Internalization of the Human *N*-Formyl Peptide and C5a Chemoattractant Receptors Occurs via Clathrin-Independent Mechanisms†

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ABSTRACT: After stimulation by ligand, most G protein-coupled receptors (GPCRs) undergo rapid phosphorylation, followed by desensitization and internalization. In the case of the *N*-formyl peptide receptor (FPR), these latter two processing steps have been shown to be entirely dependent on phosphorylation of the receptor's carboxy terminus. We have previously demonstrated that FPR internalization can occur in the absence of receptor desensitization, indicating that FPR desensitization and internalization are regulated differentially. In this study, we have investigated whether human chemoattractant receptors internalize via clathrin-coated pits. Internalization of the FPR transiently expressed in HEK 293 cells was shown to be dependent upon receptor phosphorylation. Despite this, internalization of the FPR, as well as the C5a receptor, was demonstrated to be independent of the actions of arrestin, dynamin, and clathrin. In addition, we utilized fluorescence microscopy to visualize the FPR and β_2-adrenergic receptor as they internalized in the same cell, revealing distinct sites of internalization. Last, we found that a nonphosphorylatable mutant of the FPR, unable to internalize, was competent to activate p44/42 MAP kinase. Together, these results demonstrate not only that the FPR internalizes via an arrestin-, dynamin-, and clathrin-independent pathway but also that signal transduction to MAP kinases occurs in an internalization-independent manner.

The human *N*-formyl peptide receptor (FPR)[1] is a member of the G protein-coupled receptor superfamily and is expressed predominantly on leukocytes, where it mediates cell migration and activation in the presence of agonist. Such chemoattractant receptors, which include the receptors for PAF, LTB4, the complement components C5a and C3a, and the related chemokine receptors, are critically involved in leukocyte trafficking and activation (*1*). These receptors have come under increased scrutiny since the recent discovery that HIV requires a chemokine coreceptor for infection (*2*). A cell's ability to respond appropriately to an external signal is thought to be contingent upon the capacity of its receptors to become activated, inactivated, and reactivated in the presence of agonist in an appropriate spatial and/or temporal manner. Since the ability to chemotax requires detecting a difference in agonist concentration over the length of a cell, it has been suggested that precise control of receptor inactivation and processing may play an integral part in the ability of a cell to function effectively. Furthermore, numerous pathological conditions such as arthritis, atherosclerosis, and ischaemia/reperfusion injury result in part from excess chemoattractant-mediated activation of leukocytes (*3*−*5*).

Current models of G protein-coupled receptor (GPCR) processing assert that desensitization of ligated receptors is initiated by serine/threonine kinase-mediated phosphorylation (*6*). Phosphorylation by second messenger-dependent kinases, such as cAMP-dependent protein kinase, has been shown to diminish the extent of signaling of some G protein-coupled receptors (*7*). Virtually all activated G protein-coupled receptors, however, appear to be substrates for G protein-coupled receptor kinases (GRKs). Receptor phosphorylation by GRKs is alone insufficient to produce desensitization. However, this phosphorylation allows the interaction of an auxiliary molecule, named arrestin, which serves to block receptor from binding the G protein (*8*−*10*). In addition to their central role in desensitization, arrestins have also been implicated in the targeting of receptors for endocytosis and therefore may play a dual role in both the desensitization and sequestration pathways (*11*). Receptor internalization has been shown to be involved in several processes, including receptor downregulation through degradation (*8*), novel signaling pathways through tyrosine kinases (*12*), and receptor recycling to the cell surface (*8*). It is not yet clear what role arrestin-like molecules may play in the regulation of chemoattractant receptors, such as the FPR. We have demonstrated that the desensitization (*13*) and internalization (*14*) of the FPR is completely dependent upon phosphorylation of the receptor carboxy terminus. However, further analysis revealed that the two processes of desensitization and internalization must occur through distinct mechanisms (*15*). Through the characterization of partially defective phosphorylation mutants of the FPR, we have shown that a mutant which was unable to undergo desensitization was however unaltered in its ability to be internalized. Further-

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[1] Abbreviations: β_2AR, β_2 adrenergic receptor; FPR, *N*-formyl peptide receptor; fMLF, *N*-formyl-methionyl-leucyl-phenylalanine; G protein, guanine nucleotide-binding regulatory protein; GPCR, G protein-coupled receptor; GRK, G protein-coupled receptor kinase.

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more, the recent characterization of the FPR mutant, R123G, demonstrated that internalization can occur in the absence of arrestin binding (*16*). This revealed the independence of the two mechanisms and led to the question about the roles of accessory molecules involved in mediating desensitization and internalization of the FPR and related chemoattractant receptors.

The prototypic nonvisual GPCR is the β_2-adrenergic receptor (β_2AR). Once this GPCR is activated by an agonist, it becomes phosphorylated on its cytoplasmic tail by GRK2 (17). This phosphorylated form of the receptor tightly binds arrestins, which functionally uncouple the receptor from the G protein (*18*), yet activates downstream signaling pathways through Src binding, resulting in the activation of MAP kinases (*12*). Studies utilizing dominant negative forms of β-arrestin, which no longer mediate receptor translocation to clathrin-coated pits, have demonstrated that the binding of β-arrestin is required for sequestration of the β_2AR (*12*, *19*). β-Arrestin contains a clathrin-binding domain, consisting of the 100 carboxy-terminal amino acids (*11*). This domain presumably facilitates the recruitment of the desensitized β_2-ARs to clathrin-coated pits. Another critical step in the internalization of the β_2AR involves the physical pinching off of clathrin-coated pits to form endosomes. This process is dependent upon dynamin, a GTPase that is integral to the regulation and/or physical process of clathrin-coated pit cleavage from the membrane (*20*). Coexpression of the β_2-AR with a dominant negative form of dynamin (a GTPase-deficient K44A mutation) further indicates that the β_2AR is internalized through clathrin-coated pits in a dynamin-dependent manner (*20*, *21*). Both arrestin and dynamin mutants have been shown to block the activation of MAP kinases, reflecting an essential role for arrestin in Src activation and both proteins in receptor internalization.

Like the β_2AR, the FPR is phosphorylated by GRK2 in response to stimulation with an agonist (*13*); however, it is unclear whether the FPR internalizes using the same machinery as the β_2AR. In this study, we investigate whether the class of chemoattractant receptors as represented by the FPR and C5a receptor internalize via mechanisms similar to those of the β_2AR. Utilizing flow cytometry to evaluate receptor internalization, we determined through the use of dominant negative mutants of arrestin, dynamin, and clathrin that the FPR does not internalize through clathrin-coated pits. Furthermore, visualization of the FPR and β_2AR expressed in the same cell demonstrated that the two receptors do not colocalize as they internalize. Last, we demonstrated that the activation of p44/42 MAP kinase by the FPR is independent of arrestin binding and receptor internalization. Together, these results confirm that, unlike the β_2AR, chemoattractant receptors internalize via pathways independent of arrestin-mediated translocation to clathrin-coated pits.

MATERIALS AND METHODS

Materials. The cDNA encoding the FPR was obtained from a human HL-60 granulocyte library as described previously (*22*). The cDNAs encoding the FLAG epitope-tagged β_2-adrenergic receptor (β_2AR), wild-type arrestin-2, wild-type arrestin-3, the carboxy-terminal fragment of arrestin-2 ($arr^{319-418}$), wild-type dynamin, and the dominant negative dynamin mutant (K44A) were generously supplied by J. Benovic at Thomas Jefferson University (Philadelphia, PA). The clathrin hub cDNA construct was supplied by F. Brodsky at University of California at San Francisco (San Francisco, CA) (*23*). The C5a antibody against amino acids 9−29 was a generous gift from T. Hugli at the Scripps Research Institute (La Jolla, CA) (*24*). All of the constructs were in vector pcDNA3 (or pCDM8 for the hub construct). The dynamin proteins contain the HA peptide sequence (YPYDVPDYA) and were detected with the 12CA5 monoclonal antibody (BabCo). Anti-T7 tag antibody for detection of the hub construct was from Novagen. The Texas Red-conjugated goat anti-mouse antibody was from Vector Laboratories. C5a, isoproterenol, fMLF, and the M2 anti-FLAG monoclonal antibody were purchased from Sigma.

HEK 293 Cell Culture and Transfection. Cells were grown in RPMI 1640 medium supplemented with 10 mM Hepes, 10% (v/v) bovine calf serum (Hyclone), 100 units/mL penicillin and streptomycin, and 2 mM L-glutamine. Approximately $2-3 \times 10^6$ human embryonic kidney (HEK-293) cells were plated per 60 mm dish (Corning) 1 day prior to transfection. Using lipofection (Lipofectamine, Gibco BRL), each plate was transfected with a total concentration of 2.5 μg of DNA, which included the receptor construct and either an accessory protein construct, the dominant negative phenotype of that protein or vector only as a control. For internalization assays 2 days after transfection, cells were removed from the plates with cell dissociation buffer (Sigma), washed once, resuspended in Hank's buffered saline solution (HBSS, Gibco BRL), and placed on ice.

Equilibrium Binding. Transiently transfected HEK 293 cells were harvested as described above, washed, and resuspended in PBS on ice. Equilibrium binding of the agonist *N*-formyl-Met-Leu-Phe-Phe-fluorescein was carried out for 45 min on ice in the absence and presence of a 1000-fold molar excess of fMLF, to determine the levels of total and nonspecific binding, respectively. Ligand-stained cells were analyzed for mean fluorescent intensity on a FACScan flow cytometer (Becton Dickenson) with dead cells excluded by a gate on forward and side scatter. Data analysis was performed with Prism (Graphpad). Total receptor numbers per cell were estimated by comparison of the fluorescent intensity of stained cells with fluorescein standard beads (Bangs Laboratories).

Receptor Internalization. The level of receptor internalization was determined as the level of loss of receptor from the cell surface. Transiently transfected HEK 293 cells were harvested, washed, and resuspended in serum-free RPMI for the β_2AR and C5a receptor (C5aR) or TBS for the FPR. Cells were treated with a saturating dose of ligand (10 μM isoproterenol for the β_2AR, 500 nM C5a for the C5aR, and 10 μM fMLF for the FPR) and allowed to internalize (45 min for the β_2AR and the C5aR and 30 min for the FPR) at 37 °C, unless otherwise indicated. The ligand was removed (with three centrifugation washes of incubation buffer), and the cell surface receptors were labeled. For cells expressing the FPR, the remaining cell surface receptors were determined with 10 nM *N*-formyl-Nleu-Leu-Phe-Nleu-Tyr-Lys-fluorescein or a monoclonal antibody (5F1-9, Leukosite Inc.) directed against the extracellular portions of the FPR. For cells expressing the β_2AR, a monoclonal antibody against the FLAG epitope was used to determine the remaining

surface receptors. C5aRs were labeled with the antibody against amino acids 9−29. Antibody-bound receptors were then incubated with a fluorescein-conjugated goat secondary antibody for detection. Ligand- or antibody-stained cells were analyzed for fluorescent intensity on a FACScan flow cytometer (Becton Dickenson) with dead cells excluded by a gate on forward and side scatter. The level of receptor internalization was determined relative to cells that had not been treated with ligand.

Western Blot Analysis. Proteins were separated by SDS−PAGE and transferred to PVDF membranes (Gelman) with a semidry transfer apparatus (Owl Scientific). Membranes were blotted with antibody against the specified tag or protein followed by an HRP secondary antibody. The blots were developed using ECL Plus (Amersham) and imaged using a Phosphoimager (Molecular Dynamics Storm 860).

Fluorescence Microscopy. HEK 293 cells were transiently cotransfected to express both the FPR and β_2AR. Receptor internalization was stimulated by incubating with ligand, 10 nM fMLFK-FITC and 10μM isoproterenol for the FPR and β_2AR, respectively, for the indicated times. Cells were then placed on ice, fixed with 2% PFA for 30 min, and permeabilized with 0.02% saponin. Cells were then incubated with the M2 FLAG primary antibody to detect the β_2AR epitope tag, followed by a goat anti-mouse secondary antibody conjugated to Texas Red. Cells were washed three times, resuspended in Vectashield (Vector Laboratories), and placed on a slide. Fluorescence images were acquired on a Zeiss LSM 510 confocal microscope to localize both the FPR (green) and β_2AR (red) in cotransfected cells.

MAP Kinase Assays. Transiently transfected HEK cells expressing either the wild type or ΔST mutant form of the FPR were harvested, resuspended to a density of 8×10^6 cells/mL in PBS, and stimulated with 1 μM fMLF at 37 °C for the indicated time. Samples were quenched with the addition of an equal volume of ice-cold PBS, pelleted, solubilized with Laemmli sample buffer, and sonicated to break up chromosomal DNA. SDS−PAGE and Western blots were performed as described above using antibodies and reagents for the detection of nonphosphorylated and phosphorylated p44/42 MAP kinase (New England Biolabs).

RESULTS

When the formyl peptide receptor (FPR) is stably expressed via transfection in U937 promonocytic cells, it demonstrates ligand binding and physiological properties characteristic of the natively expressed FPR in neutrophils (*25*). Our previous work, examining the properties of phosphorylation-deficient FPR mutants expressed in U937 cells, demonstrated an absolute requirement for receptor phosphorylation in the process of internalization (*14*) but revealed an apparent distinction between the mechanisms utilized for desensitization versus internalization (*15*). In the interest of determining the role of accessory proteins in the processing of the FPR after activation by ligand, we have expressed the FPR and various accessory proteins transiently in the HEK 293 cell line. These cells have repeatedly been shown to express G protein-coupled receptors in a functional state, amenable to molecular analysis (*26*, *27*). Furthermore, the transient nature of the expression allows mutant proteins such as dominant negative dynamin, which would be toxic if stably expressed, to be analyzed.

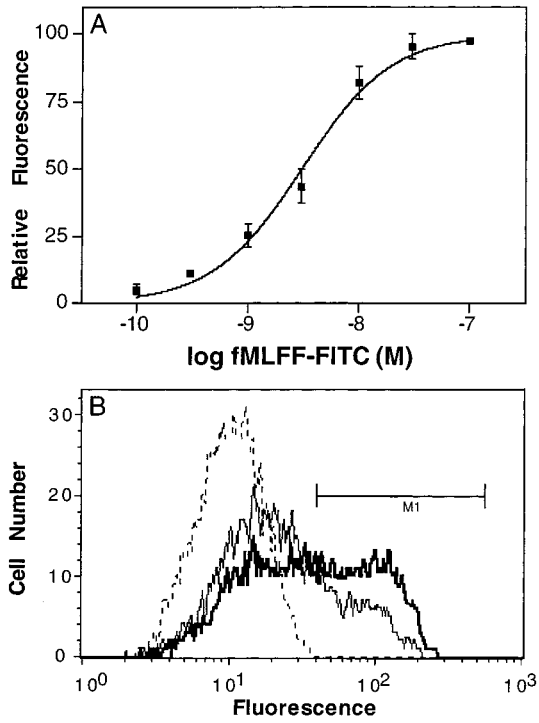

FIGURE 1: Flow cytometric analysis of FPR ligand binding and internalization. (A) The level of equilibrium binding of the agonist *N*-formyl-Met-Leu-Phe-Phe-fluorescein was determined for HEK cells transiently transfected with the FPR. Nonspecific fluorescence was determined by including a 1000-fold molar excess of fMLF. (B) The level of internalization of cell surface receptors was determined by measuring the decrease in the extent of cell surface expression of the FPR following ligand stimulation. Cells were treated with either buffer alone (solid thick line) or 1 μM fMLF (solid thin line) for 30 min at 37 °C to induce internalization of the FPR. Following washes to remove agonist, remaining cell surface receptors were quantified with *N*-formyl-Nleu-Leu-Phe-Nleu-Tyr-Lys-fluorescein. Nonspecific fluorescence was determined by including a 1000-fold molar excess of fMLF (dashed line), and a marker (M1) was set to distinguish the expressing cells. Data are representative of controls performed for each assay.

To confirm the expression and functional binding of the FPR in transient transfections of HEK 293 cells, equilibrium binding was carried out using a fluorescent formyl peptide ligand (Figure 1A). Flow cytometric determination of the level of binding of the high-affinity agonist *N*-formyl-Met-Leu-Phe-Phe-fluorescein revealed a K_d of 3.0 ± 0.8 nM. Comparison of the mean fluorescence of the transfected cell population to standard fluorescent beads demonstrated that the FPR-transfected cells expressed an average of 500 000 receptors per cell (data not shown). Internalization of the receptors in response to ligand stimulation was also assayed in a flow cytometer by determining the level of depletion of surface receptors in response to agonist stimulation. Nonspecific binding was quantitated by labeling cells in the presence of an excess of nonfluorescent ligand (Figure 1B, dashed line), and a marker was set to exclude this cell population (M1). Approximately 30−50% of the cells transfected with the FPR construct express a high level of the receptor as determined by labeling with a fluorescent formyl peptide (solid thick line). Internalization was assessed in a sample of cells that was exposed to fMLF at 37 °C for 30 min to induce internalization. Remaining surface-bound fMLF was removed with extensive washing, and the

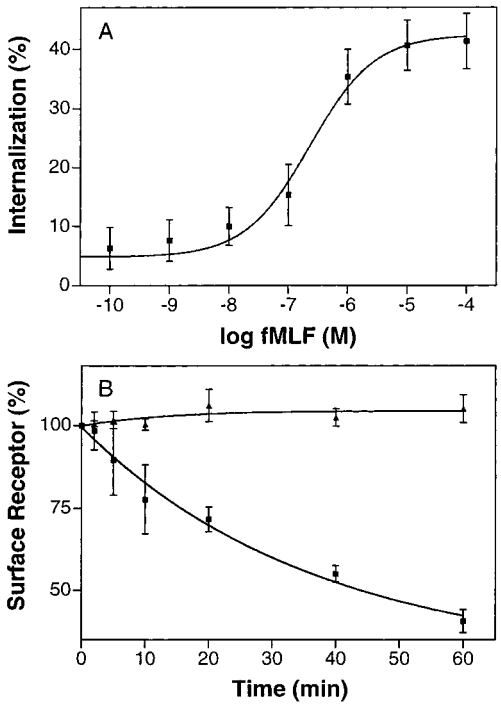

FIGURE 2: Characterization of FPR internalization in transiently transfected HEK 293 cells. (A) Response of FPR-transfected HEK cells to increasing concentrations of fMLF. Samples were treated as described in the legend of Figure 1 with fMLF concentrations varying from 0.1 nM to 100 μM. Cell surface expression of the remaining receptors was assessed with 10 nM *N*-formyl-Nleu-Leu-Phe-Nleu-Tyr-Lys-fluorescein. (B) Time course of internalization of the wild-type FPR (■) compared to ΔST mutant FPR (▲). Receptor internalization was assessed as a function of time by detecting the level of cell surface expression as outlined above following exposure of the cells to 1 μM fMLF. Data represent the mean of three separate experiments (± standard error of the mean).

fluorescent peptide was added to quantify the remaining surface-bound receptors (solid thin line). Internalized receptors are quantified by calculating the fraction of cells in the agonist-treated population compared to the cell number in the untreated population. When this procedure is carried out on ice, where internalization is prevented, there is no evidence of receptor internalization (data not shown).

Using the procedure outlined above, we evaluated the time course and dose−response characteristics of internalization of the FPR expressed in HEK 293 cells (Figure 2). On the basis of the dose−response curve in Figure 2A, we subsequently used 1 μM fMLF as the ligand in the internalization assays that followed. Internalization occurred with a half-time of approximately 25 min following the addition of agonist. This rate is somewhat slower than what we have shown for the FPR that has been stably transfected into U937 cells (*14*) and that has been reported for the FPR in neutrophils (*28*). It is however comparable to the rate of internalization of other G protein-coupled receptors, including the β_2AR, expressed in HEK cells (*29*). Since the level of receptor expression is high in some of the transfected cells, the internalization machinery may be a limiting factor in these assays, resulting in slower overall internalization rates with somewhat higher concentrations of fMLF required to induce internalization. Despite this, internalization of the FPR in HEK 293 cells remained dependent on receptor phosphorylation. We demonstrated this by transfection of the previously described ΔST mutant form of the FPR, in which all 11 of the serine and threonine residues of the carboxy terminus have been mutated to alanine or glycine (*14*, *30*). In HEK 293 cells, as in myeloid U937 cells, this mutant does not undergo internalization (Figure 2B), further confirming the appropriateness of the HEK cell system as a model system for chemoattractant receptor processing.

Much of what is known about GPCR internalization has been elucidated from studies with the prototypic GPCR, the β_2AR. Arrestins have been demonstrated to have adapter functions that couple the β_2AR directly to clathrin (*11*) and AP-2 (*31*) and when overexpressed can increase the level of receptor internalization. An arrestin domain from the C-terminal region of the protein (amino acids 319−418) that binds clathrin but does not bind the phosphorylated receptor has been shown to inhibit agonist-stimulated internalization of the β_2AR (*32*). Expression of dynamin proteins, implicated in the pinching off of vesicles from the plasma membrane, defective in their GTPase activity (dynamin K44A) also results in inhibition of β_2AR internalization (*21*). Last, it has recently been shown that overexpression of a portion of the clathrin molecule, derived from the central hub region of the triskelion, can disrupt clathrin-mediated internalization (*33*) by competing with intact clathrin for the newly synthesized light chain (*23*). Through the combined use of these reagents, it is possible to gain substantial insight into the mechanisms involved in receptor processing.

To investigate if the mechanisms of FPR internalization are similar to those known for the β_2AR, we cotransfected HEK 293 cells with GPCRs and the molecules, described above, implicated in internalization through clathrin-mediated mechanisms (Figure 3). As a control, we first expressed the arrestin, dynamin, and clathrin hub proteins with the β_2AR (Figure 3, top panel). In cells expressing only the β_2AR, stimulation of the receptor with isoproterenol resulted in a 40% decrease in the level of surface-expressed receptors. Although coexpression of arrestin-2 and arrestin-3 did not enhance this internalization, coexpression of the arrestin$^{319-418}$ fragment resulted in a significant decrease in the level of internalization (60−70%). Coexpression of the wild-type dynamin protein also had no effect on the basal level of internalization, whereas coexpression of the K44A mutant completely prevented internalization. Similarly, coexpression of the clathrin hub domain completely abolished β_2AR internalization. These results established that β_2AR internalization followed the expected paradigm for clathrin-mediated internalization.

We next performed identical experiments replacing the β_2-AR with the FPR (Figure 3, middle panel). Unlike the results obtained with the β_2AR, internalization of the FPR demonstrated no significant inhibition by either the arrestin$^{319-418}$ fragment as compared to the arrestin-2 control or the dynamin K44A mutant as compared to the wild-type dynamin protein. There was, however, inhibition of FPR internalization observed with expression of the wild-type dynamin protein, which was not seen with the β_2AR. Internalization of the FPR in the presence of the clathrin hub fragment was inhibited by only ∼20%, compared to the >95% inhibition observed with the β_2AR. These results strongly suggest that the FPR undergoes internalization utilizing mechanisms not involving arrestin, dynamin, or clathrin. To investigate whether this mode of internalization is utilized by other

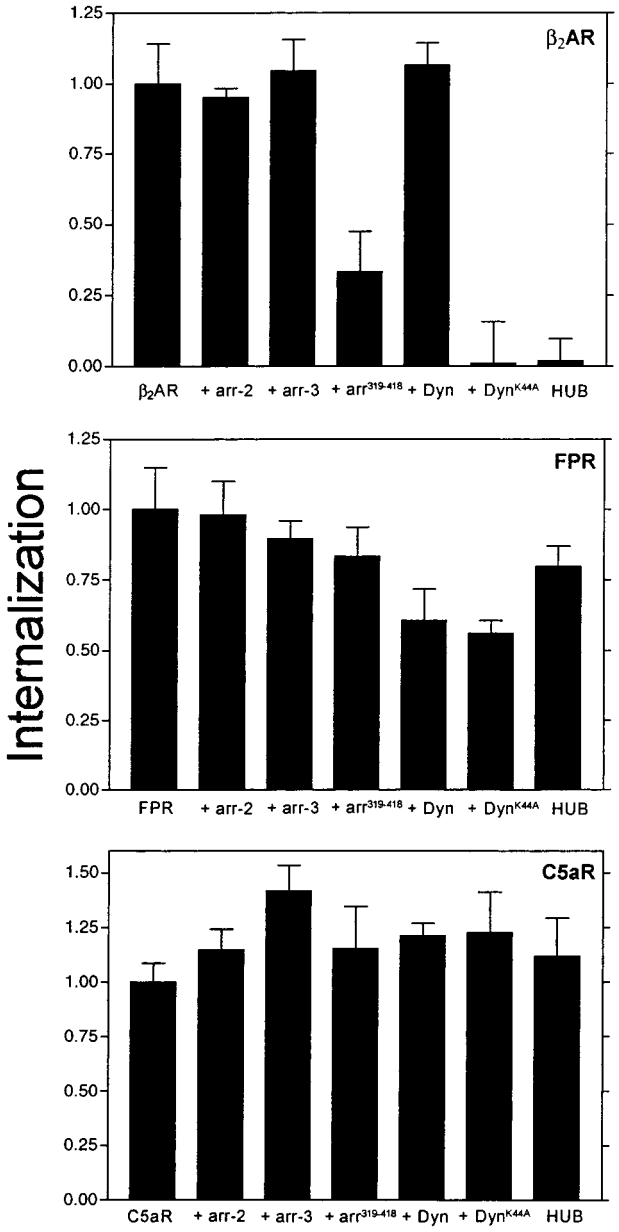

FIGURE 3: Effect of arrestin, dynamin, and hub cotransfection on wild-type β_2AR, FPR, and C5a receptor internalization. HEK 293 cells were cotransfected with either the β_2AR (top), the FPR (middle), or the C5aR (bottom) and an arrestin or arrestin mutant, a dynamin or dynamin mutant, or the clathrin hub protein. Samples were treated with ligand (10 μM isoproterenol for the β_2AR, 1 μM fMLF for the FPR, and 500 nM C5a for the C5aR) and allowed to internalize at 37 °C for 45 min (30 min for the FPR). Cell surface expression of the remaining FPR was assessed with 10 nM *N*-formyl-Nleu-Leu-Phe-Nleu-Tyr-Lys-fluorescein. The remaining β_2AR was labeled with an antibody to the FLAG epitope tag, and the C5a receptors were labeled with antibody directed to the receptor amino-terminal domain. Secondary fluorescein-labeled antibodies were used to determine the remaining level of receptor expression. Data are normalized to the receptor only transfected cells (see Materials and Methods) and represent an average of at least three separate experiments (± standard error of the mean).

chemoattractant receptors, we also investigated whether internalization of the C5a receptor displayed a dependence on these cellular components. Figure 3 (bottom panel) shows that the C5a receptor, like the FPR, undergoes internalization in an arrestin-, dynamin-, and clathrin-independent manner. Together, these data indicate that chemoattractant receptors

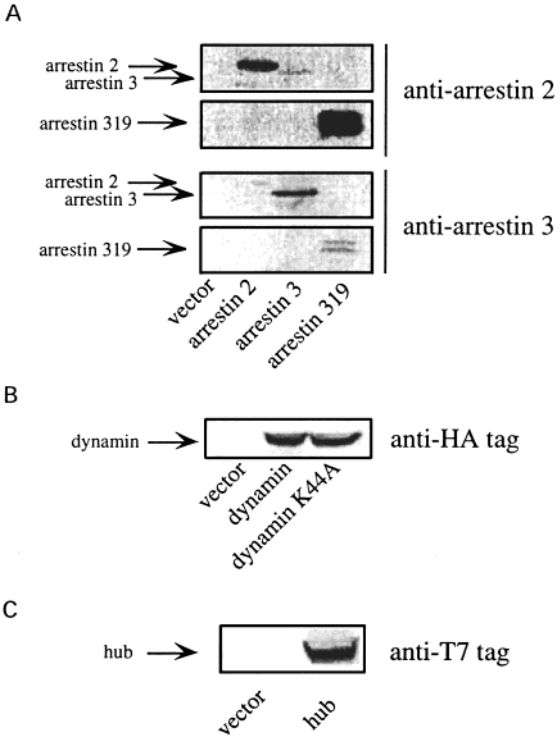

FIGURE 4: Intracellular expression of transfected proteins determined by Western blot analysis. Approximately 2×10^6 cells cotransfected with the FPR and the specified protein were solubilized in SDS sample buffer and sonicated to disrupt the DNA. Approximately 10^6 cells were loaded per lane, run on a 10% gel, and transferred to a PVDF membrane. Immunoblots were detected with a phosphoimager using the ECL Plus system. (A) Arrestin-2, arrestin-3, the arrestin$^{319-418}$ fragment, and a vector control were blotted with an antibody against arrestin-2 (top two panels) or arrestin-3 (bottom two panels). (B) Dynamin, the dominant negative mutant dynamin K44A (both containing the HA epitope tag), and a vector control were blotted with an antibody against the HA tag. (C) The T7 epitope-tagged hub protein and vector control were blotted with an antibody to the T7 tag. Each blot is representative of three separate assays.

utilize internalization machinery completely distinct from that utilized by the β_2AR.

To confirm that the transfected cells were expressing the desired proteins, we analyzed the cellular content of representative FPR-cotransfected cells by Western blotting. Figure 4A demonstrates the presence of arrestin-2, arrestin-3, and the arrestin$^{319-418}$ fragment utilizing antibodies against both arrestin-2 and arrestin-3. These antibodies exhibit a low level of cross-reactivity between anti-arrestin-2 and arrestin-3 and between anti-arrestin-3 and arrestin-2. Utilizing conditions to visualize the transfected proteins, endogenous levels of arrestins were not detectable. Figure 4B demonstrates that similar levels of transfected, tagged dynamin and the dynamin K44A mutant are expressed in transfected cells, indicating that the mutation does not affect the expression level of this protein. Finally, transfection with the clathrin hub fragment yields a unique band in samples probed with the anti-T7 tag antibody.

On the basis of these results, we speculated that if the FPR utilizes discrete mechanisms of internalization compared to the β_2AR, it would be possible to determine visually whether the FPR and β_2AR localize to distinct regions of the cell during internalization. Figure 5 shows representative

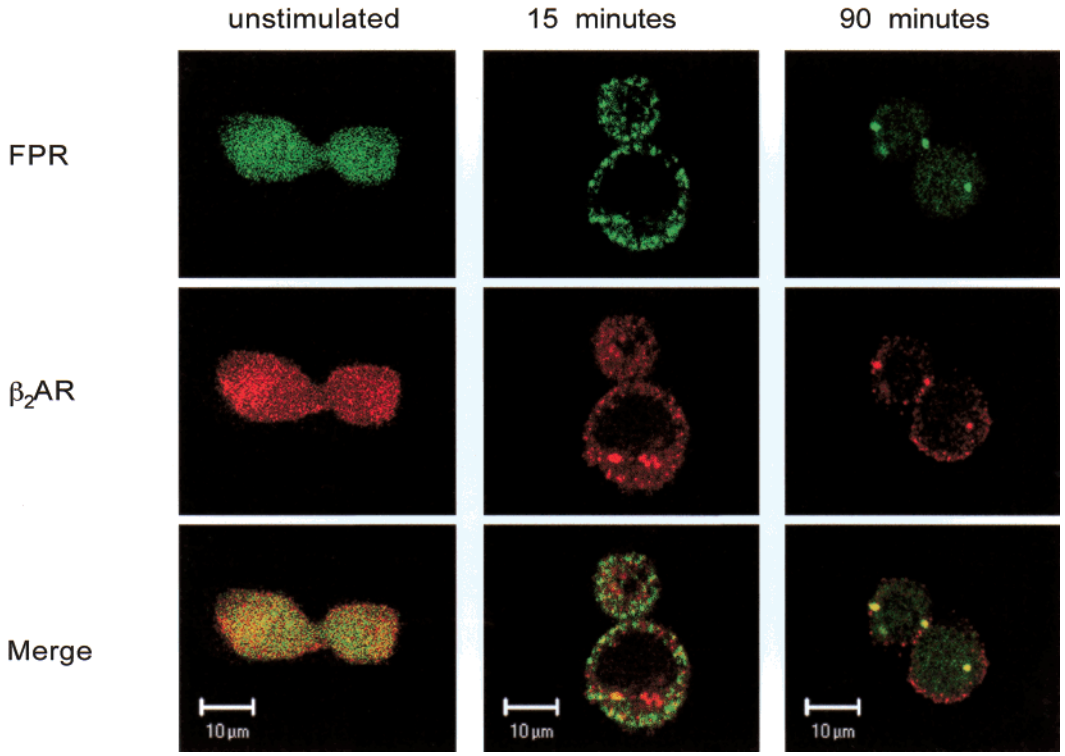

FIGURE 5: Distribution of the FPR and β_2AR following stimulation with agonist. HEK 293 cells were transiently cotransfected with the FPR and FLAG epitope-tagged β_2AR. Cells were stimulated with 10 nM *N*-formyl-Met-Leu-Phe-Lys-fluorescein and 10 μM isoproterenol for the specified amounts of time. Cells were then placed on ice, fixed with 2% PFA, and permeabilized with 0.02% saponin. Primary antibody to the FLAG epitope followed by a secondary antibody conjugated to Texas Red was used to visualize the β_2AR. Confocal fluorescence microscopy was used to analyze the simultaneous internalization of the FPR (green) and β_2AR (red) in the same cells. Images are representative of three separate assays.

fields of HEK 293 cells cotransfected with the FPR and β_2-AR undergoing internalization as visualized using confocal fluorescence microscopy. Cells were incubated with the fluorescent ligand to label the FPR and with the anti-FLAG antibody after fixation to label the β_2AR. In unstimulated cells, both receptors are found in a diffuse pattern on the cell surface. Following 15 min of stimulation with ligands, it is clear that the regions of internalization of the two receptors exhibit almost no overlap. By 90 min, however, the two receptors partially colocalize within the cell, suggesting the eventual fusion of endosomal compartments. These results are entirely supportive of our conclusion that the FPR is internalized via mechanisms distinct from the β_2AR.

Last, it has been shown that arrestin binding and receptor internalization can be required for the activation of p44/42 MAP kinase by the β_2AR (*12*). We therefore investigated whether internalization of the FPR was required to activate MAP kinases. For this purpose, we utilized the ΔST mutant form of the FPR, which is not phosphorylated and therefore not internalized, but is fully capable of activating G proteins. Figure 6A shows that both wild-type FPR- and ΔST-expressing cells are capable of activating the MAP kinase pathway in HEK cells to approximately the same extent. Furthermore, the time course of activation and inactivation is very similar for the two receptors. Since the ΔST mutant FPR is also incapable of undergoing desensitization due to its lack of phosphorylation sites, the decline in MAP kinase activity seen with this mutant suggests that there are redundant downstream mechanisms for the desensitization of this response. In all, these results lend further credence to the conclusion that FPR-mediated signaling, and internalization, are arrestin-, dynamin-, and clathrin-independent events.

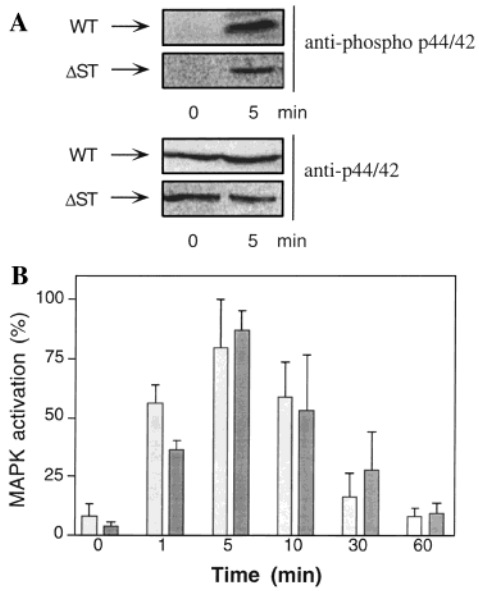

FIGURE 6: Effect of FPR internalization on the activation of MAP kinase. HEK cells transiently transfected with either the wild-type FPR (light bars) or the ΔST mutant FPR (dark bars) were activated with 1 μM fMLF for the specified time. Cells were lysed with SDS sample buffer and analyzed by SDS−PAGE followed by Western blotting for total and activated p44/42. Data are normalized to the maximal response within each paired assay and then averaged. Data are representative of three separate experiments (± standard error of the mean).

DISCUSSION

Previous studies from our laboratory have suggested that internalization of the FPR can occur in the absence of receptor desensitization (*15*), suggesting a divergence in the mechanisms utilized for these two responses. We hypothesized that a lack of receptor desensitization implied a lack of arrestin binding, and therefore, the observed internalization would likely be occurring in an arrestin-independent mechanism (*16*). In this study, we used multiple approaches to investigate whether the FPR is processed utilizing mechanisms similar to those used by the β_2AR. The β_2AR is the best characterized GPCR and serves as a paradigm for GPCR processing and internalization through clathrin-coated pits. We have shown here that internalization of the FPR occurs in an arrestin-, dynamin-, and clathrin-independent manner. In addition, the FPR and the β_2AR are found at distinct sites within the cell during internalization as determined by fluorescence microscopy. Finally, activation of MAP kinases by the FPR is independent of receptor internalization, indicating another difference in the mechanisms of signaling between the β_2AR and the FPR.

Recent studies have suggested that, in addition to the clathrin-mediated mechanisms of internalization utilized by the β_2AR and a large number of other GPCRs, additional mechanisms of GPCR internalization must exist. These conclusions are based on the following results. In addition to the arrestin- and dynamin-sensitive mechanisms of internalization, receptors such as the endothelin B receptor and the vasoactive intestinal peptide receptor appear to internalize in an arrestin-independent but dynamin-dependent manner (*34*). It is possible that this mechanism corresponds to internalization through caveolae, which also appear to utilize dynamin in the budding off of these vesicles (*35*). Last, m2 muscarinic and angiotensin 1A receptors have been shown to internalize via arrestin- and dynamin-independent mechanisms (*34*). The presumed coat proteins and other regulatory proteins involved in this pathway are completely unknown at present. The data presented in this paper indicate that the formyl peptide and C5a chemoattractant receptors probably utilize a similar pathway. Some receptors, such as the m2 muscarinic receptor, can be forced to internalize via clathrin-coated pit mechanisms in the presence of highly overexpressed arrestin proteins (*36*). Furthermore, a phosphorylation-deficient mutant of the m2 muscarinic receptor, when expressed at low numbers per cell, is able to internalize, though at a decreased rate compared to that of the wild-type receptor (*37*). These results suggest significant flexibility is possible in the mechanisms utilized by a given receptor. In our studies, we were unable to observe any significant increase in the level of FPR internalization in the presence of overexpressed arrestin-2 or arrestin-3. A growing body of evidence suggests that even the cell in which a receptor is expressed can alter the mechanism of internalization. For example, the δ opioid receptor undergoes phosphorylation-dependent internalization in CHO cells but phosphorylation-independent internalization in HEK cells (*38*). The fact that our results are not confined to HEK cells is indicated by the insensitivity of FPR internalization in stably transfected U937 cells to 300 μM mondansylcadaverine (unpublished observation), an agent known to disrupt clathrin-dependent TNF receptor internalization in U937 cells with an EC_{50} of 30 μM (*39*).

Although sensitivity of internalization to dynamin seems to indicate pathways involving either clathrin-coated pits or caveolae, use of the clathrin hub fragment to disrupt clathrin assemblies is likely to be highly selective for the clathrin pathway. Thus, the ability of the FPR to internalize in a hub (as well as dynamin)-independent manner provides even stronger evidence that the FPR does not internalize via clathrin-coated pits. Yet another marker specific to the clathrin coated pit mechanism has just been described. The GIT1 (GRK-interactor 1) protein, which contains an active ADP ribosylation factor GTPase-activating domain, is able to disrupt internalization of receptors that internalize in an arrestin-dependent manner, but has no effect on those receptors which do not (*34*). Although it remains to be determined whether internalization of the FPR is sensitive to GIT1, this seems unlikely.

To investigate whether our results were confined to the FPR, we also examined the internalization pattern of another chemoattractant GPCR, the C5a receptor. The C5a receptor, like the FPR, is expressed predominantly on myeloid cells and is involved in chemotaxis and inflammatory reactions. Analysis of the dependence of C5a receptor internalization on arrestin, dynamin, and the clathrin hub fragment revealed that, like the FPR, it was unaffected by any of these proteins. Recent data characterizing the internalization of phosphorylation-deficient mutants of the C5a receptor indicated that this receptor, like the FPR, could internalize in the absence of desensitization, suggesting an arrestin-independent mechanism (*40*). Thus, these results are entirely consistent with the data presented in this study. Published data suggest that certain chemokine receptors (CCR5 and CXCR1, -2, and -4) internalize in a dynamin-dependent manner (*41*−*44*). It is therefore not clear whether a distinction exists for the chemokine receptor family versus the chemoattractant receptor family.

In addition to mediating desensitization and internalization, arrestin binding and receptor internalization have been demonstrated to be essential components in the signaling pathway leading to activation of MAP kinases by the β_2AR, as well as numerous other G protein-coupled receptors (*12*). This appears to involve recruitment and activation of the tyrosine kinase Src to the arrestin component of the internalizing receptor−arrestin complex. Our data demonstrate that internalization of the FPR, as well as presumably arrestin binding and therefore Src activation, is not required to activate p44/42 MAP kinase. Whether Src activation occurs via alternate pathways upon FPR activation remains to be determined. In the neutrophil, the Src-related kinase Lyn and the adapter protein Shc have been implicated in the FPR-mediated activation of the MAP kinase cascade, suggesting that parallel pathways may be activated via distinct routes (*45*). Further work will be necessary to determine if such tyrosine kinases can complex with the FPR in the absence of arrestin binding.

Our results suggest that receptors which bind arrestin, such as the β_2AR and the FPR (*16*), are still capable of utilizing distinct mechanisms for internalization. This raises the interesting question of how the different subsequent mechanisms are engaged. In the case of arrestin-3, the site of its interaction with the amino-terminal β-propeller motif of

clathrin has been determined by X-ray crystallography (*46*). How do receptors such as the FPR bind arrestin yet preclude the binding of the bound arrestin to clathrin or AP-2? Furthermore, if arrestin is indeed bound to the internalized FPR but not needed as an adapter protein for internalization, what other proteins are involved and how do they gain access to the FPR? It has also been shown recently that certain receptors internalize with bound arrestin (e.g., vasopressin V2 receptor), whereas other receptors (e.g., β_2AR) dissociate from arrestin before or during internalization through a clathrin-coated pit (*47*). These effects were also correlated with resensitization with the β_2AR recycling more rapidly to the cell surface than the vasopressin V2 receptor. It is thus clear that the interactions of arrestin molecules and receptors are very complex, regulating many aspects of a receptor's functional capacity through distinct yet redundant mechanisms.

In conclusion, we have demonstrated that members of the human, G protein-coupled, chemoattractant receptor family undergo agonist-mediated internalization via a novel mechanism that is independent of arrestin binding and the clathrin-coated pit pathway of internalization. Furthermore, these sites of internalization appear to be distinct from those of the β_2AR. Finally, we have demonstrated that signaling to the MAP kinase pathway by the FPR is independent of receptor internalization, providing support for an arrestin-independent signaling mechanism.

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