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Research Article

Agonist-induced trafficking of the low-affinity formyl peptide receptor FPRL1

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Abstract. The formyl peptide-like receptor FPRL1 is a member of the chemoattractant subfamily of G protein-coupled receptors involved in regulating leukocyte migration in inflammation. To elucidate mechanisms underlying the internalization of ligand-bound FPRL1 and possible receptor recycling, we characterized the endocytic itinerary of FPRL1. We show that agonist-triggered internalization from the plasma membrane into intracellular compartments is prevented by perturbation of clathrin-mediated endocytosis, such as expression of the dominant-negative clathrin Hub mutant, siRNA-mediated depletion of cellular clathrin and expression of a dominant-negative mutant of the large GTPase dynamin. Internalized FPRL1 co-localized with endocytosed transferrin and the small GTPases Rab4 and Rab11 in vesicular structures most resembling recycling endosomes. Recycling of FPRL1 was significantly reduced by pretreatment with PI3-kinase inhibitors. Thus, ligand-bound FPRL1 undergoes primarily clathrin-mediated and dynamin-dependent endocytosis and the receptor recycles via a rapid PI3-kinase-sensitive route as well as pathways involving perinuclear recycling endosomes.

Key words. Chemoattractant receptor; endocytosis; recycling; clathrin; dynamin.

The recruitment of leukocytes to sites of inflammation and infection is mediated through bacterial and host-derived mediators of inflammatory activation which regulate leukocyte rolling on and adhesion to the endothelium as well as subsequent extravasation from the blood vessel into the tissue [1–3]. In these events, chemoattractants such as the bacterially derived, N-formylated peptides activate their seven-transmembrane G protein-coupled receptors (GPCRs) on the leukocyte surface [4]. Two receptors have been identified for the bacterial chemotactic peptide N-formyl-methionyl-leucyl-phenylalanine (fMLF): the prototype formyl peptide receptor (FPR), which is activated by low fMLF concentrations, and a low-affinity variant, FPR-like 1 (FPRL1), which requires high micromolar concentrations of fMLF [for reviews see refs. 4, 5].

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FPRL1, also known as the lipoxin A4 receptor, has been found to be rather promiscous. It binds to a variety of unrelated agonists including the acute-phase serum protein, serum amyloid A [6], protein fragments of the HIV envelope [7], amyloid beta [8] and even the lipid lipoxin A4 [9]. FPRL1 has recently been shown to participate in lipid- and peptide-mediated anti-inflammatory circuits resulting in inhibition of neutrophil extravasation [10]. Furthermore, activation of FPRL1 has been shown to induce the downregulation of chemokine receptors and to desensitize monocytes toward chemokines [6]. Thus, FPRL1 seems to be of pathophysiological significance in infection and inflammation and is therefore a potential target for developing therapeutic agents.

Agonist-mediated signal transduction and receptor desensitization and resensitization are regulated by the release of associated G proteins, receptor phosphorylation

and receptor trafficking through the endocytic pathway, thus controlling cellular responsiveness to stimulation. Following agonist binding, receptors typically are endocytosed, sorted and either recycled to the cell surface or targeted to lysosomes for degradation. These so-called sequestration processes have been well documented for several GPCRs, and different receptors have been shown to utilize different internalization mechanisms and are directed to different endocytotic pathways [for reviews see refs. 11, 12].

Here, we elucidated the mechanisms mediating FPRL1 internalization and characterized the intracellular fate of internalized FPRL1. We found that following agonist stimulation, FPRL1 is rapidly internalized and recycled. Conditions interfering with the formation of clathrin-coated pits or the internalization of clathrin-coated vesicles strongly reduced or prevented ligand-induced internalization of FPRL1 revealing that the receptor primarily uses a clathrin-dependent endocytic pathway. Following internalization, FPRL1 co-localized with Rab4 and Rab11, indicative of trafficking through the perinuclear recycling compartment. Interestingly, recycling was partly impaired in the presence of PI3-kinase inhibitors suggesting the involvement of a separate PI3-kinase-dependent recycling pathway which bypasses perinuclear recycling endosomes and has previously been described for internalized transferrin receptors.

Materials and methods

DNA constructs

The cDNA encoding the human receptor FPRL1 was obtained by PCR using a human leukocyte cDNA library as template (BD Clontech, Palo Alto, Calif.). To create an FPRL1 fusion protein N-terminally tagged with the FLAG epitope, a PCR was performed introducing a FLAG epitope sequence immediately 5′ of the initial ATG. To ensure efficient translocation of the modified receptor into the endoplasmic reticulum (ER) membrane, a cleavable signal sequence from influenza hemaglutinin was added upstream of the FLAG epitope. The PCR products were inserted into the pcDNA3.1 vector (Invitrogen, Karlsruhe, Germany) via *Xho*I/*Eco*RI restriction sites. Rab4 and Rab11 cDNAs were amplified by PCR from a human placenta cDNA library (Clontech) and inserted into the pEGFP-C1 (Rab4) or pDsRed1-C1 (Rab11a) pasmids (Clontech). All constructs were confirmed by sequencing (SEQLAB, Göttingen, Germany).

Stable expression of FLAG-FPRL1 in HeLa cells

Plasmids encoding FLAG-tagged FPRL1 were transfected into HeLa cells by electroporation as described previously [13] and cultured in supplemented DMEM in the presence of 800 µg/ml geneticin (G418; PAA Laboratories, Cölbe, Germany) to maintain selection. Resistant clones were analyzed for receptor expression by immunofluorescence using the monoclonal M1 antibody (Sigma, Diesenhofen, Germany). This antibody recognizes the FLAG epitope only if there are no preceeding amino acids, i. e. only if the hemaglutinin signal sequence is cleaved by signal peptidase.

Cell lines and transfection

The HeLa cell line tTA-HeLa stably transformed with dyn^{K44A} [14] (kindly provided by S. Schmid) was maintained in pyruvate-containing DMEM (Gibco, Darmstadt, Germany) supplemented with 10% FCS and antibiotics (400 µg/ml G418, 1 µg/ml tetracycline, 200 ng/ml puromycin, 100 units/ml penicillin/streptomycin). For induction of mutant dynamin expression, cells were grown in the complete absence of tetracycline for 48 h.

Fluorescence microscopy analysis of receptor internalization

For internalization of Texas Red-labeled human transferrin (TxTf; Molecular Probes, Leiden, The Netherlands) or the synthetic D-methionine-containing chemotactic WKYMVm peptide (W-peptide) purchased from Advanced Biotechnology Centre (London, UK), cells grown on coverslips were first serum-starved for 30 min at 37 °C and then incubated in internalization medium (IM: MEM, 20 mM Hepes, 0.8 mg/ml sodium bicarbonate, 1 mg/ml BSA, pH 7.2) containing 25 µg/ml TxTf or 10 µM W-peptide. Although FLAG-FPRL1 internalization and physiological responses in the FLAG-FPRL1-transfected cells were already triggered by employing nanomolar W-peptide concentrations, 10 µM was used to ensure saturating ligand concentrations, thereby inducing maximal receptor internalization. Incubations were carried out as indicated. The cells were then washed with ice-cold PBS, fixed with 3% paraformaldehyde in PBS for 15 min at room temperature, quenched for 10 min in 50 mM NH_4Cl in PBS, washed three times with PBS, permeabilized with 0.2% Triton X-100 in PBS for 5 min, washed three times, and blocked with 2% BSA in PBS for 15 min. Incubation with primary antibodies in PBS containing 2% BSA was performed for 1 h at room temperature. Cells were then washed with PBS and incubated for 1 h at room temperature with the fluorescently labeled appropriate secondary antibodies in PBS containing 2% BSA. For inspection using either a Leica DM RXA epifluorescence microscope or a Zeiss LSM510 confocal microscope, cells were mounted in mowiol containing 4% n-propyl-gallate as antifade agent.

Perturbation of clathrin-mediated endocytosis

Potassium depletion was carried out as described elsewhere [15] with minor modifications. Briefly, cells grown on coverslips were washed with K^+-free buffer

(140 mM NaCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 5.5 mM glucose, 1% BSA, 20 mM Hepes, pH 7.4), incubated for 5 min at 37 °C in culture medium diluted 1:1 with water (hypotonic buffer), washed with K^+-free buffer, and incubated for 1 h at 37 °C in the same buffer prior to and during ligand internalization. As a control, the same buffer containing 10 mM KCl was used.

To test hypertonic sucrose, cells were washed with serum-free culture medium containing 450 mM sucrose and incubated in the same medium for 30 min at 37 °C. Because the effect of hypertonic sucrose on endocytosis is reversible, subsequent internalization of endocytic tracers was carried out in the presence of 450 mM sucrose. Control cells were treated omitting sucrose.

For cholesterol depletion, cells grown on coverslips were preincubated with or without 10 mM methyl-β-cyclodextrin (MβCD) in IM at 37 °C for 20 min. The internalization was continued in the absence or presence of the drug.

For transient expression of the dominant-negative clathrin Hub mutant [16], cells grown on coverslips were transfected with Effectene (Qiagen, Hilden, Germany) according to the manufacturer's protocol. Expresson of the T7 epitope-tagged mutant protein was confirmed with the T7-Tag monoclonal antibody (Novagen, Bad Soden, Germany).

For siRNA transfections, cells were grown on coverslips for 1 day prior to transfection. Annealed clathrin heavy-chain siRNA duplexes (20 nM) [17] were transfected using Oligofectamine (Invitrogen) according to the manufacturer's instructions. Both siRNA-treated and control cells were grown for 48 h at 37 °C and then processed for further analyses. Downregulation of the clathrin heavy chain was confirmed with the X22 anti-clathrin heavy-chain monoclonal antibody [18].

Flow cytometry

FLAG-tagged FPRL1 on the cell surface was detected using a FACSCalibur flow cytometer (Becton Dickinson, Palo Alto, Calif.). Cells were harvested, washed and resuspended in blocking buffer (PBS, 1% BSA). For detection of FPRL1 on the cell surface, 2×10^5 cells/ml were incubated with the anti-FLAG monoclonal antibody M1 (Sigma) diluted 1:1000 in blocking buffer for 30 min, washed and subsequently labeled with FITC-conjugated secondary antibodies (1:500, goat anti-mouse; Dianova, Hamburg, Germany) for 30 min at 4 °C in blocking buffer. After extensive washing, cells were analyzed for fluorescence intensities and the mean cell-bound fluorescence values ± SEM were calculated.

FPRL1 internalization and recycling measurements

HeLa-FLAG-FPRL1 cells were treated with 10 µM WKYMVm for the times indicated at 37 °C, washed and either resuspended in ice-cold PBS or incubated further for the indicated times (chase) to determine the recovery of the epitope-tagged receptor on the cell surface. For recycling measurements, cells were cultured for 1 h with 20 µg/ml cycloheximide (Fluka, Neu-Ulm, Germany) before the addition of ligand. Since no intracellular FPRL1 storage pools were observed in unstimulated cells, the cycloheximide treatment ensured that cell surface recovery of the receptor following agonist-induced internalization was not due to the exocytosis of newly synthesized or stored receptor but truly reflected receptor recycling. When added, 100 µM LY294002 [2-(4-morpholinyl)-8-phenyl-4H-1-benzopyran-4-one; Calbiochem] was present during the last 10 min of the internalization period (pulse) and then kept in the medium during the chase. All incubations were terminated by placing the cells on ice. Receptor internalization was determined as agonist-induced loss of antibody-labeled cell surface receptor; recycling as the recovery of cell surface receptor during the chase period.

Results

Inhibition of clathrin/dynamin-mediated endocytosis affects ligand-induced FPRL1 internalization

Clathrin and the large GTPase dynamin have been identified as essential components required for the receptor-mediated endocytosis of a number of ligands, with transferrin and the uptake through its cell surface receptor being considered a paradigm for this pathway [for reviews see refs. 19–22]. Therefore, to elucidate the contribution of clathrin/dynamin-mediated endocytosis to ligand-induced FPRL1 internalization, we perturbed clathrin coat formation and clathrin-coated vesicle internalization by different means and compared the effects observed for FPRL1 to those observed for transferrin receptor endocytosis. To follow the route of internalized FPRL1, HeLa cells expressing N-terminally FLAG-tagged FPRL1 were stimulated with the peptide WKYMVm, previously described as an FPRL1 agonist [23], and the localization of the receptor was then detected by visualizing the FLAG-tag using a specific antibody. An N-terminally FLAG-tagged receptor was used because an identical epitope tag had been used in a number of previous analyses of GPCR physiology, including that of the closely related formyl peptide receptor FPR, and the tag was shown not to interfere with internalization and intracellular routing [see, for example, refs. 24, 25]. In the control experiments, internalization of transferrin receptors was monitored by employing fluorescently labeled transferrin.

As shown in figure 1, addition of WKYMVm to HeLa-FLAG-FPRL1 cells induced receptor translocation into intracellular compartments. FPRL1 could be rapidly detected in punctate intracellular structures which at 30 min of internalization were most prominent adjacent to the

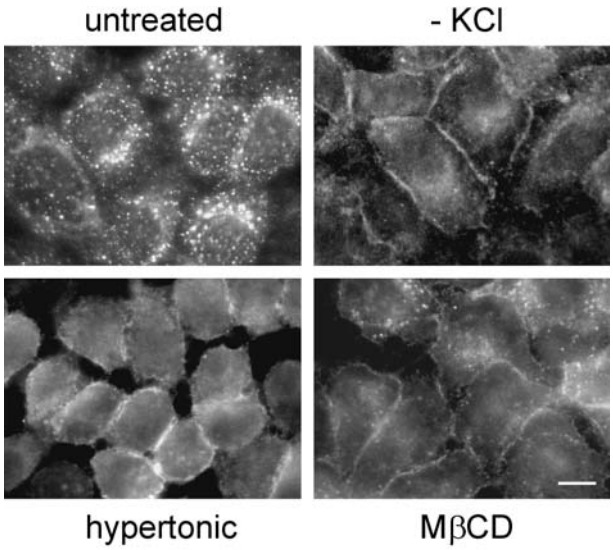

Figure 1. Perturbation of clathrin-mediated endocytosis inhibits agonist-induced FPRL1 internalization. HeLa-FLAG-FPRL1 cells were incubated either in K^+-free medium for 60 min (- KCl), hypertonic medium containing 450 mM sucrose for 30 min (hypertonic), or in medium containig 10 mM MβCD for 10 min (MβCD). Control cells were left untreated. To follow agonist-induced FPRL1 internalization, cells were then stimulated with 10 μM WKYMVm for 30 min, fixed, and FPRL1 was visualized by immunofluorescence staining using the anti-FLAG monoclonal antibody M1. Representative images are shown. Note that whereas untreated cells show intracellular vesicles loaded with FPRL1, in treated cells, the receptor remains predominantly at the plasma membrane. Bar, 10 μm.

nucleus. The same structures were labeled with internalized TxTf indicating that they resembled endosomes accessible for endocytosed transferrin receptors (not shown). K^+-depletion and the resulting change in membrane polarization was then employed to interfere with normal clathrin lattice formation [26, 27]. When K^+-depleted cells were exposed to the WKYMVm peptide, a drastic reduction of FPRL1-containing vesicles was observed and FLAG immunoreactivity remained predominantly at the cell surface (fig. 1). A similar inhibition is seen in the case of transferrin uptake in the same cells (not shown). Treatment with hypertonic sucrose was applied next to perturb clathrin-mediated entry mechanisms [28]. Again, the WKYMVm-induced internalization of FPRL1 was prevented (fig. 1) and an identical inhibition was seen for transferrin uptake (not shown). Since both hypertonic sucrose and K^+ depletion disturb normal clathrin polymerization and result in a disappearance of normal clathrin lattices and the formation of abnormal empty clathrin microcages [26–28], these observations suggest that FPRL1 is internalized by clathrin-dependent mechanisms. Cholesterol has also been shown to be essential for the efficient formation of clathrin-coated endocytic vesicles [29, 30]. Depletion of cholesterol from the plasma membrane by MβCD strongly reduces endocytosis of transferrin, whereas clathrin-independent pathways seem to be less affected [29, 30]. Therefore, we also examined the effect of cholesterol depletion on the internalization of FPRL1. Upon incubation of MβCD-treated cells with WKYMVm, internalization of the receptor was inhibited and anti-FLAG staining was primarily observed at the plasma membrane (fig. 1). Again, the uptake of transferrin was affected likewise (not shown). This again suggests the participation of clathrin-dependent pathways in FPRL1 uptake, although cholesterol depletion by MβCD treatment also affects caveolae [31, 32].

Dynamin also plays an essential role in the early stages of clathrin-mediated endocytosis. Overexpression of a dominant-negative GTPase-deficient mutant dynamin (dyn^{K44A}) has been shown to effectively block endocytosis of ligands such as transferrin and epidermal growth factor (EGF) [14], probably by inhibiting the fission of clathrin-coated vesicles from the plasma membrane. Therefore, to further confirm the clathrin/dynamin dependency of FPRL1 internalization, we transiently expressed FPRL1 in HeLa cells stably transfected with dyn^{K44A}. In this cell line, expression of the mutant dynamin is regulated by a tetracycline-sensitive promoter and can be induced upon withdrawal of tetracycline from the medium [14]. As shown in figure 2 and reported previously [14], induction of dyn^{K44A} by tetracycline deprivation results in an accumulation of TxTf at the plasma membrane. Likewise, we also observed a strong inhibition of WKYMVm-triggered internalization of FPRL1 in cells expressing dyn^{K44A} (fig. 2).

Expression of dyn^{K44A} and cholesterol depletion are not entirely specific for clathrin-mediated endocytosis and also affect other uptake routes [31–33]. To further strengthen the finding that FPR internalization is clathrin-mediated, we attempted to interfere more directly with clathrin-dependent pathways. First, we employed as a dominant-negative clathrin heavy-chain mutant, a truncated construct, also known as the Hub fragment, which specifically inhibits clathrin-mediated endocytosis [16]. Cells transiently expressing the Hub fragment were examined for uptake of labeled transferrin and agonist-induced FPRL1 internalization. Non-transfected cells (fig. 3 A, asterisks) showed perinuclear staining for both labeled transferrin and FPRL1, whereas in cells expressing the T7-tagged clathrin Hub mutant (which therefore label with the anti-T7 antibody), a marked reduction in WKYMVm-induced FPRL1 internalization was observed (fig. 3 A, arrows indicating T7-Hub-expressing cells). Again, transferrin internalization was affected in an identical manner. Next, we used small interfering RNAs to deplete cells of clathrin heavy chains. This depletion has been shown to strongly inhibit the uptake of fluorescently labeled transferrin, indicating that clathrin-mediated endocytosis is efficiently impaired [17, 34]. When clathrin heavy-chain down-regulation was

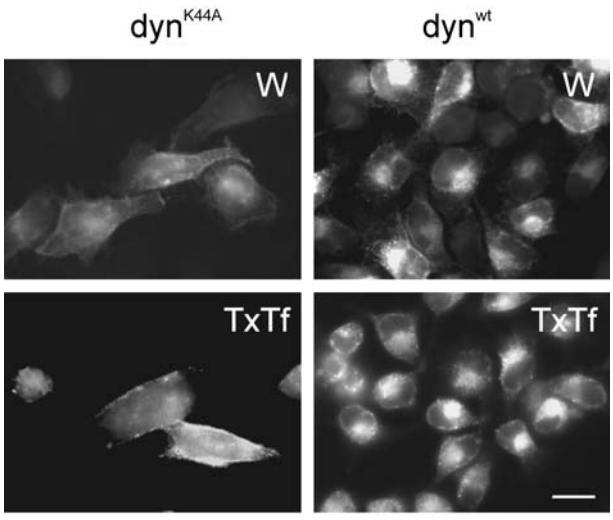

Figure 2. Overexpression of dyn^{K44A} inhibits agonist-induced FPRL1 internalization. Expression of the dominant-negative GTPase-deficient dynamin^{K44A} was induced by tetracycline withdrawal. Cells were then transiently transfected with the FLAG-tagged FPRL1 expression plasmid. Non-induced cells (dyn^{wt}) or cells grown for 48 h in the absence of tetracycline (dyn^{K44A}) were incubated for 10 min with 25 µg/ml Texas Red-labeled transferrin (TxTf) or challenged with 10 µM WKYMVm for 30 min and then processed for immunofluorescence detection of FLAG-FPRL1. In cells expressing dyn^{K44A}, both the transferrin and the FPRL1 signals accumulated at the cell periphery, whereas they were found intracellularly in the absence of dyn^{K44A}. Bar, 10 µm.

visualized with an anti-clathrin heavy chain antibody (fig. 3B, arrows), the reported inhibition of transferrin uptake was observed. The clathrin knock-down also led to a substantial reduction in WKYMVm-triggered FPRL1 internalization. As shown in figure 3B, hardly any structures resembling endosomes loaded with internalized FPRL1 following WKYMVm stimulus were evident. Taken together, these results strongly support the view that FPRL1 is taken up via clathrin-mediated endocytosis.

Internalized FPRL1 recycles via PI3-kinase sensitive and insensitive pathways

Upon 15 min of incubation with the ligand, endocytosed FPRL1 appeared in vesicular structures close to the cell nucleus. To characterize the vesicles involved in FPRL1 trafficking, in particular the perinuclear compartment positive for internalized FLAG-FPRL1, small GTPases were employed in co-labeling experiments as markers. As shown in figure 4, the FLAG signal co-localized to a large extent with GFP-Rab4 and DsRED-Rab11. Rab4 is implicated in the direct recycling from sorting endosomes back to the plasma membrane, but it also appears on perinuclear recycling endosomes enriched in Rab11 [35]. Since it is found on both recycling endosomes and peripheral sorting endosomes, endocytosed FPRL1 co-localized to a greater extent with Rab4 than with Rab11

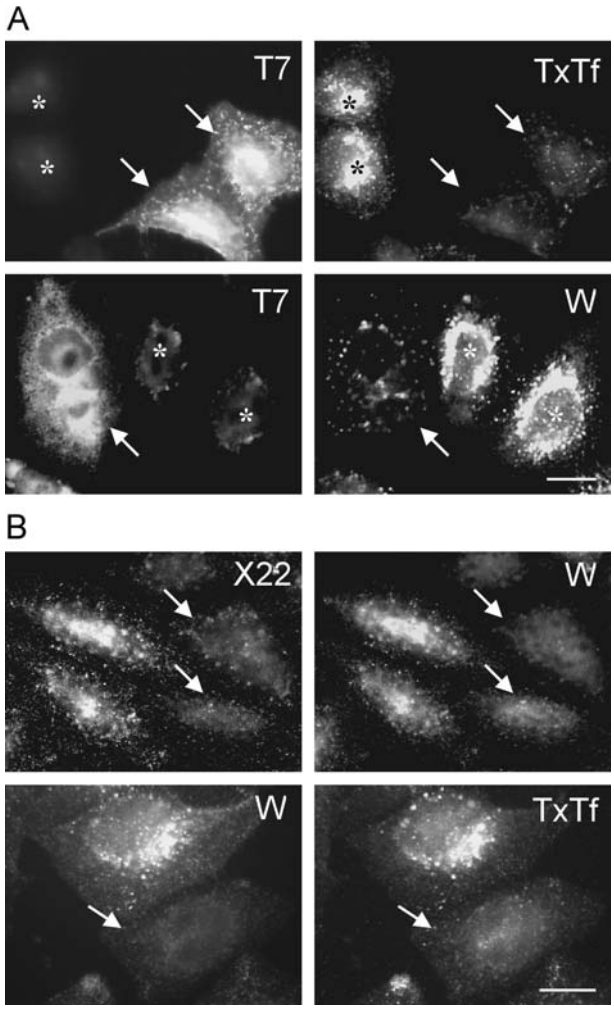

Figure 3. Interfering with endogenous clathrin impairs agonist-induced FPRL1 internalization. HeLa-FLAG-FPRL1 cells transiently expressing the T7 epitope-tagged dominant-negative clathrin Hub fragment (*A*) or transfected with clathrin heavy-chain siRNA to downregulate endogenous clathrin heavy chains (*B*) were incubated with either 10 µM WKYMVm (W) for 30 min or 25 µg/ml Texas Red-labeled transferrin (TxTf) for 10 min. Cells were then processed for immunofluorescence staining. Hub-expressing cells were detected with the anti-T7 monoclonal antibody (T7), clathrin heavy-chain reduction was confirmed with the anti-clathrin heavy chain polyclonal antibody X22 (X22), and FPRL1 was visualized with the anti-FLAG antibody M1 (panels labeled W because the receptor was stained in WKYMVm-treated cells). In (*A*), asterisks mark non-transfected cells which show no staining with the T7 antibody, and arrows indicate the position of the brightly labeled Hub-expressing cells. In (*B*), arrows indicate cells with reduced intracellular clathrin heavy-chain levels. Here FLAG-FPRL1 uptake in cells treated with WKYMVm (W) is shown to be inhibited by clathrin depletion in the co-labeled cells in the upper panels and W-peptide-triggered FPRL1 internalization is shown to be affected in an identical manner to that of transferrin in the co-labeled cells in the bottom panels. Note that both Hub expression and siRNA-mediated clathrin depletion markedly interfere with the agonist-induced uptake of both transferrin and FPRL1 into intracellular endosomes. Bar, 10 µm.

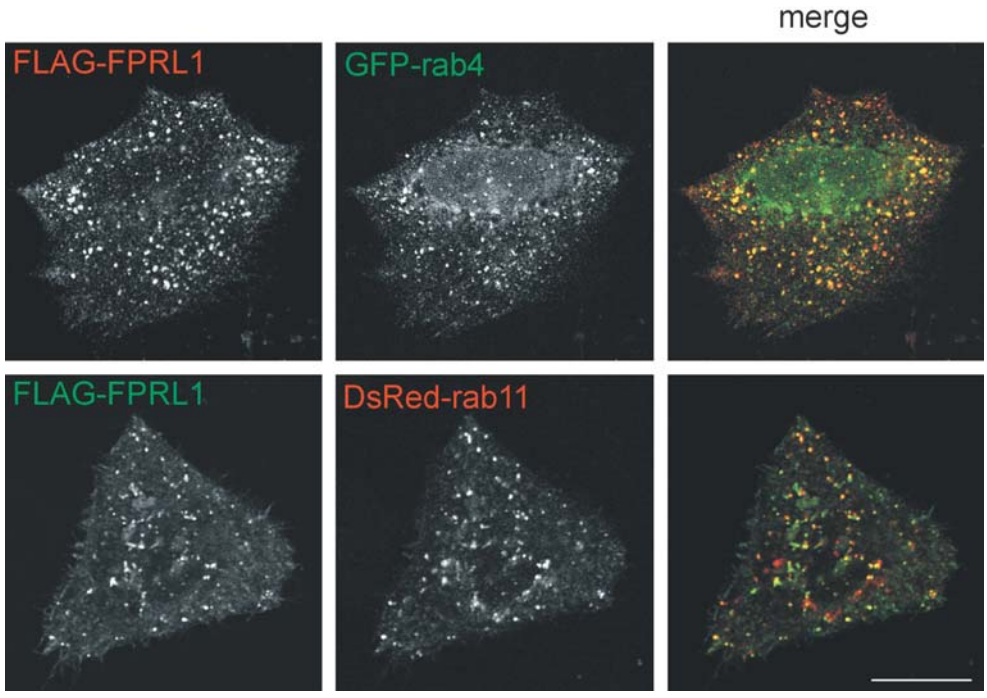

Figure 4. Agonist stimulation directs FPRL1 to vesicular compartments positive for Rab4 and Rab11. HeLa-FLAG-FPRL1 cells transiently expressing GFP-Rab4 or DsRed-Rab11 were exposed for 15 min to 10 μM WKYMVm. Co-localization of the receptor with Rab4 and Rab11 can be observed. Representative confocal images are shown. Bar, 10 μm.

which is rather specific for the perinuclear recycling compartment. The detection of internalized FPRL1 in Rab4/Rab11-positive vesicles adjacent to the nucleus suggests that the internalized receptor had entered the recycling endosomes. This is consistent with the observation that following internalization for 10–15 min, FPRL1 and transferrin were present in the same perinuclear compartment (not shown).

To analyze whether internalized FPRL1 (like transferrin-transferrin receptor complexes) can recycle back to the plasma membrane, we employed whole-cell flow cytometry measurements revealing the presence of the FLAG-tagged FPRL1 on the cell surface. When cells were exposed to WKYMVm for 30 min, a rapid loss of cell surface receptors (approx. 50% of total surface FLAG immunoreactivity, not shown) from the cell surface could be observed, indicative of ligand-induced FPRL1 internalization. Possible receptor recycling to the plasma membrane was elucidated by washing the cells following WKYMVm-induced uptake to remove remaining ligand and then incubating them for additional chase times in ligand-free medium. As shown in figure 5, the FLAG signal on the cell surface slowly recovered and after 75 min reached 80% of the initial value. Longer chase times did not further increase significantly the amount of recycled receptor (not shown). Thus most of the receptors internalized following ligand stimulation recycle back to the cell surface with the kinetics of recycling ($t_{1/2}$ of

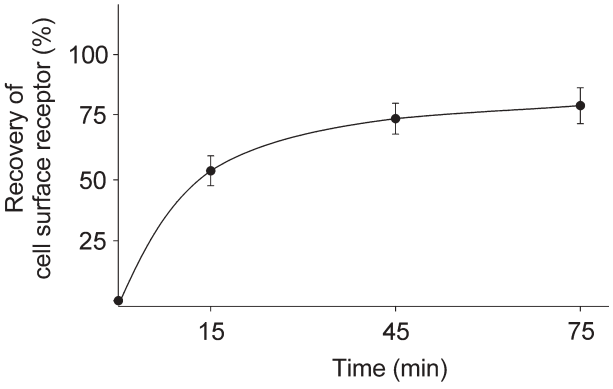

Figure 5. Recycling of internalized FPRL1. HeLa-FLAG-FPRL1 cells preincubated for 1 h with 20 mg/ml cycloheximide were treated with 10 μM WKYMVm for 30 min (pulse), washed and incubated further in the absence of ligand (pulse). Cycloheximide was added during the pulse and the chase period. At the time points indicated, cells were collected on ice, and changes in cell surface receptors were determined by flow cytometry. Receptor recycling was determined as return of cell surface immunofluorescence following agonist removal for the chase times indicated. End of pulse = time point 0. The data represent the mean ± SEM of six independent experiments.

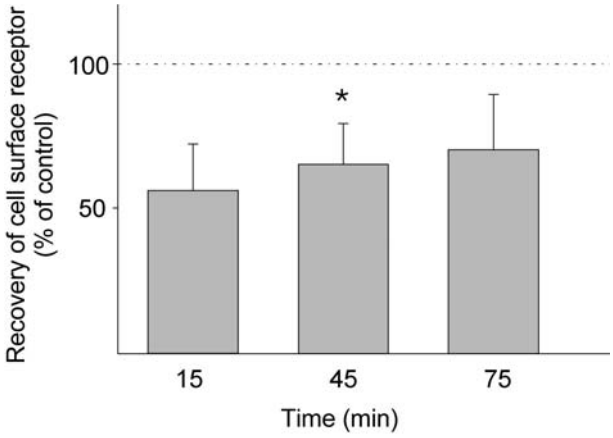

Figure 6. PI3-kinase inhibition partially inhibits FPRL1 recycling. HeLa-FLAG-FPRL1 cells were analyzed for FPRL1 recycling as described in figure 5. To inhibit PI3-kinase, 100 μM of the specific inhibitor LY 294002 was added during the last 10 min of the pulse and throughout the chase phase. FPRL1 recycling in LY 294002-treated cells was calculated as percentage of recycling observed in control cells. The data represent the mean ± SEM of four independent experiments. * $p < 0.05$.

~ 10 min) resembling those of transferrin receptors and the related formyl peptide receptor FPR [36].

Recycling pathways have been extensively characterized for the transferrin receptor and recent studies suggest that recycling proceeds via at least two routes [37–39]. Whereas only a fraction of transferrin recycles through the perinuclear recycling compartment (slow pathway), a substantial part of recycling receptors seem to use a second, more rapid, pathway that is phosphatidylinositol 3 (PI3)-kinase dependent and bypasses recycling endosomes [37]. To analyze whether FPRL1 can also utilize this rapid recycling pathway, we treated cells with LY294002, a PI3-kinase inhibitor known to block the direct recycling of transferrin from sorting endosomes [40]. Whereas FPRL1 internalization was not affected by the inhibitor (not shown), LY 294002 inhibited FPRL1 recycling (fig. 6; approx. 40% inhibition seen at 45 min chase time), suggesting that the recovery of FPRL1 at the cell surface is partially mediated by a PI3-kinase-sensitive mechanism.

Discussion

In the present study we investigated the ligand-induced endocytosis and recycling of the chemotactic receptor FPRL1. Several approaches blocking clathrin-mediated internalization (K^+ depletion, hypertonic sucrose, cholesterol depletion) and expression of the dominant-negative dynamin mutant K44A (dyn^{K44A}) impaired FPRL1 internalization. Furthermore, specifically inhibiting clathrin-mediated endocytosis by transient overexpression of the dominant-negative clathrin Hub fragment or by clathrin depletion using RNAi markedly reduced FPRL1 endocytosis. These results strongly suggest that FPRL1 is internalized by clathrin-mediated endocytosis. Other GPCRs, e.g. the β2-adrenergic receptor (AR) [41, 42] or the α_{1b}-AR [43, 44], also utilize clathrin-mediated and dynamin-dependent uptake pathways following ligand stimulation. However, different internalization mechanisms also exist for GPCRs, since certain dopamine receptors [45] or the cholecystokinin receptor [46] can be endocytosed utilizing pathways not requiring clathrin and/or dynamin [for a review see refs. 12]. Interestingly, a homologue of FPRL1, the prototypic N-formyl peptide receptor FPR, was shown to be internalized by clathrin- and dynamin-independent mechanisms in HEK 293 cells [47]. To compare the internalization mechanisms used by these two closely related receptors in our cell system, we also carried out internalization experiments employing HeLa-FLAG-FPR cells. Similar to the observations for FPRL1, ligand-induced FPR internalization was also inhibited by clathrin siRNA and overexpression of the dyn^{K44A} mutant (data not shown). This indicates that both receptors can utilize clathrin-dependent internalization mechanisms, at least under the conditions used in this study. The observation of Gilbert et al. [47] that FPR can be internalized in cells expressing dyn^{K44A} or the clathrin Hub mutant indicates that this receptor can perhaps use different mechanisms of internalization under certain conditions. Interestingly and in contrast to many other GPCRs, FPR does not require arrestin binding for this type of internalization, although its recycling depends on arrestin binding most likely occurring in the endosomal system [36].

Recent studies on transferrin receptor recycling suggest that only a fraction of the internalized transferrin receptor recycles through a perinuclear subpopulation of endosomal structures termed recycling endosomes [48]. A major part of internalized transferrin takes a fast recycling route from sorting endosomes directly back to the plasma membrane [38]. This direct route that bypasses recycling endosomes appears to depend on PI3-kinase since it is inhibited by wortmannin [37]. FRPL1 recycling shown here to occur following ligand-induced internalization is also sensitive toward PI3-kinase inhibitors. This indicates that a significant fraction of internalized FPRL1 is recycled via the fast route.

Rab proteins are thought to act in organizing membrane domains on endocytic vesicles, thereby regulating the transport of cargo during its sequential passage through the different intracellular compartments. Rab4 is believed to control the rapid recycling from sorting endosomes back to the cell surface, whereas Rab11 seems to operate on the slower recycling pathway involving the transit through the perinuclear compartment of recycling endosomes. The latter are enriched in Rab11, but are also Rab4 positive [38, 49–51]. Our localization studies identified

internalized FPRL1 in such recycling endosomes. Thus, although FPRL1 recycling is partially sensitive to LY 294002, indicative of the fast recycling route dependent on PI3-kinase, the observed co-localization with Rab11 indicates that the receptor also reaches the endosomal recycling compartment. These observations are consistent with the model of a dual recycling mechanism (rapid PI3-kinase-dependent route and slower pathways through the Rab11-enriched recycling endosomes), which has also been proposed for other GPCRs [11, 40, 52–54].

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