N-Formyl Peptide Receptors Internalize but Do Not Recycle in the Absence of Arrestins

Received for publication, July 3, 2003, and in revised form, August 4, 2003 Published, JBC Papers in Press, August 28, 2003 DOI 10.1074/jbc.C300291200

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Arrestins mediate phosphorylation-dependent desensitization, internalization, and initiation of signaling cascades for the majority of G protein-coupled receptors (GPCRs). Many GPCRs undergo agonist-mediated internalization through arrestin-dependent mechanisms, wherein arrestins serve as an adapter between the receptor and endocytic proteins. To understand the role of arrestins in N-formyl peptide receptor (FPR) trafficking, we stably expressed the FPR in a mouse embryonic fibroblast cell line (MEF) that lacked endogenous arrestin 2 and arrestin 3 (arrestin-deficient). We compared FPR internalization and recycling kinetics in these cells to congenic wild type MEF cell lines. Internalization of the FPR was not altered in the absence of arrestins. Since the FPR remains associated with arrestins following internalization, we investigated whether the rate of FPR recycling was altered in arrestin-deficient cells. While the FPR was able to recycle in the wild type cells, receptor recycling was largely absent in the arrestin double knockout cells. Reconstitution of the arrestin-deficient line with either arrestin 2 or arrestin 3 restored receptor recycling. Confocal fluorescence microscopy studies demonstrated that in arrestin-deficient cells the FPR may become trapped in the perinuclear recycling compartment. These observations indicate that, although the FPR can internalize in the absence of arrestins, recycling of internalized receptors to the cell surface is prevented. Our results suggest a novel role for arrestins in the post-endocytic trafficking of GPCRs.

G protein-coupled receptors (GPCRs) are seven transmembrane receptors that couple to heterotrimeric GTP-binding (G) proteins. Agonist binding to the receptor triggers exchange of GDP for GTP on the Gα-subunit of the G protein and consequently the dissociation of the α subunit from the βγ-dimer. Both subunits can regulate the activity of multiple effectors such as adenyl cyclase and phospholipase C. Within seconds of receptor activation, the receptor becomes phosphorylated by GPCR kinases, which increases the affinity of the receptor for cytosolic proteins called arrestins (1).

Arrestin binding to GPCRs prevents receptors from further association with G proteins thereby playing an important role in GPCR desensitization. In addition, arrestin binding can subsequently target receptors to coated pits via associations with clathrin, the clathrin adaptor AP-2, and the intracellular transport ATPase N-ethylmaleimide-sensitive factor (2–4). Arrestins have also been shown to couple GPCRs to G protein-independent signaling pathways such as the activation of Src (5–7), c-Jun NH2-terminal kinase 3 (8), and extracellular signal-regulated kinase (9, 10).

The N-formyl peptide receptor (FPR) is a well described member of the chemotactant subfamily of GPCRs (11). Previous work by our laboratory has demonstrated that while arrestins colocalize with the FPR during receptor internalization, a role for arrestins in the trafficking of the FPR has remained elusive (12–15). Overexpression studies in human embryonic kidney (HEK) cells employed the clathrin-binding domain of arrestin (arrestin319–418). This arrestin fragment, however, displayed no effect on the internalization of the FPR in HEK293 cells (14). While it is thought that normal arrestin functions are blocked by expression of this domain, since HEK cells express endogenous arrestin, it remains unclear whether the FPR is capable of internalization via an arrestin-dependent pathway that is not blocked by the arrestin319–418 peptide. Additional studies using FPR mutants containing only distinct subsets of the potential phosphorylation sites within the carboxyl terminus of the FPR, which do not form stable associations with arrestin, are still capable of ligand-induced internalization (13, 16). However, although no binding of arrestins to these mutant receptors was observed either in vitro or in vivo, it is unclear whether these mutants could form a low affinity transient association sufficient to permit internalization.

Two arrestins are ubiquitously expressed in mammalian cells, arrestin 2 and arrestin 3 (17). Differential roles and cellular distributions have been observed for arrestin 2 and arrestin 3, demonstrating these two proteins exhibit distinct properties (18–20). To define the physiological roles of arrestins 2 and 3 in the trafficking of the FPR, we have used cell lines generated from arrestin 2 and arrestin 3 knock-out mouse embryonic fibroblasts (MEFs). Previous studies using such cell lines have demonstrated that the β2-adrenergic receptor exhibits an absolute requirement for arrestin in internalization, whereas internalization of the angiotensin II type 1A receptor is reduced by ~75% in the absence of arrestins (20). Studies of...
Arrestin-dependent Recycling of the Formyl Peptide Receptor

The protease-activated receptor 1 (PAR1 or thrombin receptor) demonstrated that internalization of this receptor was not dependent on the presence of arrestin (21). Using these MEF cell lines that lack arrestins, we have analyzed the contribution of arrestins to both the internalization and recycling of the FPR.

EXPERIMENTAL PROCEDURES

Materials—fMLF (N-formyl-methionyl-leucyl-phenylalanine) and N-fNleLFNleYK (6 pep) were purchased from Sigma. N-fNleLFNleYK-fluorescein (6 pep-FITC) was purchased from Molecular Probes Inc. N-fNleLFNleYK-Alexa546 and 633 (6 pep-Alexa546 and 633) were synthesized as described previously (22). Dulbecco’s modified Eagle’s medium (DMEM) was purchased from Sigma.

DNA Constructs and Generation of Cell Lines—The cDNA encoding the FPR was obtained from a human HL-60 granulocyte library (23). The two wild type, Arr 2/−, Arr 3/− and the Arr2/−/3/− double knock-out MEF parental cell lines were generated as described previously (20). Plasmid cDNA encoding the FLAG-tagged β2-adrenergic receptor and arrestin 2 (generously provided by Dr. Jeffrey Benovic), the wild type FPR, or the ΔST mutant FPR was subcloned into the pLHCX retroviral vector (Clontech) and the resulting virus transduced into MEF cells and selected as described previously (24). Surviving cells were pooled and FPR expression was confirmed by measuring the binding of 10 nM N-fNleLFNleYK-fluorescein by flow cytometry.

Internalization and Recycling Assays—Cells were harvested, rinsed in serum-free DMEM, and allowed to equilibrate to 37 °C for 10 min. Receptor internalization was initiated by the addition of unlabelled 1 μM fMLF. At the indicated time points (or for 60 min for the recycling assays), cells were transfected with ice cold DMEM and the unbound ligand was removed by four washes in ice-cold serum-free DMEM. For recycling assays, cells were resuspended in prewarmed (37 °C) DMEM and allowed to incubate for the indicated time periods. The receptors on the surface of the cells were labeled with 6 pep-FITC. In the recycling assays, when cells had been transfected with arrestin 2-GFP or arrestin 3-GFP (kindly provided by Dr. Jeffrey Benovic), 6 pep-Alexa633 was used to detect recycled cell surface receptors. The receptors were quantified by flow cytometry on a FACSCalibur (BD Biosciences). Where appropriate GFP-expressing cells were gated in FL1 and analyzed for expression with fluorescent anti-FLAG antibody and quantitated by flow cytometry.

Confocal Fluorescence Microscopy—MEF cells were transfected with an FPR-mRFP1 construct (generated by PCR with mRFP1 kindly provided by Dr. Roger Tsien (33)) and a Rab11-GFP construct (kindly provided by Dr Angela Wandinger-Ness) using LipofectAMINE 2000 (Invitrogen). Transfected cells were stimulated with 10 nM formyl peptide for 30 min at 37 °C, fixed with ice-cold 2% paraformaldehyde for 30 min, permeabilized with 0.1% Triton X-100, and incubated with an anti-arrestin antibody followed by a secondary goat-anti-rabbit Cy5 antibody. Cells were mounted in Vectashield (Vector Laboratories) onto glass slides. Fluorescence images were acquired on a Zeiss LSM 510 confocal microscope.

RESULTS AND DISCUSSION

The FPR Undergoes Arrestin-independent Internalization—To examine the role of arrestin 2 and arrestin 3 in the regulation of FPR trafficking, we stably expressed the wild type FPR and a ΔST FPR mutant in five independently isolated MEF cell lines. These consisted of two wild type lines (WT1 and WT2) and MEF cell lines in which arrestin 2 (Arr2/−), arrestin 3 (Arr3/−), or both arrestin 2/arrestin 3 (Arr2/−/3/−) were homozogously deleted (20). The ΔST FPR mutant lacks all of the potential serine or threonine phosphorylation sites found in the carboxyl terminus and has been shown to mediate ligand-induced calcium fluxes but not to undergo ligand-induced internalization or desensitization (11, 16). Expression of the ΔST and wild type forms of the FPR on the cell surface was confirmed by flow cytometry using the fluorescent ligand N-fNleLFNleYK fluoroscein (data not shown). The ligand affinity for these receptors was similar in all five generated WT FPR cell lines (Kd ~1.0 nM, data not shown).

Arrestins have been suggested to mediate the internalization of many GPCRs, including the β2-adrenergic (20), angiotensin II type 1A (25), dopamine (26), CXCR4 (27), and α2-adrenergic receptors (29). To determine whether internalization of the wild type FPR was regulated by arrestins, we assessed the ability of the WT FPR to undergo ligand-induced internalization in each of the five MEF cells lines. The wild type receptors were rapidly internalized in both wild type MEF cell lines (WT1 and WT2) with t1/2 values of ~7 min and a total of 60–70% of the cell surface receptor being internalized (Fig. 1A). On the contrary, the ΔST FPR mutant demonstrated very little reduction in cell surface expression over the 30-min stimulation period. Similar rates and extents of FPR internalization were also observed in both the Arr 2/− and Arr 3/− single knock-out MEF cell lines, indicating that neither arrestin 2 nor arrestin 3 was individually required for FPR internalization (Table I). We next examined the rate of FPR internalization in the arrestin double knock-out cell line. In these cells, the WT FPR internalized to the same extent as the WT cell lines (Fig. 1B). As expected, the ΔST mutant failed to internalize.

To confirm that the MEF cell lines stably expressing the FPR were functioning as reported previously (20), we examined internalization of a GPCR which internalizes solely by an arrestin-dependent pathway. In the presence of isoproterenol stimulation, the transfected β2-adrenergic receptor was internalized in the wild type cell lines but not in the double knock-out cell line (Fig. 1C and Table I). These results confirmed that in the absence of arrestins, the FPR is capable of ligand-induced internalization.
The FPR Fails to Recycle following Internalization in Arrestin-deficient Cells—Although arrestins were not required for trafficking of the FPR during receptor internalization, arrestins have been shown to regulate the recycling of GPCRs (29–31). Studies comparing the ability of different GPCRs to recycle at 37 °C for the indicated time periods. Recycled receptor was labeled with 6pep-FITC and analyzed by flow cytometry (Fig. 2A). In contrast, the arrestin double knock-out MEF cell line failed to promote recycling of the FPR.

Reconstitution with Arrestin Restores Recycling of the FPR—Since the cell lines used in this study were spontaneously immortalized and individual isolates of MEF cells have been shown to exhibit significant variability in the extent of GPCR internalization (20), we sought to determine whether there were any differences in the rates or extents of recycling mediated by either arrestin 2 or 3. To this end, we transiently transfected the Arr2<sup>−/−</sup>/Arr3<sup>−/−</sup> FPR MEF cells with arrestin restore the FPR with either arrestin 2-GFP or arrestin 3-GFP fusion proteins or GFP alone as a control. Both arrestin 2 and arrestin 3 were capable of rescuing the recycling of the FPR 2-GFP or arrestin 3-GFP fusion proteins or GFP alone as a control. Both arrestin 2 and arrestin 3 were capable of rescuing the recycling of the FPR 2-GFP or arrestin 3-GFP fusion proteins or GFP alone as a control. Both arrestin 2 and arrestin 3 were capable of rescuing the recycling of the FPR (Fig. 3B). These results confirm that the FPR internalized in the absence of arrestin is incapable of recycling.

The FPR Accumulates in Perinuclear Recycling Endosomes in the Absence of Arrestins—To determine whether the distribution of the internalized FPR in the cell is altered in the absence of arrestins, we examined the localization of the FPR, formyl peptide ligand, arrestin 2, and Rab11, a marker of the perinuclear recycling endosome compartment (32). In unstimulated wild type MEF cells, the FPR was localized to the plasma membrane. In contrast, in the absence of arrestins, the FPR might recycle more rapidly. In the wild type MEF cell lines, the FPR recycled rapidly with a half-life of ≈5 min, while 50–60% of the internalized FPR recycled by 20 min (Fig. 2A). Fig. 3. Arrestin-deficient MEF cells accumulate the FPR in perinuclear recycling endosomes. Parental wild type (WT) and arrestin double knock-out MEF cells (KO) were transiently transfected with FPR-mRFP1 and Rab11-GFP (rows 1, 2, and 4) or FPR-mRFP1, arrestin 2, and Rab11-GFP (row 3). FPR-expressing U937 cells were transfected with Rab11-GFP (row 5). Cells were incubated with 10 nM fMLF (rows 2 and 3) or 10 nM fluorescent formyl peptide (rows 4 and 5) for 30 min at 37 °C. The cells were then fixed, permeabilized, and incubated with anti-arrestin 2 antibodies followed by Cy5-conjugated secondary antibodies. In all situations where parallel experiments were carried out with fluorescent ligand and FPR-mRFP1, extensive colocalization between the receptor and ligand was observed. Data are representative of three experiments.

| Cell line | t<sub>1/2</sub> (min) | Extent of receptor internalization (%) |
|-----------|---------------------|---------------------------------------|
| Wild type 1 FPR (WT1 FPR) | 7.4 ± 1.7 | 68 ± 8 |
| Wild type 2 FPR (WT2 FPR) | 7.1 ± 2.0 | 58 ± 8 |
| Arr2<sup>−/−</sup> FPR | 7.9 ± 1.6 | 57 ± 6 |
| Arr3<sup>−/−</sup> FPR | 5.9 ± 2.2 | 57 ± 13 |
| Arr2<sup>−/−</sup>/Arr3<sup>−/−</sup> FPR | 5.1 ± 0.7 | 49 ± 3 |
| Wild type 1 β<sub>2</sub>AR | 5.1 ± 2.7 | 28 ± 10 |
| Wild type 2 β<sub>2</sub>AR | 9.6 ± 4.6 | 35 ± 14 |
| Arr2<sup>−/−</sup>/Arr3<sup>−/−</sup>/β2AR | ND<sup>a</sup> | 0 ± 6 |

<sup>a</sup>ND, not determinable.
membrane, arrestin was found throughout the cytosol in a somewhat punctate pattern, and Rab11 existed predominantly in the perinuclear recycling endosome compartment as well as in distinct vesicles (Fig. 3). Upon stimulation of the FPR, the receptor and arrestin colocalized extensively in intracellular vesicles throughout the cytosol. A fraction of these vesicles colocalized with the Rab11 marker, both in the cytosol as well as in the perinuclear recycling compartment. A similar pattern was observed for arrestin double knockout cells transfected with arrestin 2. However, in arrestin-deficient double knock-out cells, the FPR and its fluorescent ligand were highly concentrated in the perinuclear recycling compartment with little ligand/receptor being present in vesicles throughout the cytoplasm. The distribution of Rab11 in the arrestin double knock-out cells appeared similar to that in the wild type cells. Finally, to determine whether arrestins may serve a similar function in leukocytes, the principle site of FPR expression, we examined the distribution of the FPR, arrestins, and Rab11 in U937 myeloid cells. As in wild type MEF cells, the FPR and arrestin colocalized extensively yet only a fraction of the receptor/arrestin complex was found associated with the perinuclear recycling compartment.

Conclusions—Arrestins are thought to regulate the internalization and desensitization of many GPCRs. While arrestins have been observed to cluster with the FPR in response to ligand binding and receptor activation, a role for arrestin in the regulation of the FPR has remained unclear. We found that although FPR internalization was not dependent on the presence of either arrestin 2 or arrestin 3, similar to previous results with PAR1 (21), FPR recycling to the plasma membrane was inhibited in the absence of arrestins. How arrestins are involved in regulating the recycling of the FPR is not yet known. Recycling of GPCRs is thought to occur following endosome acidification, arrestin dissociation, and receptor dephosphorylation as the receptor traffics through multiple vesicular compartments in the cytosol. Since arrestins serve as adaptors for many proteins, it is intriguing to speculate that arrestins may specifically regulate the post-endocytic intracellular trafficking of GPCRs or possibly even regulate the binding/activity of phosphatases. Thus, FPR internalized in the absence of arrestin binding may traffic improperly within the cell resulting in intracellular retention and commensurate recycling defects. In wild type cells, therefore, one role for arrestin may be the proper targeting of the wild type receptor to or from a recycling compartment. Our results suggest that in the absence of arrestins, the FPR becomes concentrated in perinuclear recycling endosomes, indicating that arrestin may have a role in the progression of the FPR through this compartment. The precise mechanisms involved in this trafficking defect, however, remain to be determined. It is interesting to note that the V2 vasopressin receptor that does not recycle upon endocytosis also accumulates in the perinuclear recycling compartment, though in this case with arrestin colocalizing with the receptor (30).

In conclusion, our results demonstrate that the associations of arrestins with GPCRs are complex and that as more GPCRs are characterized, novel modes of regulation by arrestins are likely to be described. The FPR is only the second GPCR that has been found to internalize in the absence of arrestins 2 and 3, but the first receptor demonstrated not to recycle in the absence of arrestins. We propose that the FPR may define a third class of GPCRs, which can be internalized in the absence of arrestins but requires arrestins for proper trafficking and/or recycling.
