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The *N*-formyl peptide receptors and the anaphylatoxin C5a receptors: An overview

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Abstract

Leukocyte recruitment to sites of inflammation and infection is dependent on the presence of a gradient of locally produced chemotactic factors. This review is focused on current knowledge about the activation and regulation of chemoattractant receptors. Emphasis is placed on the members of the *N*-formyl peptide receptor family, namely FPR (*N*-formyl peptide receptor), FPRL1 (FPR like-1) and FPRL2 (FPR like-2), and the complement fragment C5a receptors (C5aR and C5L2). Upon chemoattractant binding, the receptors transduce an activation signal through a G protein-dependent pathway, leading to biochemical responses that contribute to physiological defense against bacterial infection and tissue damage. C5aR, and the members of the FPR family that were previously thought to be restricted to phagocytes proved to have a much broader spectrum of cell expression. In addition to *N*-formylated peptides, numerous unrelated ligands were recently found to interact with FPR and FPRL1. Novel agonists include both pathogen- and host-derived components, and synthetic peptides. Antagonistic molecules have been identified that exhibit limited receptor specificity. How distinct ligands can both induce different biological responses and produce different modes of receptor activation and unique sets of cellular responses are discussed. Cell responses to chemoattractants are tightly regulated at the level of the receptors. This review describes in detail the regulation of receptor signalling and the multi-step process of receptor inactivation. New concepts, such as receptor oligomerization and receptor clustering, are considered. Although FPR, FPRL1 and C5aR trigger similar biological functions and undergo a rapid chemoattractant-mediated phosphorylation, they appear to be differentially regulated and experience different intracellular fates.

Keywords: Chemoattractant receptors; Anaphylatoxin; *N*-formyl peptide; Signal transduction; Phosphorylation; Internalization

1. Introduction

Polymorphonuclear phagocytes accumulate in infected and/or inflamed tissue, and are of critical importance in the initial defense against microorganisms and the pathogenesis of inflammatory diseases. Upon activation by soluble or particulate products of either exogenous or endogenous origin, they penetrate the endothelial barrier and migrate to the inflammatory sites (chemotaxis). Once neutrophils have reached the site of inflammation, they phagocyte foreign particles and invading microorganisms. Phagocytosis is accompanied by the release of reactive oxygen species (ROS) and proteolytic enzymes that play a critical role in the clearance of invading pathogens. These components are also responsible for tissue damage associated with a number of inflammatory disorders. The migration of leukocytes from the blood stream towards sites of infection and inflammation is dependent on the gradient of chemotactic factors derived from pathogens or host tissues. In the mid-1970s, it was demonstrated that small *N*-formylated peptides deriving from the catabolism of either bacterial products [1,2] or components from disrupted mitochondria [3] were potent activators of neutrophils. Since then, a growing number of chemoattractants have been discovered. These include the complement component C5a, lipid metabolites

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such as leukotriene B4 and platelet-activating factor (PAF), a large group of chemokines, and a variety of virus- and host-derived proteins and peptides. These chemoattractants engage receptors that belong to the seven transmembrane G protein-coupled receptor (GPCR) family and trigger identical neutrophil responses, i.e. chemotaxis, up-regulation of surface receptors, release of proteolytic enzymes from granules, and ROS production [4]. These responses are largely inhibited by *Bordetella pertussis* toxin, indicating that signal transduction is dependent on a heterotrimeric G protein of the Gi type.

In this review, we will present current knowledge about the peptide-induced activation of chemoattractant receptors and their regulation, with special emphasis on the human formyl peptide receptor family (FPR, FPRL1, and FPRL2) and the human receptors for the complement component C5a (C5aR and C5L2).

2. The FPR family

2.1. FPR and its homologues FPRL1 and FPRL2

The prototypical *N*-formylated peptide, *N*-formyl-methionyl-leucyl-phenylalanine (fMLF) binds with high affinity to human FPR, which was the first neutrophil chemoattractant receptor to be characterized biochemically [5]. Its primary structure remained elusive until two allelic forms (FPR26 and FPR98) were cloned from a differentiated HL-60 cell library by an expression cloning strategy [6,7]. This breakthrough opened the way for the discovery of a rapidly expanding family of myeloid cell chemoattractant receptors (reviewed in Ref. [4]). Two structurally related receptors, FPRL1 and FPRL2, were further cloned by low-stringency hybridization with the human FPR cDNA as a probe [8–10]. All three receptor genes were shown to form a cluster on chromosome 19. Despite a high degree of amino acid identity with FPR (69%), FPRL1 binds fMLF only with low affinity. It is also referred to as LXA_4R, because the lipid mediator lipoxin A_4 (LXA_4), an eicosanoid with anti-inflammatory properties, was the first endogenous ligand shown to bind with high affinity to FPRL1 [11]. The biological effects of LXA_4, and current knowledge of the mechanisms underlying its anti-inflammatory properties, are discussed in recent reviews [12,13]. The third member of the human FPR family, FPRL2, shares 56% amino acid identity with human FPR, but it does not bind or respond to fMLF. These two homologues exhibit a high degree of amino acid identity with FPR in the cytoplasmic loops, whereas the carboxyl tails are more divergent.

Although human FPR and FPRL1 originate from a common ancestor and are structurally and functionally related, they appear to have followed different evolutionary pathways. An extensive investigation of the frequency of polymorphism in the open reading frame of human *FPR* and *FPRL1* identified for *FPR* seven common sites of polymorphisms in Caucasians, Blacks, and Asians, whereas no polymorphisms could be detected for *FPRL1* in the same three racial groups [14]. The reason for the high frequency of single nucleotide polymorphisms (SNP) in the coding sequence of FPR is presently unknown. It may enable phagocytes to escape untimely activation by *N*-formyl peptides secreted by pathogens or be an adaptation that allows FPR to bind to a much wider variety of agonists. Apart from the fact that two SNPs (R190W and N192K) in the second extracellular loop of FPR appear to be associated with aggressive periodontitis [15], little is known about the consequences of SNP in FPR as regard the expression of the receptor, its function, or its affinity for the ligand. The functional analysis performed on the FPR isoforms originally isolated, namely FPR26 (V101, N192, and E346), FPR98 (L101 and A346), and FPR-G6 (V101, K192, and E346), indicates that some SNPs, like those found in FPR98 and FPR-G6 may result in a partial defect in the coupling of Gi protein to FPR26. In transfected cells, FPR26 has a high degree of constitutive activity that seems to be due to the presence of E346 [16]. These results therefore indicate that some SNPs may dramatically affect the functionality of the receptor.

2.2. Novel ligands that activate or inhibit the members of the FPR family

During the past few years, many new agonists and/or antagonistic compounds were discovered that bind to FPR, FPRL1 or FPRL2. Both FPR and FPRL1 have emerged as promiscuous receptors that can be activated by a wide range of structurally unrelated peptide agonists that are classified either as synthetic or host- and pathogen-derived agonists. FPRL2 is activated with low potency by several non-formylated chemotactic peptides that are also agonists for FPRL1 [17,18]. Recently, an acetylated amino-terminal peptide derived from the human heme-binding protein has been identified as a natural ligand specific for FPRL2 [19,20].

2.2.1. Antagonists

Antagonistic components have been identified for both FPR and FPRL1. *In vitro*, the action of fMLF can be antagonized by various peptide analogs. For many years, the *N-tert*-butoxycarbonyl-Phe-Leu-Phe-Leu-Phe-OH peptide (*t*-BOC peptide) was known as the most potent competitive antagonist [21]. Peptides with antagonistic properties were engineered by substituting the amino terminus of the tripeptide Met-Leu-Phe with branched carbamates, such as *iso*- and *tert*-butyloxycarbonyl, whereas unbranched carbamates, such as methoxycarbonyl and ethoxycarbonyl, resulted in agonist activity [22]. However, none of these antagonists is totally selective for the FPR. In 1993, the cyclic undecapeptide cyclosporine H (CsH), which is derived from the fungus *Tolypocladium inflatum*, was described as a strong inhibitor of FPR, being around 10 times more potent and more selective than previously described antagonists [23]. CsH acts as an inverse agonist, switching the receptor from the active state (R*) to the inactive state (R) [24].

Peptides or proteins with antagonist properties were found to be secreted by pathogens. Peptides derived from the membrane proximal region of fusion proteins of human immunodeficiency virus 1 and 2, Ebola virus, coronavirus 229E, and severe acute respiratory syndrome (SARS) coronavirus act as potent antagonists of FPR [25]. A 121-residue chemotaxis inhibitory protein secreted by *Staphylococcus aureus*, known

as CHIPS, binds to FPR and thereby specifically blocks phagocyte responses and suppresses the first line of defense against this pathogen [26]. A 105-residue FPRL1 inhibitory protein, referred to as FLIPr, that presents 28% homology with CHIPS was also isolated from *S. aureus* [27]. Thus, by blocking these two chemoattractant receptors, *S. aureus* appears to have engineered mechanisms to escape the first line of defense constituted by phagocytes.

Recently, ligand-based virtual screening was combined with high-throughput flow cytometry to identify novel non-peptidic antagonists to FPR [28]. Such compounds may prove to have pharmacological use. In a search for FPRL1 antagonists in hexapeptide libraries, a novel peptide, Trp-Arg-Trp-Trp-Trp-Trp-$CONH_2$ (WRWWWW), was identified that showed the most potent activity in terms of inhibiting agonist binding to FPRL1 [29]. The hexapeptide WRWWWW is presently one of the rare compounds that specifically blocks the activation of FPRL1. Recently, PBP10, a cell-permeable rhodamine B-coupled polyphosphoinositide-binding peptide (QRLFQVKGRR), derived from gelsolin (region 160–169) [30], was found to block FPRL1-mediated signalling [31] This blockage appears to be specific, since it has no inhibitory effect on the neutrophil response mediated through FPR, C5aR, or CXCR1/2 [31].

2.2.2. Synthetic agonists

By screening a random peptide library, Ryu et al. identified two amidated synthetic hexapeptides, Trp-Lys-Tyr-Met-Val-Met-NH_2 (WKYMVM) and Trp-Lys-Tyr-Met-Val-D-Met-NH_2 (WKYMVm), that differed in their ability to activate the three FPR receptors [32,33]. While the D-methionine-containing peptide activated all three receptors with a markedly higher efficacy for FPRL1, the peptide containing the L-isomer had lost most of its ability to activate FPR [18,34,35]. Another small, unrelated, peptide, called MMK-1 (LESIFRSLLFRVM), which was also derived from a random peptide library, was found to specifically activate FPRL1 [36].

2.2.3. Pathogen-derived agonists

The pathogen-derived agonists include peptide domains from virus and bacteria. Several cryptic peptides of HIV-1 envelope proteins have been shown to activate myeloid cells via FPR and/or FPRL1. For example, T20/DP178, a peptide fragment located in the C-terminal part of HIV-1_{LAV} envelope protein gp41 (aa 643–678) is a functional ligand for FPR, whereas two partially overlapping peptides, T21/DP107 (aa 558–595) and N36 (aa 546–581), in a leucine zipper-like domain of gp41 of HIV-1_{LAV}, activate FPRL1 [37,38]. Two peptides, named F and V3, derived from the HIV-1_{LAV} envelope protein gp120, are also good activators of FPRL1 [39,40]. Like the prototypical *N*-formylated peptide fMLF, the synthetic peptides fMIVIL and fMIVTFL, that represent putative degradation fragments from *Listeria monocytogenes* components were found to be 100-fold more active on FPR than on FPRL1, and completely inactive on FPRL2 [41]. Hp(2–20), an antibacterial, cecropin-like peptide derived from the N-terminal sequence of *Helicobacter pylori* ribosomal protein L1, activates both calcium mobilization and the NADPH oxidase in neutrophils via FPRL1 and to a lower extent in monocytes via FPRL2 [17,42]. Using overlapping synthetic peptides to scan the secreted glycoprotein G from Herpes virus type 2 (HSV-2), Bellner et al. [43] have identified a 15 amino acid long peptide (gG-2p20, aa 190–205) that serves as a chemoattractant for both neutrophils and monocytes through the FPR. The ROS secreted in response to binding of Hp(2–20) to FPRL1 and FPRL2 or gG-2p20 to FPR were shown to specifically inhibit NK cell cytotoxicity and to induce the apoptosis of these cells. This immune escape might be of importance for the pathogenesis of *H. pylori* and HSV-2.

2.2.4. Host-derived agonists

Since the identification of the lipid mediator lipoxin A_4 as a high-affinity agonist for FPRL1 [11], numerous host-derived agonists have been identified. Amyloidogenic proteins, or fragments of such proteins, have been found to activate myeloid cells through FPRL1. Serum amyloid A (SAA), a protein secreted during the acute phase of inflammation and involved in chronic inflammation-associated systemic amyloidosis, was the first amyloidogenic ligand found to be specific for FPRL1 [44]. Further, it has been shown that FPRL1 also serves as a receptor mediating the proinflammatory responses elicited by the fragment 1–42 of amyloid β ($A\beta_{42}$), a protein that plays an important role in neurodegeneration in Alzheimer disease [45]. The reader is referred to a recent review that discusses the role of FPRL1 in microglial cell responses in Alzheimer disease [46]. Finally, the neurotoxic prion peptide fragment $PrP_{106–126}$, which is also an amyloidogenic polypeptide, was found to activate FPRL1 [47]. In addition to these protein fragments, the neuroprotective peptide, humanin, a 24-aa peptide detected in the occipital region of the brain, uses FPRL1 as a functional receptor [48]. Although humanin and $A\beta_{42}$ are both able to activate FPRL1, only $A\beta_{42}$ causes apoptotic death of the cells. From these observations, Ying and coworkers [48] have suggested that humanin may exert its neuroprotective effect by competitively inhibiting the access of FPRL1 to $A\beta_{42}$. This is the only one possible mechanism, since humanin has also been shown to bind to the apoptosis-inducing protein Bax in the cytoplasm and, thereby suppress apoptosis by preventing Bax translocation from the cytoplasm to the mitochondria [49].

Additional host-derived peptides and proteins that act as FPR/FPRL1 agonists were reported over the past few years. LL-37, a 37-residue fragment of a human antimicrobial protein of the cathelicidin family, was found to have chemotactic properties on neutrophils, monocytes, and T cells, via the activation of FPRL1 [50]. However, the fact that micromolar concentrations of LL-37 are required to mediate chemotaxis in leukocytes and calcium mobilization in FPRL1-transfected 293 cells and monocytes is indicative of a low affinity of LL-37 for FPRL1. The proinflammatory and antimicrobial neutrophil granule protein cathepsin G is also a chemotactic agonist for the FPR, but unlike fMLF it does not trigger calcium mobilization in phagocytes, although it is able to rapidly induce mitogen-activated protein (MAP) kinase phosphorylation [51]. This also suggests that cathepsin G is a low-affinity agonist and that a threshold of occupied receptor has to be

reached for calcium mobilization to occur. Thus, antimicrobial proteins released from neutrophil granules or peptides deriving from such proteins may establish a link between innate and adaptative immunity through their ability to recruit dendritic cells via the activation of the *N*-formyl peptide receptors. Formylated hexapeptides derived from the N-terminal region of subunits 4 (fMLKLIV) and 6 (fMMYALF) of human mitochondrial NADH dehydrogenase are potent activators of calcium mobilization through both FPR and FPRL1 [41]. These peptides may play a crucial role in the clearance of necrotic cells. Annexin I, a protein that is localized in the cytosol of resting neutrophils, is known to inhibit neutrophil extravasation in models of acute and chronic inflammation and is believed to act on the neutrophil in an autocrine/paracrine manner to reduce cell extravasation. Annexin I and its bioactive N-terminal peptides (Ac2–26 and Ac9–25) activate different signalling pathways in neutrophils in a dose-dependent manner through the FPR family members [52]. While annexin I binds to FPRL1 only, Ac2–26 binds to both FPR and FPRL1 [53]. In contrast, the annexin I peptide Ac9–25 activates phagocyte ROS production exclusively through FPR [54]. Finally, a recent study demonstrates that the pituitary adenylate cyclase-activating polypeptide 27 (PACAP27), a multifunctional neuropeptide hormone that influences the immune system by both suppressing and activating inflammation through the regulation of several interleukins, activates neutrophils via FPRL1 stimulation [55].

It is likely that this family of receptors plays a critical role in the regulation of the inflammatory processes associated with host defense, tissue damage, and neurodegenerative diseases. The role of the FPR in host defense against bacterial infections is supported by the observation that the invalidation of FPR in mice confers to the animals a higher susceptibility to infection when they are challenged with *L. monocytogenes* [56]. The observation that the orthologue of FPRL1 in mice is unable to compensate for the loss of FPR is consistent with the much lower affinity of the *Listeria*-derived peptides for FPRL1 [41]. The engagement of the FPR family by pathogen-derived agonists may have different consequences depending on the amount of chemoattractant that is released. At low concentrations of agonist, the engagement of the FPRs could contribute to the infiltration of phagocytes to the local site of infection, where they degrade the invading pathogens. The process might be amplified by the release of host-derived molecules that would attract more phagocytic cells, thereby leading to an enhanced inflammation and tissue damage. At high concentration of agonist, phagocytic cells are fully activated and release reactive oxygen species (ROS) that may locally create an immunosuppression. In addition, the activated FPR and FPRL1 become down-regulated and/or desensitized through phosphorylation and internalization (see Section 6). Both FPR and FPRL1 have also been shown to down-regulate other chemotactic receptors that participate in host defense. These different mechanisms might allow pathogens to escape and propagate, as seems to be the case for *H. pylori* and Herpes simplex virus-2.

3. The complement C5a receptor (CD88) and C5L2

3.1. The C5a receptor (C5aR)

The complement system is part of the humoral innate immune system. Its activation in a cascade via three convergent pathways leads to the generation of small fragments (10 kDa) of C3, C4, and C5 components that are referred to as anaphylatoxins C3a, C4a, and C5a, respectively. All three anaphylatoxins contain a carboxyl-terminal arginine residue that is rapidly cleaved by serum carboxypeptidase to yield the desarginine (desArg) derivatives. The desArg forms exhibit a dramatic decrease in biological activity. The C5a anaphylatoxin is a potent proinflammatory mediator which exerts its effect by binding to the C5aR. Elucidation of the amino acid sequence of the C5aR was independently achieved by two groups using totally different strategies [57,58]. The overall protein presents 34% amino acid sequence identity with that of FPR. The reader is referred to several reviews that described in details the structure and function relationships of the C5aR [4,59]. Interestingly, the C5aR is encoded by a single copy gene that is located on chromosome 19 close to the genes for FPR, FPRL1, and FPRL2. In contrast to FPR, only two single nucleotide polymorphisms have been identified for C5aR. One silent nucleotide change (C450T) is located in the coding sequence [60]. The second site is located in the promoter region at position −245 (T/C) and is apparently not associated with a particular disorder [61]. The very low frequency of single nucleotide polymorphism in C5aR suggests a selective pressure for the maintenance of a conserved receptor structure. Indeed, in contrast to the FPR, many engineered single point mutations in C5aR generally affect ligand binding, G protein activation, or surface expression [62].

The function of C5aR in host defense was clearly demonstrated by gene deletion in mice. In a model of *Pseudomonas aeruginosa*-mediated lung infection, mice deficient in C5aR have a higher rate of mortality [63]. Furthermore, in the model of *Escherichia coli*-induced inflammatory response, the activation of C5aR is required for the *E. coli*-induced up-regulation of CR3 and the subsequent superoxide production and phagocytosis [64]. The inappropriate activation of the complement system leads to inflammatory disorders and a number of pathologies. For instance, during sepsis, an excessive generation of the C5a anaphylatoxin and activation of C5aR has detrimental effects [65,66]. In experimental sepsis, the blockade of C5a by infusion of antibody against C5a has been shown to improve hemodynamic parameters [67]. In the cecal ligation/puncture rodent model for sepsis, preventing the interaction of C5a with its receptor greatly improves the survival of the animals [65]. Further work points out the role played by the couple C5a/C5aR in the dysfunction of cardiomyocyte contractility during sepsis [68]. In this context, the reader is referred to a recent review in which current knowledge regarding the pathophysiology underlying cardiac dysfunction during sepsis is discussed [69]. Thus, the blockade of C5a or its receptor may be a promising strategy for the treatment of sepsis in humans.

3.2. The anaphylatoxin C5a-binding protein, C5L2

The gene encoding the orphan receptor C5L2 localizes to the same region of chromosome 19 as *C5aR* and the different members of the FPR family. C5L2 was shown to be co-expressed with C5aR on leukocytes and immature (but not mature) dendritic cells [70] as well as in astrocytes where it is up-regulated by noradrenaline [71]. This receptor, which presents 36% amino acid identity with the C5aR (Fig. 1), was found to be a high-affinity receptor for C5a and $C5a_{desArg}$ [72,73]. However, unlike C5aR, C5L2 exhibits an apparent absence of coupling with the heterotrimeric Gi protein, and it is not internalized [72,73]. A recent study using mice deficient in C5L2 shows that bone marrow cells derived from $C5L2^{-/-}$ mice are more responsive to C5a as compared to cells derived from wild type animals in *in vitro* chemotaxis assays [74]. Furthermore, immune complex pulmonary injury in $C5L2^{-/-}$ mice results in both a greater influx of inflammatory cells and a greater release of IL-6 and TNF-α as compared to wild type animals. This study suggests that C5L2 acts independently of the C5aR to counteract the C5a/C5aR-mediated inflammation. It is, nevertheless, unclear as to how C5L2 mediates its anti-inflammatory activity. Several hypotheses that are not mutually exclusive have been proposed. C5L2 may act as a decoy receptor that modulates the biological activity of C5a during the inflammatory process or it may form oligomers with the C5aR. Alternatively, C5L2 may trigger the activation of a non-conventional anti-inflammatory signalling pathway.

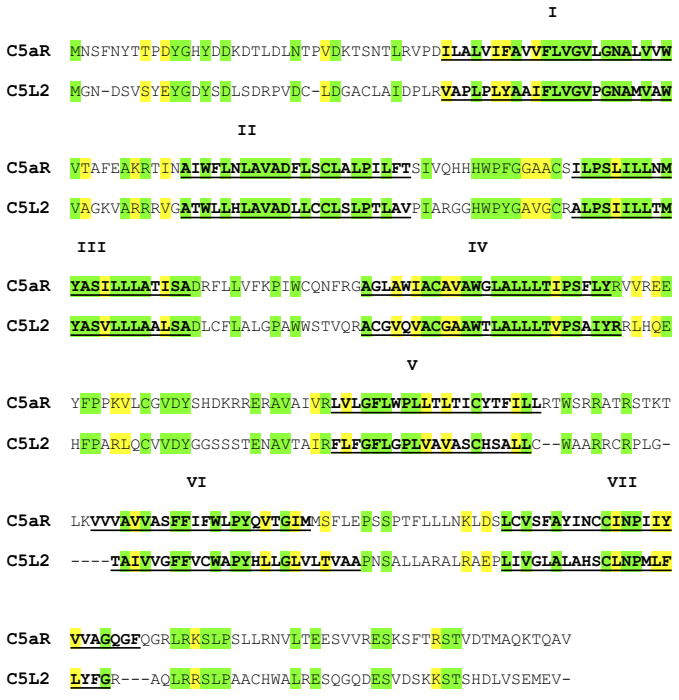

Fig. 1. Alignment of the amino acid sequence of the C5a receptor and C5L2, a non-signalling C5a receptor. Residues that are identical are highlighted in green, whereas homologous amino acids are highlighted in yellow. The transmembrane α-helices are underlined.

3.3. C5aR antagonists

The C-terminal domain of C5a contains the determinants responsible for C5aR activation and it has been used to design small peptide antagonists. A short peptide, methyl-Phe-Lys-Pro-D-Cyclohexylamine-Trp-D-Arg (MeFKPdChaWr) has been demonstrated to antagonize C5a-mediated polymorphonuclear activation, albeit with a low potency [75]. A step towards the finding of a potent and selective antagonist was achieved with the design of a restrained structure, acetyl-Phe[Orn-Pro-D-Cyclohexylamine-Trp-Arg] (AcF[OPdChaWR]), which was found to be 30 times more potent than MeFKPdChaWr for blocking the C5aR [76]. The 121-residue chemotaxis inhibitory protein, an excreted virulence factor of *S. aureus* (CHIPS), represents a new class of antagonists that suppresses the fMLF- and C5a-mediated activation of neutrophils and monocytes by binding and blocking specifically the FPR and the C5aR [77].

4. Cell type distribution of the members of FPR and C5aR families

As more specific ligands were discovered and the immunological tools refined, FPR family members and C5aR were found to be expressed differently by a variety of cell types and not restricted to phagocytes as previously thought (Table 1). FPRL2 is present in monocytes/macrophages but not in neutrophils, whereas FPR, FPRL1, and C5aR are expressed in neutrophils and monocytes/macrophages. Human dendritic cells express FPRL2 and C5aR throughout maturation, whereas FPR is only present in immature dendritic cells [78,79]. No functional FPRL1 could be detected in either immature or mature dendritic cells [80]. The expression of the formyl peptide receptors in a variety of cells other than phagocytic cells suggests that they might have functional roles beyond that of host defense in innate immune response. C5aR expression was originally described in myeloid cells, including neutrophils, monocytes, eosinophils, basophils, immature and mature dendritic cells. The C5aR has also been found in glial cells [81,82], cerebellar granule cells [83], vascular endothelial cells [84–86] and in cells of liver and lung [87,88]. Its expression is up-regulated in regenerating hepatocytes [89] and, during the early phase of sepsis, in heart tissue [90]. C5L2 transcripts were observed in many organs and tissues including heart, lung, spleen, liver, placenta, skeletal muscle, bone marrow [73], and several regions of the brain [91].

5. Chemoattractant-mediated intracellular signalling

5.1. Signal transduction

Chemoattractant receptors, including C5aR and the members of the FPR family, are coupled to the heterotrimeric G proteins of the G_i subtype as evidenced by the observation that chemoattractant-mediated neutrophil functions, i.e. chemotaxis, degranulation, and superoxide production, are largely inhibited by treatment of cells with pertussis toxin (PTX) [92].

Table 1
Cell type and tissue expression of FPR, FPRL1, FPRL2, C5aR, and C5L2

Receptors	Cells and tissues	Methods of detection
FPR	Neutrophils; monocytes/macrophages; differentiated U937, HL-60 and NB4	Ligand binding
	Immature dendritic cells; astrocytes (HSC2), microglia, neuroblastoma [82]; glioblastoma [198]; hepatocytes [199]; Kupffer cells; endothelial cells; lung carcinoma cells; epithelial enzyme-secreting cells, smooth muscle cells of muscularis propria and of mucosa of ileum, arterioles, retina, some neurons [200,201]	Flow cytometry, immunofluorescence, RT-PCR, and immunohistochemistry
FPRL1	Neutrophils; monocytes/macrophages; differentiated HL-60 cells	Ligand binding and calcium mobilization
	Colonic epithelial cells (T84, HT29, Caco-2, CL. 19A) [202]; medial tissue of coronary arteries [203]; astrocytoma cell lines [204]	Immunohistochemistry ligand binding RT-PCR
FPRL2	Monocytes; immature and mature dendritic cells; lung; medial tissue of coronary arteries [203]	Immunohistochemistry, Northern blot
C5aR	Neutrophils; monocytes/macrophages	Ligand binding
	Immature and mature dendritic cells	Chemotaxis, RT-PCR, Flow cytometry
	Bronchial epithelial cells [205], vascular endothelial cells [84–86]; brain endothelial cells in inflamed tissue [206]; cardiomyocytes [68]	Flow cytometry and RT-PCR, immunohistochemistry
	Astrocytes and glial cells [81,82]; cerebellar granule neurons [83]	Flow cytometry, RT-PCR, immunocytochemistry
C5L2	Granulocytes; immature dendritic cells [70], astrocytes [71], heart, lung, spleen liver, placenta, skeletal muscle, bone marrow	Northern blot, flow cytometry

A schematic view of the main signalling pathways stimulated downstream of the G protein is presented in Fig. 2.

Upon chemoattractant binding, receptors undergo a conformational change that enables them to interact with the Gi_2 protein, thereby triggering both the exchange of GDP to GTP in the G protein α subunit and the dissociation of the βγ complex from the α subunit [93]. In neutrophils, monocytes and differentiated HL-60 cells, a small PTX-resistant fMLF- or C5a-mediated activity is often observed that may result from the coupling of FPR and C5aR to residual molecules of G_{16}, a promiscuous G protein of the G_q class, restricted to a subset of myeloid cells in the early steps of differentiation. In co-expression systems, FPR and C5aR can also couple to G_{i1}, G_0 and a PTX-resistant G protein G_z [94]. Many studies aimed at defining the contact sites

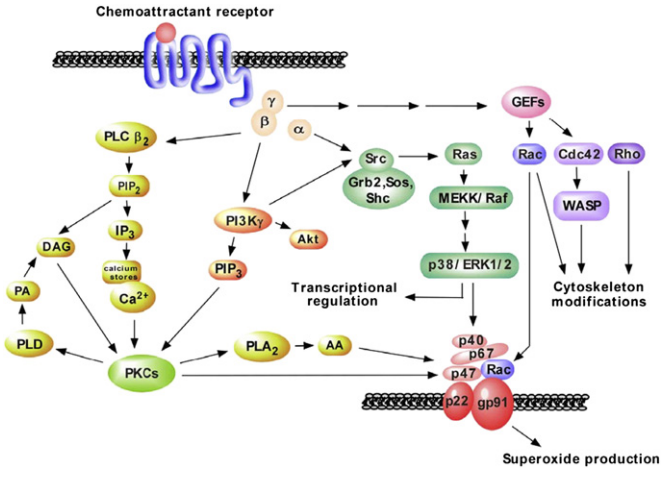

Fig. 2. Schematic summary of the main signalling pathways initiated by C5aR and *N*-formyl peptide receptors in myeloid cells. Agonist binding to the receptors results in dissociation of heterotrimeric G protein into Gα-GTP and Gβγ subunits which activate downstream effectors and signalling cascades (see text for details) involved in the regulation of cellular functions (chemotaxis, superoxide production and release of inflammatory mediators). AA, arachidonic acid; PA, phosphatidic acid. Other abbreviations are mentioned in the text.

responsible for coupling of G proteins to chemoattractant receptors have led to the notion that G_{i2} protein–receptor interactions most likely involves multiple contact sites with intracellular loop 1, the boundaries of intracellular loops 2 and 3 and restricted regions in the carboxyl tail, as well as in the transmembrane regions [95–99]. A recent study by Matsumoto and coworkers [100,101] provides a comprehensive functional map of the intracellular surface of C5aR. They identified the residues essential for G protein activation in the amino terminal half of intracellular loop 2 and the carboxy-terminal half of intracellular loop 3 as well as the adjoining transmembrane α-helices. The main determinants for G protein specificity are clustered in the carboxy-terminal tail and carboxy-terminal half of intracellular loop 2.

Following its dissociation from the α subunit, the G protein βγ subunits activate the phospholipase $C\beta_2$ ($PLC\beta_2$) [102] and the phosphoinositide 3-kinase γ(PI3Kγ) [103]. PI3Kγ converts the membrane phosphoinositol-4,5-bisphosphate (PIP_2) into phosphoinositol-3,4,5-trisphosphate (PIP_3). Deletion of PI3Kγ in mice has revealed that this enzyme is required for both the directed migration of neutrophils in a gradient of fMLF [104,105] and the generation of superoxide mediated by the stimulation of chemoattractant receptors [106]. $PLC\beta_2$ hydrolyzes plasma membrane PIP_2 into diacylglycerol (DAG) and inositol trisphosphate (IP_3). The latter causes the release of calcium from the intracellular calcium stores. The interplay of calcium and DAG leads to the activation of protein kinase C (PKC) isoforms. Neutrophils and neutrophil-like differentiated HL-60 cells [107,108] express the classical PKC isoforms α, βI, and βII, the novel PKC isoforms δ, and the atypical PKC isoforms ζ. Studies concerning the role of PKC isoforms in neutrophil functions are somewhat contradictory and assignment of PKC isoforms in specific biological functions of neutrophil are relatively scarce. *In vitro* studies or reconstituted superoxide generation systems indicate that several PKC isoforms are involved in the activation and

regulation of the NADPH oxidase. *In vitro*, the cytosolic factor $p47^{phox}$, a key component in the assembly of the superoxide generating complex, is a substrate for PKCα, βII, δ and ζ [109]. However, in myeloid cells, the identity of the PKC isoforms involved in the phosphorylation of $p47^{phox}$ and the triggering of superoxide production is controversial. Based on the inhibitory effect of antisense RNA to the beta isotype, it has been proposed that PKCβ regulates superoxide generation in neutrophils and neutrophil-like differentiated HL-60 cells. It is likely that isotypes other than PKCβ are also involved, as indicated by the fact that neutrophils from PKCβ knockout mice exhibit only a 50% decrease in the level of superoxide production as compared to neutrophils from normal mice [110]. PKCα has been implicated in the generation of superoxide in monocytes [111]. The reconstitution of FPR-induced NADPH oxidase activation in transgenic COS-phox cells has indicated that PKCδ, but not PKCα, βII, and ζ, is involved in the generation of superoxide [112].

Activation of several other kinases is also intimately involved in chemoattractant signalling. The extracellular signal-regulated kinases (ERK1/2) and the stress-activated p38 MAP kinase are activated by chemoattractants in neutrophils. These two signalling pathways are thought to participate at different degrees in adherence, chemotaxis and superoxide production. The pathway leading to p38 activation has not been completely deciphered, whereas that for ERK1/2 activation has been reconstituted [113]. The G protein βγ subunits recruit PI3Kγ to the plasma membrane, thereby enhancing the activity of Src-like tyrosine kinases, which phosphorylate docking proteins such as the Shc adaptor protein. A functional association between Shc, Grb2 and SOS follows, leading to the activation of the Ras-Raf-MEK-ERK pathway.

Intracellular effectors coupled to the FPR or C5aR signalling cascade include, among others, phospholipase A_2-α (PLA_2-α), phospholipase D (PLD), mitogen-activated protein kinases (MAPK), and c-Src family kinases [114–116]. PLA_2-α is phosphorylated by MAP kinases and is translocated to the plasma membrane in a calcium-dependent manner [117]. The requirement of PLA_2-α for the activation of the NADPH oxidase had been proposed on the basis of the inhibition of superoxide release after targeting of PLA_2 with antisense RNA in differentiated HL-60 cells. The resulting defect can be compensated for by the addition of arachidonic acid [118]. However, these results are contradicted by a recent study showing that PLA_2 gene disruption in mice did not affect superoxide generation, even though the release of arachidonic acid was totally suppressed [119].

The stimulation of C5aR or *N*-formyl peptide receptors leads to the activation of low molecular weight G proteins of the Rho family (Rho, Rac and Cdc42), via the activation of guanine-nucleotide exchange factors (GEFs) such as Vav1 or pRex1 [120,121]. The Rho GTPases are key regulators of many leukocyte functions, including adhesion to endothelial cells, chemotaxis and superoxide generation (for a review see Ref. [122]). RhoA is thought to be involved in chemoattractant-mediated triggering of adhesion through integrins. The Rho GTPase Rac2 plays a pivotal role in the formation of an active NADPH oxidase complex (for a review see Ref. [123]), whereas Rac1 and Cdc42 are involved in the remodelling of the actin cytoskeleton at the leading edge of migrating neutrophils. The activation of Cdc42 is thought to release the auto-inhibited conformation of the Wiskott–Aldrich syndrome protein (WASP), a multidomain protein that is an activator of the nucleating Arp2/3 complex [124,125]. It has been shown that the C-terminal cytoplasmic tail of the C5aR interacts with WASP when the auto-inhibited conformation is released [126]. This interaction could provide the C5aR with a mean of controlling the precise area at the cell surface where actin has to polymerise during the directed migration of leukocytes along a gradient of C5a.

5.2. FPR and FPRL1 induce intracellular calcium mobilization through different pathways

Although many functions of the neutrophil, including chemotaxis, exocytosis, and superoxide generation, can take place in calcium-depleted cells, it is clear that a transient rise in intracellular calcium is required, albeit not sufficient, for triggering optimal chemoattractant-mediated responses [127]. Despite the fact that FPR and FPRL1 share a high degree of amino acid identity and trigger the same neutrophil functions with a similar efficacy through the activation of a PTX-sensitive G_i protein, there is a fundamental difference between the two receptors as to their ability to transduce intracellular signalling. For instance, the mechanisms by which they raise intracellular calcium appear to be different. Recent studies indicate that chemotaxis and calcium responses to the engagement of FPR and FPRL1 are differentially regulated by cyclic adenosine 5′-diphosphate ribosyl cyclase (CD38) [128,129]. The cyclic adenosine 5′-diphosphate ribose (cADPR) has been described as a regulator of calcium signalling [130,131]. The engagement of a subset of chemoattractant receptors, including C5aR, FPR, and the IL-8 receptors (CXCR1/2) results in a cADPR-independent intracellular calcium rise mainly via the release of calcium from the IP_3 receptor-gated stores in the endoplasmic reticulum and secondarily through a modest influx of extracellular calcium. Conversely, FPRL1, CXCR4, CCR1, and CCR5 mobilize calcium in a cADPR-dependent manner and do so primarily through a strong influx of extracellular calcium with a minimal release of calcium from the intracellular stores. Chelating the extracellular calcium abolishes the calcium response mediated by this subset of receptors. Thus, despite the fact that both FPR and FPRL1 couple to the G_i type of heterotrimeric G protein, they activate neutrophil responses through partially overlapping intracellular pathways. It has to be noted here that a unique type of hierarchy inside the FPR family has been recently unveiled [132]. Although FPR and FPRL1 have no ability to cross-desensitize each other, desensitization experiments based on the release of superoxide have indicated that an agonist such as WKYMVm, which has the ability to activate neutrophil responses via both FPR and FPRL1, utilizes the FPR-mediated signalling pathways only when FPRL1 is blocked [132]. One possible mechanism that could explain this hierarchy is that

the agonist-occupied FPRL1 exhibits a higher affinity for the G protein than activated FPR. Consequently, FPR would take over only when FPRL1 is blocked. The difference between FPR and FPRL1 is also particularly well illustrated by the fact that the cell-permeable gelsolin-derived phosphoinositide-binding peptide PBP10 does not block FPR-mediated cellular signalling, whereas it inhibits certain pathways activated by FPRL1, namely granule secretion and the release of superoxide [31]. However, PBP10 has no effect on the mobilization of calcium mediated by FPRL1 [31]. Thus, the PBP10-mediated inhibition is unlikely to take place at the receptor level. The mechanism underlying this selective inhibition is unknown but might possibly result from differences in the localization of the two receptors in membrane micro-domains, in their oligomeric state or in the different interactions of their intracellular regions with the cytoskeleton.

5.3. Differential signalling mediated by peptide ligands

Several reports have demonstrated that distinct peptide agonists can activate different functions via the use of FPRL1 [133–135]. For instance, the activation of FPRL1 by the high-affinity hexapeptide HRYLPM was found to stimulate chemotaxis and superoxide production, whereas the low-affinity peptide HEYLPM and the amyloidogenic protein SAA were good inducers of chemotaxis but poor agonists for the triggering of superoxide release [133]. Similarly, a differential response was observed when FPRL1 was stimulated with WKYMVm or SAA, despite the fact that both ligands led to the phospholipase A_2-mediated release of arachidonic acid, a precursor of leukotriene B4 (LTB_4) and prostaglandin E_2 (PGE_2) biosynthesis [134]. The engagement of FPRL1 with WKYMVm results in a strong increase in LTB_4 production without affecting PGE_2 production. When FPRL1 is stimulated with SAA, the opposite effect is observed. SAA has no effect on LTB_4 production, but it induces a strong stimulation of cyclooxygenase-2 expression and PGE_2 production. The molecular mechanisms underlying this differential signalling are not completely understood. It is generally assumed that every agonist–receptor–G protein ternary complex has equal ability to activate the G protein and to transduce intracellular signals. However, GPCRs pass through a collection of conformations and it has been demonstrated that distinct GPCR conformations can stimulate distinct intracellular signals [136]. Distinct ligands are likely to have different efficacy [137]. They recognize specific receptor states and shift the receptor to conformations with different efficiency to produce G protein activation, and different affinity for other partners [138]. For instance, FPRL1 is a promiscuous receptor that recognizes many structurally unrelated ligands susceptible to induce distinct conformations that may condition the intracellular signalling and fate of the receptor. Weak agonists may be sufficiently potent to activate the few intracellular pathways required for chemotaxis but only potent agonists can fully trigger the activation of all the different intracellular pathways which are required to orchestrate a robust superoxide release. Ligands that have a poor efficacy for the production of cell responses may paradoxically have strong abilities to trigger receptor phosphorylation and internalization. For instance, we have previously observed that the low-affinity peptide WKYMVM has a higher efficacy than the high-affinity peptide WKYMVm for inducing FPRL1 phosphorylation and internalization [18]. Different agonists may also bring about particular FPRL1 conformations that preferentially activate a different G_i subtype of G protein (e.g., G_{i1} or G_{i3}) or a different set of G_{i2} with a particular combination of $\beta\gamma$ subunits. This may result in a differential ability to activate intracellular signalling pathways.

A puzzling observation regarding the behavior of FPR and FPRL1 is that several ligands, including LXA_4, SAA, and annexin I, are capable of inducing pro- and anti-inflammatory activities through the activation of either FPRL1 or FPR. It is not known how some agonists of FPR/FPRL1 can induce contrary signals. One possibility is that the inhibitory signal involves a non-identified receptor that cross-reacts with these agonists. This aspect has been discussed in detail in a recent review in the case of LXA_4 [13] and will not be developed here. It is interesting to note that agonists inducing pro- and anti-inflammatory signals are not among the most potent. They apparently activate distinct signalling pathways at low and high concentrations. This is clearly illustrated in the case of annexin I, which elicits a transient calcium release at low concentrations without fully activating the MAP kinase pathways [52]. This may be sufficient to down-regulate the receptor and cross-desensitize other chemoattractant receptors with, as a primary consequence, the paralysis of phagocytes when further challenged by chemoattractants. Likewise, SAA triggers neutrophil chemotaxis but is a weak inducer of superoxide production. A recent study with human monocytes indicates that SAA stimulates the production of TNF-α and IL-10 which are pro- and anti-inflammatory cytokines, respectively [135]. TNF-α production required the PI3K/p38 pathway, whereas the PI3K/ERK pathway is essential for IL-10 production. TNF-α secretion precedes IL-10, production and requires SAA concentrations around 10 times lower than those necessary for IL-10 production. In addition, the level of IL-10, which is still maximal when TNF-α is declining, may counteract the proinflammatory action of TNF-α. Thus, the differential activation of two signalling pathways that are involved in the production of two cytokines with opposite actions, as well as the concentration dependence and time-lag in their expression, could explain how the same receptor can generate contrary immune responses.

The functional role of a ligand can also vary with the cell type that expresses the receptor. For instance, LXA_4 has been shown to lack the ability to mobilize intracellular calcium in neutrophils, whereas it stimulates an increase of calcium in monocytes [139,140]. This is particularly puzzling because FPRL1 is expressed in both cell types and triggers a robust calcium response in neutrophils and monocytes when occupied by the FPRL1 specific hexapeptide WKYMVM. Likewise, in the case of C5a, opposite effects have been described in neuronal cell death. While C5a mediates apoptosis in neuroblastoma cells [141,142], it antagonizes the neurotoxic effect of the amyloid peptide $A\beta_{42}$ in differentiated neuroblastoma cells

[143,144] and is a potent inhibitor of apoptosis in cultured granule neurons [83].

6. Regulation of chemoattractant receptor functions

6.1. Modulation of signalling

Defining the events that localize and restrict signalling activity is of particular interest in chemotaxis and in the bactericidal activity of leukocytes. The rapid modulation of signalling at the level of the receptor may be critical for the ability of chemoattractant receptors to sense small changes in chemoattractant concentrations as cells move along a chemotactic gradient. Conversely, the attenuation of leukocyte responses such as degranulation and superoxide production is of major importance in controlling inflammation and preventing tissue damage.

6.1.1. Chemoattractant receptor oligomerization

A growing body of biochemical and biological evidence has accumulated recently, which supports the idea that most GPCRs can form dimeric structures or higher order oligomeric complexes (reviewed in Refs. [145–147]). Receptor oligomerization appears to have physiological relevance, as it seems to be an early event in receptor biosynthesis and of critical importance for receptor trafficking. The association of two or more receptors may have consequences for specific signal transduction pathways, thus explaining why a wide variety of responses appear to be differentially regulated via the same receptor. Furthermore, a rapidly increasing number of GPCRs are now demonstrated to form not only homo-dimers but also hetero-dimers. Hetero-dimerization may result in novel agonist specificities and contribute to the modulation of receptor functions.

Different approaches have been used over the years to detect GPCR oligomerization (reviewed in Refs. [145,148]). Separation of membrane proteins by sodium dodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE), combined with Western blotting and immunodetection, has led to the identification of molecular species of higher molecular mass than expected for receptor monomers. More specific techniques involve the co-immunoprecipitation, with or without cross-linking, of two receptors tagged with different epitopes. New biophysical techniques, such as bioluminescence resonance energy transfer (BRET) and fluorescence resonance energy transfer (FRET), are now available to monitor GPCR oligomerization. The functional effect of oligomerization can be studied with peptides that block dimerization, or by the co-expression of a non-functional mutant with the wild type receptor, or by the co-expression of mutants bearing different mutations.

FRET analyses have shown that the C5aR self-assembles into dimers and higher order oligomers *in vivo* when expressed in *Saccharomyces cerevisiae* [149]. The formation of these oligomers is observed in a standard yeast strain and thus does not require accessory mammalian proteins. The specificity of the oligomerization is supported by the fact that C5aR does not form hetero-dimers with the pheromone receptor present in yeast. Several investigations of GPCR oligomerization have demonstrated very different effects of ligand binding on the extent of GPCR oligomerization, with an increase, a decrease, or no change, depending on the type of receptor. In the case of C5aR, oligomerization appears to be constitutive, as suggested by the observation that the FRET signal is unaffected by binding of the ligand. Subcellular fractionation studies revealed that FRET occurs to a similar extent in the membrane-enriched fraction and in the endoplasmic reticulum- and Golgi-enriched fraction. These observations suggest that C5aR oligomerization occurs early in the biosynthesis of the receptor and that constitutive oligomers transit through the secretory pathway. Oligomerization may thus be important in the terminal glycosylation of the C5a receptor and for its effective delivery to the cell surface and possibly, to storage organelles, such as secretory vesicles in neutrophils. Thus, C5aR oligomerization may play a key role in the proper cellular targeting of the receptor and in receptor functions.

Unlike C5aR, FPR does not seem to form homo-dimers. Gripentrog et al. [150] have reported that the co-expression of a signalling defective mutant with wild type FPR does not show any inhibitory effect on ligand-induced intracellular calcium release, chemotaxis and activation of the extracellular signal-regulated kinases ERK1/2. Wild type FPR, co-expressed with another mutant that has a defect in receptor endocytosis, is efficiently internalized and fails to correct the mutant defect. However, the possibility that the mutations themselves might prevent receptor dimerization cannot be excluded. In any case, no oligomerization products could be visualized when wild type receptors tagged with different epitopes are co-expressed.

For most GPCRs the domains involved in dimerization remain elusive. Several models have been proposed for GPCR oligomerization [151], incorporating a contact dimerization model in which non-covalent lateral contacts between transmembrane segments of two independently folded receptor monomers maintain the dimeric structure [152]. A disulfide trapping strategy has been used to probe the intermolecular contact surfaces involved in C5aR dimerization [153]. In this study, the cross-linking of C5a receptors is observed not only in transfected mammalian cells but also in human neutrophils. The results suggest two possible helical orientations, a symmetric helix-4 dimer or a helices-1,2-symmetric dimer. However, the participation of a cysteine in the second intracellular loop is not adequately explained by either model and can be best explained by higher order oligomers, like tetramers. A preferential role for helices 1 and 2 in the putative dimerization domain is supported by the identification, by genetic mapping, of transmembrane amino acids critical for C5aR functions [154].

6.1.2. Role of chemoattractant receptors in the desensitization of chemokine receptors

Cell responses to chemotactic factors are tightly controlled by up-regulation through priming or down-regulation by desensitization/internalization (for a review see Ref. [155]). Homologous desensitization occurs when a receptor is occupied by its cognate ligand and phosphorylated by GPCR kinases (GRKs)

[155]. The phosphorylated receptor associates with β-arrestins and undergoes uncoupling from the G protein and internalization. Heterologous desensitization corresponds to a decrease in receptor responsiveness to its ligands following phosphorylation by second messenger-triggered kinases, such as protein kinase C (PKC) or protein kinase A (PKA), that have been activated by other receptors and signalling cascades [156].

C5aR and the *N*-formyl peptide receptors are structurally and functionally closely related to chemokine receptors. Both homo- and hetero-dimerization were demonstrated for CC and CXC chemokine receptors. In a recent study, Huttenrauch et al. [157] have shown that the chemokine receptor CCR5 forms hetero-oligomeric complexes with C5aR. Stimulation of cells co-expressing CCR5 and C5aR with C5a induced cross-phosphorylation of CCR5 not only by well-established PKC-mediated mechanisms, but also by GRKs. These data suggest that unligated CCR5 receptors are substrates for GRKs if they form hetero-complexes with an agonist-activated receptor. Co-expression of C5aR promoted CCR5 co-internalization upon C5aR stimulation. Cross-phosphorylation and co-internalization of unligated CCR5 receptors within hetero-oligomeric complexes thus lead to down-regulation of the chemokine receptor.

Down-regulation of several chemokine receptors by receptors of the FPR family has also been reported. The activation of FPR results in the cross-desensitization of CXCR1 and CXCR2 [156]. Similarly, the activation of either FPR or FPRL1 leads to the desensitization of CCR5 as a result of a PKC-dependent phosphorylation of CCR5 on serine residues [158]. FPRL1 agonists also induce a PKC-dependent phosphorylation of CXCR4 and its down-regulation [159]. There is no information about the ability of FPR and FPRL1 to form hetero-oligomers with chemokine receptors, but heterologous desensitization seems to be mediated, in these cases, by activation of second messengers. The desensitization of other neutrophil/monocyte chemoattractant receptors by FPR and FPRL1 suggests the existence of a hierarchical cross-talk between different groups of receptors.

6.1.3. Receptor clustering

The activation of membrane receptors with a point source of chemoattractant induces a cell polarization that dictates the direction of movement. Polarization of chemoattractant-stimulated leukocytes correlates with an asymmetric redistribution of distinct subtypes of lipid raft domains to the leading (GM1 glycolipid-rich raft domains) and trailing edges (GM3 glycolipid-rich raft domains) [160]. The segregation of lipid rafts may provide an organizing platform for signalling during gradient sensing and cell polarization. After stimulation with a point source of agonist, an enrichment of the C5aR has been observed at the leading edge [161]. This increase in C5aR concentration reflects the accumulation of highly folded plasma membrane ruffles at the leading edge rather than a real accumulation of receptors [161].

The lipid rafts are thought to participate in membrane reorganization during receptor internalization. It has been shown that, following a uniform agonist stimulation, FPR concentrates into patches/clusters on the plasma membrane in the initial phase of the internalization process [162]. The receptor has been shown to translocate to lipid rafts and to co-localize with GM1 glycolipid-rich domains following ligand binding and prior internalization. Depletion in cholesterol inhibits signalling in response to ligand stimulation, as well as clustering and internalization of the receptor. The fact that FPR clustering is not affected by treatment with pertussis toxin and is still observed with a non-phosphorylatable mutant suggests that clustering precedes receptor phosphorylation and is independent of receptor signalling. However, switching the receptor to an active conformation is required to maintain the receptor in lipid raft domains as evidenced by the fact that an FPR mutant that binds the ligand but is incapable of transitioning to an active state fails to form clusters. In addition, FPR clustering is reversible [162]. Displacement of the ligand from the clustered receptor, by treatment of the cell with an antagonist, results in the loss of clusters and the cessation of signalling activity [162]. Similarly, FPRL1 (personal observation) and C5aR [161] form patches at the plasma membrane. This is observed very shortly after stimulation with a uniform concentration of agonist and before internalization occurs. Receptor clustering may act as a mechanism to assemble signal amplification centres where receptors, G proteins and downstream effectors, by virtue of proximity, can more efficiently and specifically transduce signals. Clusters may also represent specific sites where receptors become phosphorylated and subsequently internalized.

6.2. Receptor inactivation: a multi-step process

Termination of the chemoattractant-mediated responses makes cells refractory to a second stimulation with the same agonist. Several mechanisms that are not mutually exclusive may coexist and be responsible for this loss of responsiveness. It is clearly established that the agonist-mediated phosphorylation of the receptor leads to the high-affinity binding of accessory molecules such as β-arrestins. β-Arrestins sterically interfere with G protein coupling and thereby deactivate receptor signalling. However, this concept has recently been challenged in the case of FPR *in vitro*. Indeed, it has been shown in a soluble reconstituted system that FPR phosphorylation can block the interaction of the G protein independently of β-arrestin binding [163]. In neutrophils, the cytoskeleton is also thought to take part in the deactivation of the FPR-mediated superoxide production through a direct interaction of actin filaments with the C-terminal domain of the receptor [164]. Deactivated receptors, including FPR, FPRL1, and C5aR but not the receptors for IL-8 and the platelet-activating factor, can be reactivated by cytochalasin B, a drug that binds to and blocks the reorganization of actin filaments [165].

6.2.1. Receptor phosphorylation

C5aR and FPR have been shown to become rapidly and differentially phosphorylated in an agonist-, time-, and concentration-dependent manner [166–168]. While FPR phosphorylation is resistant to the PKC inhibitor staurosporin, the phosphorylation of C5aR is partially inhibited. Furthermore,

phorbol 12-myristate 13-acetate (PMA) causes no phosphorylation of FPR but does phosphorylate C5aR. The third cytoplasmic loop of C5aR has potential phosphorylation sites in the context of a consensus sequence (R-X-X-S-X-R-X) for phosphorylation by PKC. However, the major phosphorylation sites appear to be restricted to the carboxyl-terminal region of C5aR [169]. The nature of the kinases that are physiologically involved in the agonist-dependent phosphorylation of C5aR remains controversial. In one study, the overexpression of the G protein-coupled receptor kinases GRK2 and GRK3, in cells that expressed C5aR, resulted in the C5a-dependent hyperphosphorylation of the receptor [170]. However, no effect was observed in another study in which the dominant-negative form of GRK2 and GRK3 were co-expressed [171]. The C5a-stimulated receptor is primarily phosphorylated on serine residues (Ser^{314}, Ser^{317}, Ser^{327}, Ser^{332}, Ser^{334}, and Ser^{338}) [169] and to a much lower extent, on threonine residues that have not been identified [157] (Fig. 3). Mutants with combined amino acid substitutions exhibit different capacities to incorporate phosphate on the remaining serine residues [172,173]. The phosphorylation of either of the two serine pairs (Ser^{332} and Ser^{334}, or Ser^{334} and Ser^{338}) is a prerequisite for full receptor phosphorylation. They serve as primary phosphorylation sites that are required for the phosphorylation of the other serine residues. This support the notion that phosphorylation of C5aR occurs via a hierarchical process. The phosphorylation of either pair of serines is sufficient to allow receptor internalization by the formation of a stable complex with β-arrestins and co-trafficking in intracellular vesicles [174]. Phosphorylation-deficient receptors are not internalized [173] and trigger sustained intracellular signalling events that result in a significant increase in calcium mobilization and production of superoxide [172]. Interestingly, these desensitization-defective receptors were still able to recruit the β-arrestins at the plasma membrane [174].

The carboxyl tail of the FPR contains 11 serine and threonine residues (Fig. 3). Both serines and threonines are phosphorylated [175]. The use of phosphorylation-deficient mutants has

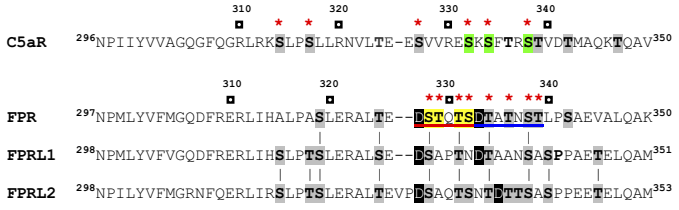

Fig. 3. Alignment of the carboxyl-terminal portions of the C5aR and the members of the FPR family. Serine and threonine residues are indicated in bold. The amino acids identified as the major sites of phosphorylation upon agonist binding are pointed with a red star in the case of C5aR and FPR. The sites phosphorylated in FPRL1 and FPRL2 have not yet been identified. In C5aR, serine residues at positions 332, 334, and 338 (highlighted in green) are critical for both C5a-mediated phosphorylation and C5a-mediated intracellular trafficking of the C5aR-β-arrestin complex. In FPR, the phosphorylation of serines and threonine residues at positions 328, 329, 331, and 332 (highlighted in yellow) is required for the subsequent phosphorylation of the other serine and threonine residues. The residues at positions 328, 332, and 338 are for β-arrestin binding and internalization. The two putative GRK-mediated phosphorylation sites in FPR are underlined in red and blue.

led to the conclusion that eight of these residues, located between and including Ser^{328} and Thr^{339}, are critical for the internalization and the desensitization of the FPR [176]. These residues are arranged in two domains, containing four serines or threonines and preceded by an acidic amino acid. This is a characteristic of GRK-mediated phosphorylation site [177]. Site specific mutagenesis has shown that Glu^{326}/Asp^{327} and Asp^{333} are critical for FPR phosphorylation [175]. Protein kinase A, protein kinase C and tyrosine kinase inhibitors fail to prevent agonist-mediated FPR phosphorylation. The carboxyl tail of FPR (residues 303–350) in fusion with glutathione-S-transferase (GST) can be phosphorylated by GRK2 and to a lower extent by GRK3, whereas GRK5 and GRK6 have no detectable activity [175]. Amino acid substitutions indicate that FPR phosphorylation is a hierarchical mechanism in which the phosphorylation of adjacent serine and threonine (Ser^{328}/Thr^{329} or Thr^{331}/Ser^{332}) is required for the subsequent phosphorylation of the other serine and threonine residues (Fig. 3). The phosphorylation of Ser^{328}, Ser^{332}, and Ser^{338} is critical for the internalization, desensitization, and β-arrestin 2 binding [178]. Two phosphorylation domains differentially regulate β-arrestin and agonist affinities [179,180]. The phosphorylation status of serines and threonines between residues 328 and 332 is a key determinant that controls the affinity of the FPR for β-arrestin. The phosphorylation status of the serines and threonines between residues 334 and 339 allows the receptor that is complexed to β-arrestin to form a high-affinity ternary complex with the ligand. The lack of receptor phosphorylation does not prohibit β-arrestin binding to the FPR, but such interactions are of markedly lower affinity compared with phosphorylated receptors. Phosphorylation of carboxyl-terminal serine and threonine residues may produce a localized concentration of negative charges, which facilitates ionic interactions with the positively charged phosphorylation recognition domain of β-arrestin. This may either enable a firm interaction between the two proteins or induce a β-arrestin intramolecular conformational change that stabilizes the interaction and/or exposes a secondary high-affinity binding site [181,182].

Likewise, FPRL1 is phosphorylated in an agonist-dependent manner but little is known as to the nature of the kinase(s) involved in the process. The determinants responsible for its internalization have not yet been identified. In contrast to FPR and FPRL1, FPRL2 displays a marked phosphorylation in the absence of stimulation [18].

6.2.2. Internalization, intracellular trafficking and recycling

The β-arrestin-dependent endocytic pathway is commonly used by most G protein-coupled receptors. β-Arrestins link phosphorylated receptors to the components of the endocytic machinery, including clathrin and the clathrin adaptor AP2 [183,184]. Fig. 4 illustrates the classical endocytosis/recycling pathways followed by the majority of GPCRs. The β-arrestins target the agonist-occupied receptor to pre-existing clathrin-coated pits for internalization [185]. The large GTPase, dynamin, is required in the process and plays a key role in the constriction of the coated pits [186]. Clathrin-mediated

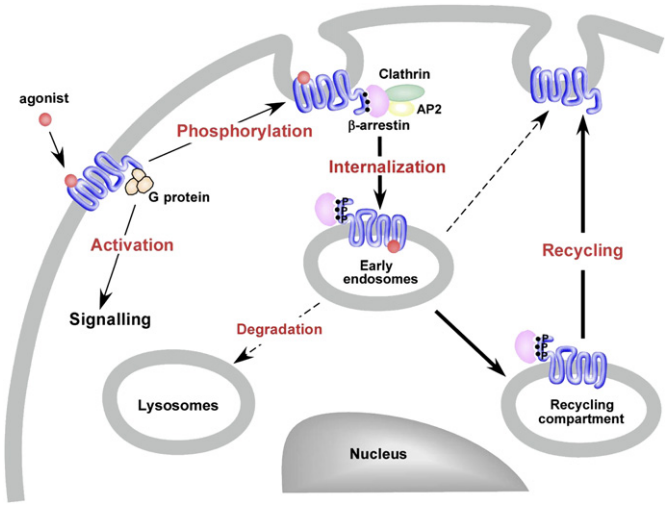

Fig. 4. Intracellular trafficking of activated receptors. Agonist dependent phosphorylation of the receptors leads to the recruitment of β-arrestins. The receptor–β-arrestin complex is targeted to clathrin-coated pits, traffics in early endosomes and accumulates in a perinuclear recycling compartment. After dephosphorylation and dissociation from β-arrestins, the receptors resensitize and recycle to the cell surface. In the case of C5aR, a fraction of the internalized receptor is targeted to lysosomes for degradation.

endocytosis is a common pathway used by GPCRs in many cell types, including myeloid cells. For example, in neutrophils, it has been shown that the ligation of the platelet-activating factor (PAF) to the PAF receptor induces the recruitment of β-arrestin and components of the clathrin-dependent endocytic machinery, namely clathrin heavy chain, α-adaptin, and dynamin [187]. Whether the clathrin-dependent endocytic pathway is the main route followed by chemoattractant receptors in neutrophils is not currently known.

The FPR appears to diverge from this standard model and is capable of using an alternative pathway independent of the action of β-arrestin, dynamin and clathrin [188,189]. Through the characterization of partially phosphorylated mutants, it has been shown that FPR mutants that do not bind β-arrestins are, nevertheless, efficiently internalized [163]. Furthermore, studies with mouse embryonic fibroblasts (MEF) from β-arrestin 1 and 2 double knockout mice [190] have established that phosphorylated FPR internalizes in a β-arrestin-independent manner [189]. Among the formyl peptide receptors, this β-arrestin-independent internalization seems to be a peculiarity of the FPR, as the absence of β-arrestins prevents the internalization of FPRL1 [207]. However, the ability of FPR to be internalized in β-arrestin-deficient cells does not exclude the possibility that a β-arrestin-dependent pathway is involved when β-arrestins associate with the phosphorylated receptor. In β-arrestin-deficient MEF cells, FPR is nevertheless directed to and trapped in the perinuclear recycling compartment [189]. Reconstitution of the β-arrestin-deficient cells with either isoform of β-arrestin restores the return of FPR to the plasma membrane. Recycling thus appears to be β-arrestin-dependent, therefore assigning an expanded role for β-arrestin in receptor trafficking. β-Arrestin binding most likely occurs in the endosomal system, but how β-arrestins regulate the recycling of FPR is still unknown. In contrast, partially phosphorylated FPR mutants do not require β-arrestins for recycling [180]. A constitutively active form of β-arrestin 2 with high affinity for FPR inhibits the recycling of wild type FPR but not the recycling of a partially unphosphorylatable mutant that displays a low affinity for the constitutively active form of β-arrestin 2 [180]. The inhibition of receptor recycling thus appears to correlate with ability of *N*-formyl peptide, FPR and active β-arrestin 2 to form a stable ternary complex. This also suggests that the pattern of phosphorylation/dephosphorylation of the receptor determined its transport from recycling endosomes back to the plasma membrane.

Agonist-triggered internalization of FPRL1 follows a clathrin-dependent endocytic pathway. Internalization from the plasma membrane into intracellular compartments is prevented by conditions interfering with the formation of clathrin-coated pits or the internalization of clathrin-coated vesicles, such as expression of a dominant-negative clathrin mutant, siRNA-mediated depletion of cellular clathrin, and expression of a dominant-negative mutant of dynamin [191]. Internalized FPRL1 co-localizes with Rab11, indicating trafficking through the perinuclear recycling compartment ([191] and our personal observation). A separate, PI3K-dependent, recycling pathway bypassing perinuclear recycling endosomes has also been observed [191].

Similar to FPR, FPRL1 is associated to β-arrestins in the perinuclear recycling compartment [207]. For a growing number of GPCRs that form a long-lasting complex with β-arrestins, it is established that β-arrestins function as signal transducers to connect the receptors with diverse signalling pathways. β-Arrestins have been demonstrated to bind components of the MAP kinase cascades, leading to their activation [192]. β-Arrestin-mediated scaffolding and activation of MAP kinases appear to be linked to the endocytosis of the receptor-β-arrestin complex. The stable association of the receptor and β-arrestins is thought to stabilize cytosolic activity of phosphorylated ERK1/2, while inhibiting ERK1/2 translocation to the nucleus and nuclear ERK1/2 activity [193]. Several recent studies indicate that ERK1/2 activation through β-arrestins does not apply to chemoattractant receptor signalling. Through the use of a collection of FPR mutants, it has been shown that activation of ERK1/2 is independent of receptor phosphorylation, β-arrestin binding, and receptor endocytosis but is conditioned by the activation of the Gi protein [188,208]. Similarly, we found that FPRL1-mediated ERK1/2 activation is not dependent on β-arrestin and occurs primarily through G protein signalling [207]. Since β-arrestins sequester phosphorylated ERK1/2, preventing nuclear translocation and transcriptional activation, it seems highly beneficial for chemoattractant receptors to signal essentially through the G protein, and not through β-arrestins, to ensure the activation of transcription factors that regulate the synthesis of modulators of inflammatory and immune responses.

It has been originally reported that C5aR was not internalized through clathrin-coated pits [188]. However, more recent studies have provided evidence that the C5aR is internalized via the classical clathrin-dependent pathway [174,194]. In neutrophils and transfected CHO cells, C5aR appears to follow

a route divergent from that followed by the formyl peptide receptors after reaching early endosomes. After long-term exposure to the ligand, the C5aR mainly co-localizes with the late endosomal/lysosomal marker LAMP2, and Western blot analyses revealed that a fraction of C5aR is degraded in lysosomes [194]. Nevertheless, a substantial fraction of the receptor recycles to the plasma membrane in the cell type used in this study. The degradation of internalized C5aR appears to be cell type-dependent, since efficient recycling and the absence of degradation has been observed in RINm5F cells ([173] and Boulay, unpublished results). Thus, depending on the cell type, C5aR appears to be submitted to a differential sorting. Ubiquitination of cytoplasmic lysines in the β_2-adrenergic receptor is required for its degradation after prolonged exposure to ligand [195] and three lysine residues in the C-terminal domain of C5aR are potential ubiquitination sites. Alternatively, the C5aR might be directed to the degradation pathway through the interaction with lysosomal targeting sorting proteins such as sorting nexin 1, involved in the sorting of the protease-activated receptor PAR1 [196], or the GPCR-associated sorting protein GASP for the δ-opioid receptor [197]. Whatever sorting machinery is used to target a fraction of C5aR to lysosomes, the nature of the structural determinants involved remains to be defined.

7. Conclusion

Since their identification and molecular cloning, a decade ago, a large body of knowledge has accumulated concerning the biological roles, the intracellular signalling, and the regulation of chemoattractant receptors. Their pathophysiological role has been shown to extend beyond host resistance against microbial infection. The ability of FPR to interact with high affinity with agonists derived from pathogens suggests that this receptor plays a critical role in innate immunity. It has been suggested to behave as a pattern recognition receptor. It is puzzling, but perhaps of pathophysiological relevance, as to how this receptor escapes the classical mode of regulation that applies to its homologue FPRL1, and to C5aR. FPR, and especially FPRL1, can now be considered as promiscuous receptors, with affinity for apparently unrelated agonists. The use of FPRL1 by host-derived agonists, and its association with amyloidosis and multiple inflammatory neurodegenerative diseases, indicates that this receptor may play a crucial role in the regulation of the inflammatory process associated with tissue damage and neurodegeneration. Therefore, it seems of importance to consider chemoattractant receptors as potential targets in the search for specific anti-inflammatory drugs and for the development of new therapeutic strategies.

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