP may well be prevented by WT-sensitive mechanisms, our data suggest that the effect of WT is more complex than stabilization of Rab5GTP. The ability of Rab5S34N to prevent the formation of large vesicles after WT treatment indicates the need for continuous Rab5-assisted transfer of the receptor–ligand complex from internalized vesicles to early endosomes. However, not all of the enlarged vesicles contained wild-type Rab5–GFP in WT-treated cells, suggesting that Rab5 must be released from at least some of the vesicles despite the presence of WT. Moreover, Rab5Q79L caused the accumulation of large clusters of Rhod–Ang II and HA–AT_1R within the enlarged vesicles, a feature that was not seen in vesicles of WT-treated cells. Furthermore, although expression of Rab5Q79L was without effect on TfR recycling (Ceresa et al., 2001; Volpicelli et al., 2001), we observed that WT treatment inhibits early recycling of the AT_1R. These data suggest that although the abnormal vesicles induced by WT or expression of Rab5Q79L share many similarities, they are not identical. Inhibition of H^+-ATPase by bafilomycin prevented the separation of AT_1R from its ligand, and also prevented the WT-induced enlargement of AT_1R-containing vesicles. These findings are similar to those observed with major histocompatibility complex (MHC) class II molecules in melanoma cells (Fernandez-Borja et al., 1999), and indicate that acidification is a prerequisite for the maturation of endosomes as well as for the dissociation of the ligand–receptor complex.

Based on the present results and data available in the literature, a proposed model depicting the intracellular traffick-

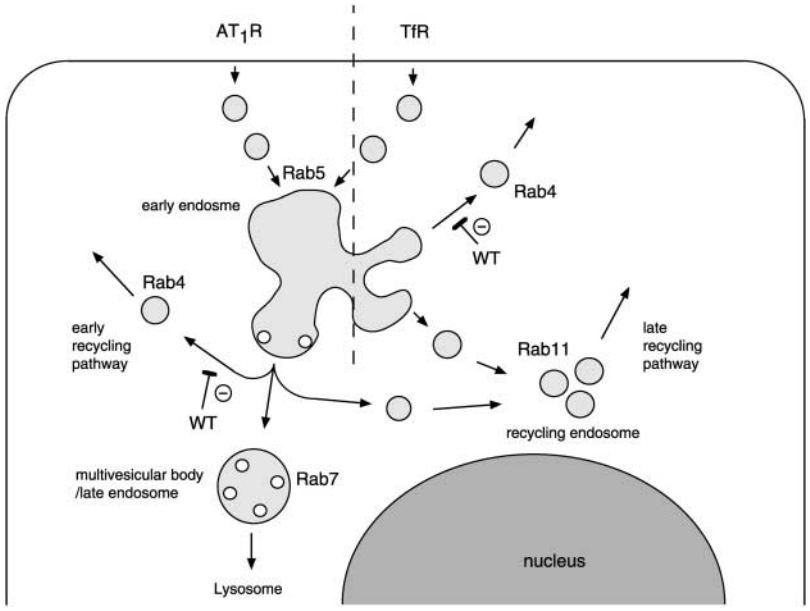

Figure 10. **Trafficking steps after internalization of the AT_1R.** After ligand binding, AT_1Rs undergo clustering into clathrin-coated pits at the plasma membrane followed by endocytosis. After uncoating, endocytic vesicles fuse with early endosomes. Some of the internalized receptors are processed toward a compartment (most likely the MVB) where, due to acidification, the ligand dissociates and the receptor rapidly recycles back to the plasma membrane. During this process, some of the ligand is sorted into lysosomes and some is recycled with the receptor and leaves the cells. Inhibition of PI 3-kinases by WT attenuates this pathway, leading to the accumulation of an intermediate vesicular compartment that contains the AT_1R and its ligand. Internalized receptors also traffic toward a juxtanuclear recycling compartment, from which both the receptor and the ligand are processed slowly. This recycling pathway remains functional during WT treatment.

ing of AT_1R and possibly other GPCRs is shown in Fig. 10. Agonist-induced internalization of the AT_1R is rapidly followed by its appearance in early endosomes. Unlike the TfR, which leaves early endosomes and recycles via Rab4- and Rab11-positive recycling pathways, most of the AT_1R initially follows a route leading to the formation of MVBs. Some of the AT_1R and its ligand could bud off by a mechanism that is similar to that of the TfR. However, both can leave the vesicles by other means and may utilize mechanisms that are also involved in the formation of small internal vesicles. WT treatment causes multiple defects in early endosome maturation and processing. These include the inhibition of early recycling of TfR, probably via Rab4, with less impairment of the Rab11-positive slower recycling pathway. WT also inhibits the processing of AT_1R toward the MVB, leading to the enlargement of this vesicular pool that contributes to the inhibition of recycling of receptor and ligand to the surface. The slow recycling of the receptor during WT treatment involves a distinct mechanism that proceeds via the Rab11 recycling pathway. Further studies are required to pinpoint the exact site(s) and enzyme(s) that are targeted by WT, and to validate the relevance of this model to the trafficking of other GPCRs.

Materials and methods

Materials and DNA constructs

The cDNAs of the rat vascular smooth muscle AT_{1A} receptor, and type A cholecystokinin receptor–GFP were provided by K.E. Bernstein (Emory University, Atlanta, GA) and S.A. Wank (National Institute of Diabetes and Digestive and Kidney Diseases), respectively. Rhod–Ang II was obtained from NEN Life Science Products. ^{125}I-Ang II, ^{125}I-[Sar^1, Ile^8]Ang II, and ^{125}I-Tf were purchased from NEN Life Science Products. Lysotracker, Alexa®594–Tf and the Alexa®594 secondary antibody were from Molecular Probes. The chimeric AT_1R–GFP chimera was constructed by linking the cDNA of an enhanced fluorescent mutant of GFP (EGFP) (Tarasova et al., 1997) to the 3′ end of the coding region of the AT_1R. The FYVE–GFP plasmid was created by subcloning the human EEA1 cDNA sequence (amino acids 1252–1411) between the BglII and EcoRI sites of the pEGFP–C1 plasmid. GFP-tagged versions of the Rab proteins (Rab4, Rab5, Rab7, and Rab11) were created in the pEGFP–C1 plasmids (CLONTECH Laboratories, Inc.) after PCR amplification of the Rab proteins from cDNAs prepared from MDCK cells. Mutations were generated with two-step PCR. All constructs were verified with dideoxy sequencing.

Confocal analysis of receptor endocytosis

HEK 293 cells stably expressing the AT_1R–GFP chimera were plated onto polylysine-coated 25-mm-diameter glass coverslips at a density of 5×10^5 cells/dish and cultured for 3 d before analysis. HEK 293 cells stably expressing the wild type, HA–AT_1R were transfected with selected GFP constructs using the lipofectamine reagent for 24 h. Cells were washed twice with Dulbecco's PBS containing 0.1% BSA before analysis and treated with the indicated stimuli or inhibitors at 37°C. The coverslip was placed into a chamber mounted on a heated stage with the medium temperature kept at 33°C. Cells were incubated in 1 ml buffer and examined in an inverted microscope under a 40× oil immersion objective (Nikon) and a Bio-Rad Laboratories laser confocal microscope (MRC-1024). For double labeling experiments the 488- and 568-nm laser lines were used with 525 ± 20-nm and 580-nm-long pass filters for the detection of GFP and Alexa®, respectively.

Immunocytochemistry

HEK 293 cells grown on glass coverslips were transiently transfected with HA–AT_1R and the respective Rab–GFP constructs for 24 h. Cells were stimulated with Ang II for the times indicated and were washed twice with PBS before fixation with 4% paraformaldehyde in PBS (pH 7.4) for 20 min at room temperature. After treatment with sodium borohydride (1 mg/ml) for 15 min, cells were permeabilized with 0.1% Triton X-100 in PBS and incubated in blocking solution for 30 min (1% BSA in PBS), before incubation with anti-HA antibody (1:200 in blocking solution) for 1 h at room temperature. This was followed by two washes with TBST and incubation with rhodamine-conjugated anti–mouse antibody (1:200) for 1 h. The coverslips were mounted using Dako fluorescence mounting medium. Images were acquired with a ZEISS LSM 510 confocal laser scanning microscope in multi track mode, using the 488- and 543-nm laser lines with 500–550-nm band pass and 560-nm long pass filters for the detection of GFP and rhodamine, respectively.

Recycling of the AT_1R in HEK 293 cells

HEK 293 cells stably expressing AT_1R–GFP were cultured on 24-well plates and preincubated in medium 199 (Hank's salts) containing 20 mM Hepes (pH 7.4) and 1 g/liter BSA in the absence or presence of Ang II (1 μM) for 30 min at 37°C. Cells were then washed four times with 1 ml ice cold Dulbecco's PBS, and the incubation continued for the indicated times in medium 199 containing 20 mM Hepes (pH 7.4) and 1 g/liter BSA at 37°C. Inhibitors were added 10 min before the end of the preincubation and were readministered with medium 199 after washing the cells to remove Ang II. Incubations were terminated by placing the cells on ice and rapidly washing them twice with ice cold Dulbecco's PBS. The surface

binding of the AT_1R was then determined at equilibrium by incubating the cells in the presence of ^{125}I-[Sar^1,Ile^8]Ang II (0.05 μCi/well) and unlabeled [Sar^1,Ile^8]Ang II (2 nM) at 4°C overnight. The cells were washed twice with 1 ml Dulbecco's PBS before solubilization with 0.5 M NaOH, 0.05% SDS for determination of cell-associated radioactivity.

Internalization kinetics of prelabeled AT_1R or TfR

HEK 293 cells were cultured in four-well plates and were incubated in the presence of ^{125}I-Ang II or ^{125}I-Tf (0.05 μCi/well) in 0.5 ml medium 199 containing 20 mM Hepes (pH 7.4) and 1 g/liter BSA at 4°C for 4 h (Ang II) or 90 min (Tf) to permit binding to the cell surface receptors without internalization. The unbound tracer was removed by washing the cells twice with 1 ml ice cold Dulbecco's PBS. At this point, 0.5 ml warm (37°C) medium was added, and the cells were incubated for the indicated times at 37°C to allow receptor internalization and subsequent processing to proceed. In the case of TfR studies, 100 μM desferroxamine and 100 nM unlabeled Tf was added after 5 min to prevent possible reuptake of the recycled ligand. Incubations were terminated by placing the cells on ice, and the medium containing the radioligand released during the incubation was collected and replaced with 0.5 ml ice cold acid wash solution (150 mM NaCl and 50 mM acetic acid) to remove the surface-bound radioligand (Hunyady et al., 1994). After a 10-min incubation on ice, the extracellular (acid sensitive) tracer was collected. Cells were then lysed with 0.5 M NaOH and 0.05% SDS to determine the internalized (acid resistant) radioactivity. The total binding was calculated as the sum of the released (into the medium), extracellular (acid sensitive), and internalized (acid resistant) radioactivities. The internalized radioactivity was expressed as a percent of the total binding at each time point. Recycling of internalized TfR was also studied after loading cells with ^{125}I-Tf for 30 min at 37°C and following the release of internalized ^{125}I-Tf at 37°C after washing off the noninternalized tracer in the presence of desferroxamine.

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