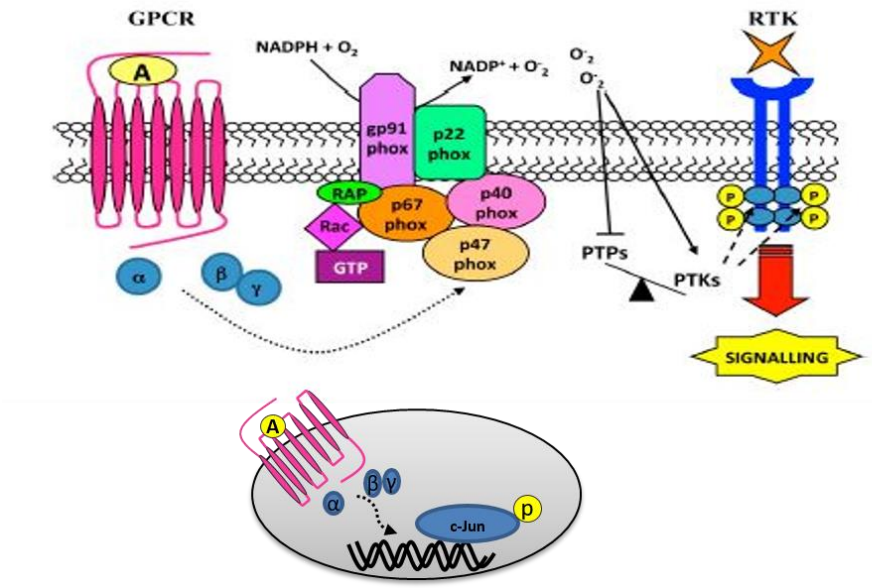
UNIVERSITA' DI NAPOLI FEDERICO II

DOTTORATO DI RICERCA IN BIOCHIMICA E BIOLOGIA CELLULARE E MOLECOLARE XXVIII CICLO

Melania Parisi

New insights in Formyl-peptide Receptors functions



Anno accademico 2014/2015

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New insights in Formyl-peptide Receptors functions

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"Non permettere mai a nessuno di dirti che non sai fare qualcosa. Se hai un sogno tu lo devi proteggere. Se vuoi qualcosa, vai ed inseguila."

Melania

Riassunto

La famiglia dei recettori accoppiati a proteine G (GPCR) comprende la maggior parte dei recettori noti, in grado di convertire un'ampia varietà di stimoli extracellulari in risposte intracellulari attraverso l'attivazione delle proteine G eterotrimeriche, costituendone una categoria importante. Per il loro coinvolgimento in diversi processi fisiologici e patologici rappresentano target ideali per lo sviluppo di nuovi agenti terapeutici. Il recettore per N-formil peptidi FPR1 e le sue varianti FPR2 e FPR3 appartengono alla famiglia dei recettori a sette tratti trans-membrana accoppiati a proteina G_i, sensibili all'azione della tossina della pertosse (PTX). La loro espressione è stata individuata inizialmente in cellule del compartimento polimorfonucleato dove tali recettori mediano la migrazione dei fagociti presso i siti di infezione batterica e/o di danno tissutale, contribuendo alla risposta umorale innata. I recettori accoppiati a proteina G sono in grado di transattivare i recettori tirosina kinasi presenti sulla membrana cellulare. Questo meccanismo di cross-talk tra differenti sistemi di signaling gioca un ruolo chiave nel coordinare la pletora di stimoli extracellulari ai quali la cellula è sottoposta in condizioni fisio-patologiche. Abbiamo analizzato la capacità di FPR2 di transattivare il recettore per il fattore di crescita epatico (c-Met) in cellule epiteliali di prostata (PNT1A) e di FPR1 di tranattivare il recettore per il fattore di crescita dell'endotelio vascolare (VEGFR2) in cellule endoteliali (ECV). I risultati hanno mostrato che WKYMVm, agonista di FPR2 induce la fosforilazone delle tirosine 1313, 1349 e 1356 di c-Met e N-fMLP agonista di FPR1 determina la fosforilazione del residuo di tirosina 996 di VEGFR2. Come risultato della transattivazione, le tirosine fosforilate di questi due recettori forniscono sito di attracco per il reclutamento di molecole trasduttori di segnale evocando l'attivazione di STAT3, PLC-γ/PKCα e del pathway PI3K/AKT. La generazione di specie reattive dell'ossigeno da parte della NADPH ossidasi gioca un ruolo chiave in questo meccanismo di cross-talk, infatti, agenti in grado di bloccare l'attività della NADPH ossidasi prevengono la trasattivazione dei due recettori tirosina kinasi nonché i meccanismi molecolari a valle.
Modelli correnti indicano che ciascun GPCR ed il set di proteine eterotrimeriche ad essi associate sulla superficie cellulare innescano

una o più vie di segnalazione intracellulare attraverso complessi di GTPasi, chinasi e proteine ad essi legati. Recenti studi di "trafficking" di alcuni GPCRs hanno mostrato, che in seguito a stimolazione con specifici ligandi o ad attivazione attraverso meccanismi ligando-indipendente, i GPCRs possono essere esposti sulla membrana nucleare per periodi prolungati, ed innescare eventi di segnalazione diversi ed in alcuni casi opposti rispetto a quelli mediati dai GPCR di membrana. Abbiamo dimostrato mediante esperimenti di western blot, immonofluoscenza e saggi di binding che l'isoforma FPR2, ma non FPR1 ed FPR3, è espressa sulla membrana nucleare di cellule di cancro anaplastico del polmone (Calu6) e di adenocarcinoma gastrico (AGS). FPR2 nucleare è un recettore funzionale, in quanto partecipa al signaling intra-nucleare, come dimostrato dalla diminuzione della proteina G_i associata al recettore, dalla fosorilazione di ERK2, c-Jun e c-Myc, in seguito a stimolazione di nuclei intatti con WKYMVm, agonista selettivo di FPR2. Abbiamo quindi ipotizzato che la sequenza amminoacidica di FPR2 potesse contenere una sequenza di localizzazione nucleare (NLS) che giustificasse la sua dislocazionesulla membrane nucleare. Attraverso analisi bio-informatiche in silico ed un programma di predizione di sequenze contenenti NLS, abbiamo identificato la sequenza (227-KIHKK-231) presente nel terzo loop intracellulare del recettore, costituita da amminoacidi basici (lisina, isoleucina, istidina, lisina, lisina). Sono stati condotti quindi esperimenti di mutagenesi sito-diretta della putativa NLS. La sequenza consenso è stata mutata e il cDNA è stato trasfettato in cellule HEK293, che non esprimono il recettore FPR2. L'amminoacido basico lisina, che segue l'istidina, nella sequenza -KIHKK- è stato mutato in alanina (apolare e idrofobico), originando la sequenza -KIHAK- (FPR2 mut 3). A partire da tale mutante ulteriori mutazioni sono state effettuate a carico dell'istidina e dell'ultima lisina generando il mutante -KIAAA- (FPR2 mut bis), dove due alanine hanno sostituito i due amminoacidi basici. Esperimenti di immunofluorescenza e western blot hanno mostrato che la localizzazione o traslocazione nucleare di FPR2 dipende dall'integrità dell'istidina 229 e dalla lisina 231.

Questi risultati evidenziano un ruolo inaspettato dei recettori FPRs, sottolineando che per la loro promiscuità e il legame a diversi ligandi, unita alla loro espressione in differenti tipi cellulari e tessuti, tali recettori possono avere funzioni diverse in contesti biologi differenti.

Una migliore comprensione dei meccanismi molecolari potrebbe portare all'identificazione di targets terapeutici per lo sviluppo di nuovi farmaci.

Summary

GPCRs (G-protein-coupled receptors) are versatile signaling molecules at the cell surface and are the largest and most diverse family of membrane receptors in the human genome. They convert a large variety of extracellular stimuliinto intracellular response through the activation of heterotrimeric G-proteins, which constitute the key regulatory elements in a broad range of normal and pathological processes and the most important targets for pharmaceutical drug discovery. The human formyl-peptide receptor FPR1 and its variants FPR2 and FPR3 belong to the Gi-protein coupled seven transmembrane receptor family sensitive to pertussis toxin (PTX)as indicated by the total loss of cell response to their agonists upon exposure to pertussis toxin.They are expressed in several cell types and their crucial biological functions are supported by the identification of high affinity host-derived agonists. An important feature of GPCR is their ability to transactive tyrosine kinase receptor. This cross-communication between different signaling systems plays a key role to coordinate the plethora of extracellular stimuli to which a cell is subjected under several physiological or pathological conditions. We investigated the ability of FPR2 to transactivate the RTK Hepatocyte growth factor receptor (c-Met) in PNT1A cells and of FPR1 to cross-talk with the vascular endothelial growth factor receptor (VEGFR) in ECV cells. We show that WKYMVm an FPR2 agonist induces the phosphorylation of Y1313/1349/1356 of c-Met and the FPR1 agonist N-fMLP triggers VEGFR2 activation by phosphorilation of Y996 residue.As a result of transactivation, phosphotyrosine residues of two receptors provide docking sites for recruitment and triggering of STAT3, PLC-γ/PKCα and PI3K/Akt pathways in both cell lines. The critical role of NADPH oxidase-dependent superoxide generation in this cross-talk mechanism is supported by the finding that blockade of NADPH oxidase function prevents trans-phosphorylation of two TRKs and the resulting downstream signalling cascade.

Current model indicate that each GPCR and associated sets of heterotrimeric G proteins on the cell surface had been proven to initiate one or more chains of intracellular signaling events mediated by complexes of GTPases, kinases, and linking proteins.Recentstudies

of intracellular trafficking of a few different GPCRs, evoked by exposure to ligand or by cellular activation through ligand-independent mechanisms, have shown that GPCRs may be inserted into nuclear membranes for prolonged periods, compose distinctive signaling units there, and respond to specific intracellular ligand by transducing nuclear transcriptional signals that differ from those sent by their plasma membrane complexes.We show by western blot analysis, immunofluorescence experiments and radioligand binding assays that FPR2, but not FPR1 and FPR3, is expressed at nuclear level in CaLu-6 and AGS cells. Nuclear FPR2 is a functional receptor, since it participates in intra-nuclear signaling, as assessed by decreased G protein-FPR2 association and enhanced ERK2, c-Jun and c-Myc phosphorylation upon stimulation of intact nuclei with the FPR2 agonist, WKYMVm. We analyzed FPR2 sequence for the search of a nuclear localization sequence (NLS) and we found a stretch of basic aminoacids (227-KIHKK-231) in the third cytoplasmic loop of the receptor. We performed single (K230A) and multiple (H229A/K230A/K231A) mutagenesis of NLS. The constructs were individually overexpressed in HEK293 cells and immunofluorescence and western blot analysis showed that nuclear localization or translocation of FPR2 depends on the integrity of the H229 and K231 residues within the NLS.

Taken together, our data indicate that FPRs play new unexepected roles in cell signaling and function. The promiscuity of these receptors in binding different ligands, coupled with their presence in different cells and tissues, indicates a diverse role in multiple biological settings. A better understanding of these fundamental functions could lead to the identification of new therapeutic targets for drug development.

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1. Introduction

1.1. Structure and function of G-protein-coupled-receptor

GPCRs (G-protein-coupled receptors) are versatile signaling molecules at the cell surface and constitute the largest and most diverse family of membrane receptors in the human genome. They convert a large variety of extracellular stimuli into intracellular response through the activation of heterotrimeric G-proteins. GPCRs represent key regulatory elements in a broad range of normal and pathological processes as well as the most important targets for pharmaceutical drug discovery [1].They share a common seven-transmembrane (7TM) topology. Diversity of the extracellular ligands is reflected in the structural diversity of more than 800 human GPCRs, which can be grouped in families and numerous subfamilies on the basis of their aminoacid sequences [2]. Variations in extracellular loops, TM helices, and side chains create a remarkable variety of sizes, shapes, and electrostatic properties of the ligand binding pockets in different GPCR subfamilies, reflecting the diversity of their corresponding ligands [2]. G proteins, form hetero-trimers composed of Gα, Gβ and Gγ subunits. G protein α subunits, possess an intrinsic GTPase activity, which enables them to act as time switches: hydrolysis of the bound GTP to GDP promotes the re-association of the α subunit with the βγ dimer and renders the G protein in an inactive form [3]. Gα subunits are grouped into four families on the basis of their amino acid similarity and function. These include, Gαs and Gαi/o which stimulate or inhibit an adenylate cyclase, respectively, Gαq/11 which stimulates a phospholipase C, and the less characterized Gα12/13 family that activates the Na+/H+ exchanger pathway [4]. GPCRs interact specifically with the α subunits of the G proteins through their intracellular domains, however the same G protein may be activated by several receptors and the same receptor may couple to different G proteins, under different circumstances [4]. GPCR activity represents a coordinated balance between molecular mechanisms governing receptor signaling, desensitization, and resensitization. The desensitization is the consequence of a combination of different mechanisms. These mechanisms include the uncoupling of the receptor from heterotrimeric G proteins in response

to receptor phosphorylation [5], the internalization of cell surface receptors to intracellular membranous compartments, and the downregulation of the total cellular complement of receptors due to reduced receptor mRNA and protein synthesis, as well as both the lysosomal and plasma membrane degradation of pre-existing receptors [5]. The extent of receptor desensitization varies from complete termination of signaling, to the attenuation of agonist potency and maximal responsiveness. Three families of regulatory molecules are known to contribute to the GPCR desensitization process: second messenger-dependent protein kinases, G protein-coupled receptor kinases (GRKs) and arrestins [6]. The most rapid means by which GPCRs are uncoupled from heterotrimeric G proteins is through the covalent modification of the receptor as a consequence of phosphorylation by intracellular kinases. The GRK family of kinases is comprised of seven family members (GRK1-GRK7) that share significant sequence homology. GRK family members selectively phosphorylate agonist activated receptors, thereby promoting the binding of cytosolic cofactor proteins called arrestins, which sterically uncouple the receptor from heterotrimeric G protein [7]. GRKs phosphorylate GPCRs at both serine and threonine residues localized within either the third intracellular loop or carboxyl-terminal tail domains. The relative contributions and mechanisms by which second messenger-dependent protein kinases and GRKs regulate GPCR desensitization are not fully understood. Beta-arrestin is a small protein that can regulate GPCRs desensitization and internalization [8]. An other important aspect of GPCR desensibilization is the internalization or sequestration of agonist-activated receptors into the intracellular membrane compartments of the cell. Different receptor types utilize distinct pathways for removal from the cell surface, such as clathrin-coated pits or caveolae-mediated endocytosis. Once internalized, receptors traffic in intracellular vesicles through a series of intracellular vesicular compartments that eventually lead to release of the receptor back onto the cell surface in an activatable state [9].

1.2. N-formil peptide receptors

The human formyl-peptide receptor FPR1 and its variants FPR2 and FPR3 belong to the Gi-protein coupled seven transmembrane receptor family sensitive to pertussis toxin [10], as indicated by the total loss of cell response to their agonists upon exposure to pertussis toxin (PTX). Pertussis toxin catalyses an ADP-ribosylation of Gα, arresting Gα in the GDP-bound state and, in turn, prevents the protein to transduce signals [10]. In human three genes encoding two functional receptors, FPR1and FPR2, and the putative receptor FPR3 have been identified. Human FPR1 was first defined biochemically, in 1976, as a high affinity binding site on the surface of neutrophils for the prototypic N-formyl peptide formyl-methionine-leucyl-phenylalanine (fMLP). It was then cloned in 1990, from a differentiated HL-60 myeloid leukemia-cell cDNA library [11]. Functionally, FPR1 is activated by picomolar to low nanomolar concentrations of fMLP, and is therefore also identified as the high-affinity fMLP receptor. It is encoded by a 6 kb single copy gene [12], the open reading frame is intronless but the 5% untranslated region resides in three exons. The start sites for transcription and translation are separated by approximately 5 kb. The proposed promoter contains a nonconsensus TATA box and an inverted CCAAT element. FPR1 is a 350-residue protein with seven hydrophobic segments [12]. A subsequent study characterized FPR1 as a seven-transmembrane receptor with the N-terminus and three loops exposed on the cell surface for ligand interactions and the C-terminus and the remaining loops found within the cytoplasm necessary for intracellular signaling [13]. FPR1 expression has initially been described in phagocytic leukocytes, monocytes and neutrophils. It was later observed in immature dendritic cells (DCs), microglial cells, platelets, spleen and bone marrow. It is also described in non-hematopoietic cell populations and tissues, such as hepatocytes, fibroblasts, astrocytes, neurons of the autonomic nervous system, lung and lung carcinoma cells, thyroid, adrenals, heart, the tunica media of coronary arteries, uterus, ovary, placenta, kidney, stomach and colon. Using low-stringency hybridization with FPR1 cDNA as the probe, two separate but relatively conserved low-affinity receptors, were cloned from

amRNA library of neutrophil-like promyelocytic HL-60 cells. These receptors have been named FPR2/ALX and FPR3, respectively, as more has become known about their distinct biochemical and physiological roles. All three receptors are clustered together on chromosome 19q13.3 and share significant sequence homology [14]. The sequence similarity between FPR1 and FPR2 is 69% at the aminoacid level. Although there is a large sequence similarity also between FPR2 and FPR3 (83% identity at the aminoacid level) [15] the latter can not bind formylated peptides. The similarities between the receptors are even larger when only the intracellular signalling transducing domains are compared [15]. FPR2 is considered a low-affinity fMLP receptor based on observations that high concentrations of fMLP could elicit only Ca^{2+} mobilization but not chemotaxis through this receptor and compared to FPR1. FPR2 is also expressed in a large variety of cells and organs, including monocytes, macrophages, neutrophils, T and B lymphocytes, microglial cells, platelets, hepatocytes, epithelial cells, microvascular endothelial cells, fibroblasts, spleen, lung, testis, placenta, brain and bone marrow, as well as astrocytoma and neuroblastoma cells [16]. FPR3 transcripts were detected in a wide variety of tissues, including spleen, lymphnodes, lung, trachea, liver, adrenal gland, small intestine, placenta, and other tissues at lower levels. FPR3 is the only receptor expressed by both immature and mature dendritic cells.

Classic studies suggested that the N-formyl group was a crucial determinant of ligand binding to FPRs. Because bacterial and mitochondrial proteins [11, 14] are the only sources of N-formyl peptides in nature, it has been widely thought that these receptors evolved to mediate trafficking of phagocytes to sites of bacterial invasion or tissue damage. Nevertheless, in the last years a large number of non-formylated peptide ligands for FPR1 and FPR2 have been identified [15].

1.3. Agonists and antagonists

Since FPRs initial characterisation, and the discovery of several members in this receptor family, it has become evident that a variety of biological functions could potentially be driven by the large

number of molecules able to agonise or antagonise these GPCRs. The FPR1 is a high-affinity pattern recognition receptor with the ability to track bacteria that release formylated peptides [17]. The receptor also binds formylated peptides of mitochondrial origin [16]. Recent work reveals that FPR1 also recognizes nonformylated peptides, as well as peptides with several other modifications which all bind with high affinity and activate the receptor. In addition, FPR1 recognizes and is activated by peptides that lack all sequence similarities with the peptides originally isolated from bacteria [18]. FPR1 binds N-fMLP with high efficiency, whereas FPR2 is defined as a low-affinity N-fMLP receptor, based on its activation only by micromolecolar concentrations of formyl-peptide. FPR2 is the only receptor whose ligands include both a formyl-peptide and a lipid, as demonstrated by the observation that it also binds. Lipoxin A4 (LXA4) [19]. FPR3 does not respond to formyl-peptides. FPR2 also shows the ability to bind microbe-derived peptides, such as those derived from Helicobacter pylori and HIV-1, but it can also respond to mitochondrial peptides and synthetic peptides [20].LL37 is a 37 amino-acid peptide naturally cleaved from the C-terminus of the anti-microbial protein cathelicidin, which is present in human neutrophil granules and epithelial cell surfaces. LL-37 is microbicidal, neutralizes endotoxin by direct binding and, in addition, has chemotactic activity for human neutrophils, monocytes, and resting T cells interacting with FPR2 [20]. Therefore, LL37 could act to amplify the innate immune response. FPRs have also been implicated in host responses during HIV-1 infection because several synthetic peptides, corresponding to amino-acid sequences of HIV-1 envelope proteins gp41 and gp120, are chemotactic agonists for FPR2. Annexin I (lipocortin I), lipoxin A4 (LXA4) and urokinase-type plasminogen activator receptor (uPAR) comprise a second group of newly identified host-derived agonists for FPR1 and FPR2, which have suggested novel roles for these receptors in the regulation of acute and chronic inflammation. From the pathogenic point of view, amyloidogenic molecules have been studied as interesting ligands for FPR2. This group comprises the acute phase protein serum amyloid A (SAA), β-amyloid peptide 42 (Aβ42) [21] and the prion protein-

derived peptide PrP106-126. The observation that Aβ42 is chemotactic and activating for monocytes and microglia cells through FPR2, suggests that this receptor is at least partly responsible for proinflammatory destructive activity in brain tissue during Alzheimer's disease. The PrP106-126 prion-peptide fragment activates FPR2 to induce human monocyte migration and to increase the production of the proinflammatory cytokines tumor necrosis factor (TNF)-α and IL-1β, both of which are implicated as neurotoxic mediators [22]. This suggests that FPR2 might participate in inflammatory pathology seen in prion diseases. A number of synthetic peptides are agonists for FPR2. The first synthetic peptides proposed as a specific FPR2 agonist is the WKYMVm (Trp-Lys-Tyr-Met- Val-D-Met) peptide. It was isolated by screening a synthetic peptide library composed of random sequences of hexapeptides and contains a D-methionine at position 6 that greatly enhances its biological activity [20]. WKYMVm binds to FPR2 with high efficiency and with lesser efficiency to FPR1 and FPR3 [23], thereby activating neutrophil and monocyte functions, including chemotaxis, mobilization of complement receptor-3, cytokine release and activation of NADPH oxidase, which results in the respiratory burst. WKYMVm activates neutrophils through FPR1 only when signaling through FPR2 is blocked, which is indicative of a receptor switch.FPR1 and the closely related FPR2 have been linked, in different animal models, to chronic inflammation and auto-immune diseases. Through this type of research few inhibitors/antagonists have been identified and characterized [24].FPR1 is activated by formylated peptides, and already 25 years ago it was shown that replacement of the formyl group by a tert-butyloxycarbonyl (Boc) group generates a receptor-specific antagonist wich inhibit chemotactic peptide-induced cell activation [25]. An other antagonists of Boc group selective for FPR2 is the peptide FLFLF (also called Boc-2) containing a tert-butyloxylcarbonyl (Boc) group at the N-terminus. This peptide antagonize the FPR2-agonist serum amyloid A (SAA). Endogenous bile acids such as deoxycholic acid (DCA, 1) and chenodeoxycholic acid (CDCA, 2) are reported to antagonize FPRs at pathophysiological concentrations . These peptides block the access of agonists to the

receptor and may interfere with conformational movements of extracellular loops linked to receptor activation. Among the novel peptides, Trp-Arg-Trp-Trp-Trp-Trp-CONH2 (WRW4) shows the most potent activity in terms of inhibiting WKYMVm binding to FPR2. WRW4 prevents the activation of FPR2 by WKYMVm, resulting in the complete inhibition of the intracellular calcium increase, extracellular signal-regulated kinase activation, and chemotactic migration of cells toward WKYMVm. WRW4 specifically inhibits the increase in intracellular calcium by the FPR2 agonists MMK-1, amyloid β42 (Aβ42) peptide but not by the FPR1 agonist, fMLP [26].

1.4. GPCR-mediated cell-surface receptors transactivation

Cross-communication between different signaling systems plays a key role to coordinate the plethora of extracellular stimuli to which a cell is subjected under several physiological or pathological conditions. Cell-surface receptors are the key components of these networks and the inter-receptor crosstalk acts as a general signaling mechanism connecting and diversifying signal transduction pathways. The major classes of cell surface transmembrane proteins are tyrosine kinase receptors (RTKs) and G-protein-coupled receptors (GPCRs), which are the largest group of cell-surface seven-transmembrane proteins [27]. RTKs activation is achieved by ligand binding to the extracellular domain, which can induce dimerization of the receptor and, in turn, the autophosphorylation on tyrosine residues within the cytosolic domain with the formation of Src homology 2 (SH2) or phospho-tyrosine binding (PTB) sites [28]. These represent docking sites for the recruitment of SH2-domain-containing proteins or adaptor proteins, which trigger intracellular signaling cascades. RTKs and GPCRs act in concert to regulate physiological processes and in some cases their effects are synergic whereas in others they antagonize [29]. The activation of GPCRs can stimulate the signaling activity of RTKs connecting the broad diversity of GPCRs with the potent signaling capacities of RTKs. Transactivation of RTKs by GPCRs signaling can occur through different mechanisms: in a ligand-dependent or in a ligand-indipendent manner. In the ligand-

dependent triple-membrane-passing-signal (TMPS) mechanism, GPCR-mediated RTK transactivation depends on activation of membrane-bound matrix metalloproteases (MMPs), such as the A Disintegrin And Metalloprotease (ADAM) family members wich triggers MMP mediated shedding of ligands able to activate RTKs. The ligand-independent mechanism instead suggests that GPCR stimulation triggers the activation of several second messengers such as Ca^{2+} ions, protein kinase C (PKC), the non-receptor protein tyrosine kinases Src and Pyk, β-arrestin and reactive oxygen species (ROS) which, in turn, induce tyrosine phosphorylation and subsequent activation of RTKs [30]. Several molecular mechanisms allow GPCRs to activate Src family kinases, and conversely Src activity plays a central role in controlling GPCR responses. Src family tyrosine kinases are an integral component of the signal transduction apparatus employed by RTKs, playing a key role in cellular growth and malignant transformation.In many cases Src family kinases are associated with GPCRs through direct interaction with cytoplasmic receptor domains, which contain consensus SH3 domain-binding motifs or proline-rich motifs within their third intracellular loops or C-terminal tails, or by binding to GPCR-associated proteins, such as heterotrimeric G-protein subunits or β-arrestins. Src family kinases are also activated by GPCRs [31]. Transactivation can require the generation of oxygen species. ROS can activate many protein tyrosine kinases through different mechanisms. In fact, ROS (1) may directly activate kinases by altering protein–protein interactions; (2) may directly inactivate by oxidation the cysteine residue in the catalytic site of protein tyrosine phosphatases, which in turn results in tyrosine kinases activation; (3) may stimulate proteolysis of regulatory proteins inhibiting tyrosine kinase activity. ROS are produced by a variety of extracellular stimuli such as growth factors, GPCR agonists, cytokines, ultraviolet radiation, increased osmolarity, and other cellular stresses [32]. Sources of their production include mitochondria, xanthine oxidase and NADPH oxidase (Nox) family, which generate superoxide anion (O_2^-), hydrogen peroxide (H_2O_2), and hydroxyl radical (OH·) involved in intracellular redox signaling. The pathophysiological effects of these molecules depend on their

concentration, subcellular localization and the endogenous antioxidant status. Plasma membrane-associated Nox family members are the only enzymes that generate ROS as their primary purpose in a highly regulated and spatial restricted manner, apparently suited for cell signaling and are considered the main source of ROS acutely produced upon growth factor or cytokine stimulation [33]. In several cell types seven isoforms of the catalytic subunit (Nox1–5, Duox1 and 2) of NADPH oxidase have been identified. The NADPH oxidases are multimeric protein complexes consisting of up to three cytosolic subunits (p47phox, p67phox and p40phox), a regulatory G-protein (Rac1 or Rac2) and a membrane-bound cytochrome b558 reductase domain [34]. The cytochrome b558 domain includestwo proteins: a small α-subunit, p22phox, and a larger, catalytic β-subunit that contains binding sites for NADPH and molecular oxygen, as well as flavin and heme groups to allow electron transport between the two substrates [34]. When cells are exposed to any of a very wide variety of stimuli, the cytosolic component p47phox becomes heavily phosphorylated and the entire cytosolic complex migrates to the membrane, where it associates with cytochrome b558 to assemble the active oxidase.The assembled oxidase is now able to transfer electrons from the substrate to oxygen [34]. Stimulation of GPCRs with an agonist induces p47phox phosphorylation and Nox activation, which generates reactive oxygen species (ROS) by O2 using NADPH as electron donor. ROS inactivate phosphotyrosine-phosphatases (PTPs) by oxidation of a cysteine in the catalytic domain, unbalancing intracellular phosphorylation equilibrium. The enhanced activity of phosphotyrosine-kinases (PTKs) mediates the trans-phosphorylation of tyrosines in the cytosolic region of RTK which, in turn, provide docking sites for assembly and activation of signaling complexes [35,36].

1.5. Nuclear GPCR

GPCRs comprise a large family of transmembrane proteins activated by a broad range of ligands and implicated in many patho-physiological processes [37]. Until now, each GPCR and associated sets of heterotrimeric G proteins on the cell surface had been proven

to initiate one or more chains of intracellular signaling events mediated by complexes of GTPases, kinases, and linking proteins. Occupancy of a GPCR by its complementary ligand stimulates down-regulation of expression of the GPCR, by processes that include a brief intracellular cycle through endosomes and perinuclear vesiculotubular structures, and thereby lead to proteolysis or reinsertion in the plasma membrane [38]. New studies of intracellular trafficking of a few different GPCRs, evoked by exposure to ligand or by cellular activation through ligand-independent mechanisms, have shown that GPCRs may be inserted into nuclear membranes for prolonged periods, compose distinctive signaling units there, and respond to specific intracellular ligand by transducing nuclear transcriptional signals that differ from those sent by their plasma membrane complexes [39]. Several mechanisms according to which GPCRs can significantly influence nuclear events, have been proposed. The first consists of the activation of the GPCR complex, triggering second messengers which enter in the nucleus and induce processes of cellular modification, without leave the membrane. The second consists in the entrance in the nucleus of protein fragments of GPCRs, that are able to induce ligand-independent signals, or intact GPCRs that active trascription factors that directly induce or regulate transcriptional events. In other conditions it seems that GPCRs are constitutively expressed on the nucleus. Several hypotheses have been proposed to elucidate the mechanism(s) by which membrane-bound receptors are localized in nuclei, including nuclear import of cleaved receptor fragments and chaperone proteins facilitating the removal of the receptor from a membrane-embedded state. Presently, the most plausible explanation for GPCRs in the nucleoplasm is through transport within micelles, which would retain a membrane-embedded GPCR suitable for further signaling in cells nuclei [40]. The most studied is the molecular mechanism through which intact GPCRs localize in nuclear membranes of activated cells, assemble signaling complexes there, bind their respective cognate mediators in the nuclear domain, and transduce transcriptional signals different from those sent to the nucleus by the same GPCRs in the plasma membrane. For istance, T cell proliferation by S1P has been linked to

signals from type 1 GPCR for S1P (S1P1) in the plasma membrane, whereas nuclear S1P1 transduces intracellular S1P suppression of proliferation [41].

1.6. Implications of nuclear signaling by GPCRs

The nuclear GPCR complexes signal transcriptional events different than the corresponding plasma membrane GPCRs. The potential biological importance of these GPCRs may be considered on three different levels. First, these GPCRs operate differently in pre- and post cell activation stages, both in terms of their mechanisms of signal transduction and the functional consequences. Secondly, prior to cell activation by a GPCR ligand or other mechanism, plasma membrane GPCRs send one type of nuclear signal whereas after cell activation and GPCR nuclearization the subsequent nuclear signals may have different or even opposite cellular effects [42]. Multiple GPCRs have been localized on the nuclear membrane of cardio-myocytes including angiotensin II type 1 receptor (AT1R) [43], angiotensin II type 2 receptor AT2R , α 1AR, β1-AR, β3-AR, and endothelin B receptor ETBR. Most of the downstream molecules normally associated with GPCR signalling at the plasma membrane have also been found in the nucleus. These include all three major forms of the G protein α subunit as well as βγ complexes [44]. Furthermore, a number of effector molecules have also been shown to be located at nuclear level, including cAMP , PLA2, PLCβ1 and PLCδ1 as well as a variety of ion channels, including Ca^{2+}, $K^{+}$and Cl^{-} channels [45]. Moreover, the second messengers associated with downstream signalling events, including cAMP , IP3 and DAG, are also present at the level of the nucleus or generated in isolated nuclei [46]. In addition to effectors, many of the systems involved in regulation of GPCR signalling are also be found in the nucleus, including RGS proteins, β-arrestin-1 and GRKs. This indicates that nuclear GPCR signalling is tightly regulated, in a manner analogous to their cell surface counterparts, and hence reinforces the idea that these receptors are functionally relevant. Furthermore, certain GPCRs, such as GABA B receptors, have been shown to interact directly with transcription factors [47], while nuclear bradykinin B2 and type I

gonadotropin releasing hormone receptors appear to be able to regulate histone acetylation, adding yet another potential method by which nuclear localized GPCR signalling might modulate gene transcription [48]. Nuclear GPCRs can be constitutively activated or activated by specific ligands, which are synthesized within the target cell or are internalized from the extracellular space (intacrine ligands). The potential actions of intracellular ligands include: a) binding to intracellular membrane receptors and generation of second messengers; b) binding to nuclei and nucleolus; c) binding to chromatin; d) binding to receptors on the endoplasmic reticulum (ER) immediately following their synthesis, and e) binding to intravesicular receptors with consequent generation of second messengers [42]. Intracrine ligands can be synthesized and targeted to the Golgi apparatus for secretion; however, they may also act intracellularly either before or after secretion. Many of these ligands regulate the generation of second messengers, translocate and bind to the nucleus, and can be pro-angiogenic or anti-angiogenic [49]. Although still poorly understood, the diverse functions exerted by agonists and hormones acting at intracellular GPCRs suggests that intracrine signalling may play important roles distinct from those of the same receptors activated at the cell surface. Further confirm the nuclear localization of GPCR constitutively expressed, but also internalized in a ligand depend or indipendent manner, is supported by the recognition of a nuclear localization sequence (NLS). Searches in data bank revealed 17 GPCRs with a clearly recognizable NLS motif in the eighth helix including adenosine, angiotensin, bradykinin, and endothelin receptors. However, a putative NLS motif is located at the carboxyl-terminal region of the third intracellular loop in other receptors such as apelin receptor [50].

1.7. Scientific hypothesis and aim of the work

The G-protein-coupled receptor (GPCR) comprise a large family of transmembrane proteins actived by a broad range of ligands and implicated in many phato-physiological process [27]. Despite GPCRs lack intrinsec tyrosine kinase activity, tyrosine phosporylation of RTKs occurs in response to binding of specific agonists of several

such receptors, triggering intracellular mitogenic cascades. This suggests that the notion that GPCRs are associated with the regulation of post-mitotic cell fuctions is no longer believable.GPCRs are able to transactive tyrosine kinase receptors (RTKs) and this inter-receptor crosstalk acts as a general signaling mechanism connecting and diversifying signal transduction pathways. We investigated:

- The cross-talk between FPR2 and c-Met in primary prostate epithelial cells.
- The cross-talk between FPR1 and VEGFR2 in endothelial cells.
- The role of NADPH oxidase-dependent superoxidase generation in RTKs transactivation.

Current model of GPCRs signalling describe binding of external agonists to cell surface receptors which, in turn, trigger several biological responses. New paradigms indicate that GPCRs localize to and signal at the nucleus, thus regulating distint signalingcascades. A full complement of downstream signal trasduction components is present on the nuclear membrane including G proteins and enzyme effectors. Therefore, the resulting biological effects might result from the intregation of extracellular and intracellular signalin pathways. We investigated:

- FPR2 expression on the nuclear membrane in human lung cancer (Calu6) and gastric adenocarcinoma (AGS) cells.
- The presence of a nuclear localization sequence (NLS) in FPR2 cDNA sequence.
- The ability of nuclear FPR2 to trigger specific nuclear signaling upon stimulation with its specific synthetic ligand WKYMVm.

2. Materials and Methods

2.1. Cell culture

CaLu-6 cells were obtained from ATCC (Rockville, MD, USA) were grown in Dulbecco's modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS), 100 U/ml penicillin, 100 µg/ml streptomycin, 1% l-glutamine, and 1% modified Eagle's medium. PNT1A,primary prostate epithelial and ECV304 cells were purchased from were obtained from ATCC (Rockville, MD, USA) and were grown in RPMI supplemented with 10% heat-inactivated fetal bovine serum (FBS), 100 U/ml penicillin, 100 µg/ml streptomycin and 1% L-glutamine. AGS cells are derived from poorly differentiated human gastric adenocarcinoma and were grown as monolayer in DMEM (Invitrogen, Grand Island, NY, USA) supplemented with 10% fetal calf serum, 100 U/ml penicillin G sodium and 100 µg/ml streptomycin sulfate at 37 °C in a humidified atmosphere of 5% CO2.Stable FPR2 shRNA-expressing cell lines were genereted as described [51]. In short interfering RNA experiments 4x 10^5 cells were incubated for 12 h with 5 nM siRNAs in RPMI containing 10% FBS in the presence of 20 ll HiPerFect (Qiagen, Hiden, Germany). Cells were then serum-deprived for 24 h and stimulated with WKYMVm or N-fMLP. PNT1A cells were also stimulated with 10 ng/ml PMA for different times as indicated in the figure. HEK293 cells (8x105/well) were seeded in a 6-multiwell and transfected with FPR2wt or FPR2mut3 or FPR2mutBis. Briefly, 30 µg of plasmids were diluted in 700 µl of DMEM without antibiotics and growth factors. 150 µl of this solution was incubated with 10 µl of Lipofectamine 2000 (Invitrogen, Carlsbad, CA, USA) diluted in 150 µl of DMEM without antibiotics and growth factors, for 5 min at room temperature (RT). DNA complexes were incubated with HEK293 cells for 24 h at 37 °C.

2.2. RNA isolation

Total RNA was purified with TRIZOL® (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's instructions and retrotranscribed according to the manufacturer'sinstructions by using cDNA Synthesis Kit (Invitrogen) in a final volume of 20 µl.

2.3. Protein studies

Protein extractions, immunoblotting and immunoprecipitation were carried out according to standard procedures [52].

2.4. Mutagenesis Assay

K230A point mutation and H229A/K230A/K231A triple mutant within myc-tag-FPR2 fusion protein were generated by using Geneart site-directed mutagenesis system kit (Invitrogene, Carlsbad, CA, USA) according to the supplier's instructions. The pCMV6-ENTRY vector (OriGene Technologies Inc., Rockville, MD, USA) containing wild type FPR2 sequence served as the template. The forward and reverse primers to create K230A mutant were 5'-GCAGCCAAGATCCACGCAAAGGGCATGATTAA-3' and 5'-TTAATCATGCCCTTTGCGTGGATCTTGGCTGC-3', respectively. The forward and reverse primers to produce the H229A/K230A/K231A triple change were 5'-ATTGCAGCCAAGATCGCCGCAGCGGGGCATGATTAAATC-3' and 5'-GATTTAATCATGCCCGCTGCGGCGATCTTGGCTGCAAT-3', respectively. Mutant FPR2 clones were selected with kanamycin and the resulting plasmids FPR2mut3 and FPR2mutBis were purified by maxi-prep (Qiagen, Hiden, Germany). The mutations were confirmed by DNA sequencing on an ABI 3130 xl Gene Analyzer Sequencer (CEINGE Biotecnologie Avanzate s.c.a.r.l., Naples, Italy).

2.5. Immunofluorescence

Briefly, CaLu-6 cells, or HEK293 cells transfected with FPR2wt or FPR2mut3 or FPR2mutBis were washed in PBS containing Ca^{2+} and Mg^{2+}, fixed in 4% paraformaldehyde (PFA) for 20 min at RT and quenched with 50 mM NH4Cl for 10 min at RT. After blocking with 1% BSA in PBS for 10 min, cells were permeabilized in 0.2% TritonX-100 (Sigma, Saint Louis, MO, USA) 1% BSA in PBS for 10 min at RT. Cells were then washed in 1% BSA in PBS at RT and incubated with anti-FPR2 (1:100; Thermoscientific, Waltham, MA, USA) or anti-LAP2 (1:100; Santa

Cruz Biotechnology, CA, USA) antibodies, for 20 min. Cells were washed with 1% BSA in PBS and stained with goat anti-mouse Alexa Fluor 488 (1:200; Invitrogen, Carlsbad, CA, USA) or donkey anti-goat Alexa Fluor 680 (1:200; Invitrogen, Carlsbad, CA, USA) secondary antibodies, for 1 h at RT. Staining was performed by incubating cells with DAPI (1 μg/ml; Invitrogen, Carlsbad, CA, USA) in PBS for 10 min. Cells. were then washed with 1% BSA in PBS and images were captured by using a Zeiss LSM 510 meta confocal microscope equipped with an oil immersion plan Apochromat 63×objective 1.4 NA.

2.6.Receptor binding assay

Radioligand binding assays were performed as described [53].

2.7. Statistical analysis

All the presented data are expressed as means ± S.D. and are representative of three or more independent experiments. Statistical analyses were assessed by Student's t test for paired data. Results were considered significant at $p<0.05$.

3. Results

3.1. *PNT1A cells express a functional FPR2 receptor*

Cross talk between cell surface receptors, such as G-protein-coupled receptors (GPCRs) and receptortyrosine kinases (RTKs), is a crucial signaling mechanism to expand the cellular communication network. We analyze the cross-talk between FPR2 and c-Met in PNT1A cells to identify intracellular signalling cascades triggered by the WKYMVm-mediated activation of HGF receptor.Wefirst analyzed the expression of FPR2 in membrane, cytoplasmic and total proteins purified by PNT1A cells. We detected the presence of the band corresponding to FPR2 protein at the expected molecular size on membrane extracts by using an α-FPR2 antibody (Fig. 1A). By RT-PCR we detected FPR2 but not FPR1 and FPR3, providing the first evidence of FPR2 expression in these cells. (Fig.1B). In IMR90 fibroblasts and in human lung cancer cells, stimulation of FPR2 with 10 μM WKYMVm induces p47phox phosphorylation, which is considered the key event for NADPH oxidase-dependent superoxide generation [54]. In immunoblot experiments we observed that in PNT1A cells p47phox results phosphorylated on serine residues within the first 2 min of stimulation with the FPR2 agonist (Fig.1C) and that preincubation with PTX or with the FPR2 antagonist WRW4 peptide, significantly prevents p47phox serine phosphorylation (Fig. 1D). Furthermore, stimulation of FPR2 by WKYMVm induces NADPH oxidase-dependent superoxide generation with maximal production occurring at 2 min (Fig.1E), indicating that FPR2 is a biologically functional receptor in PNT1A cells.

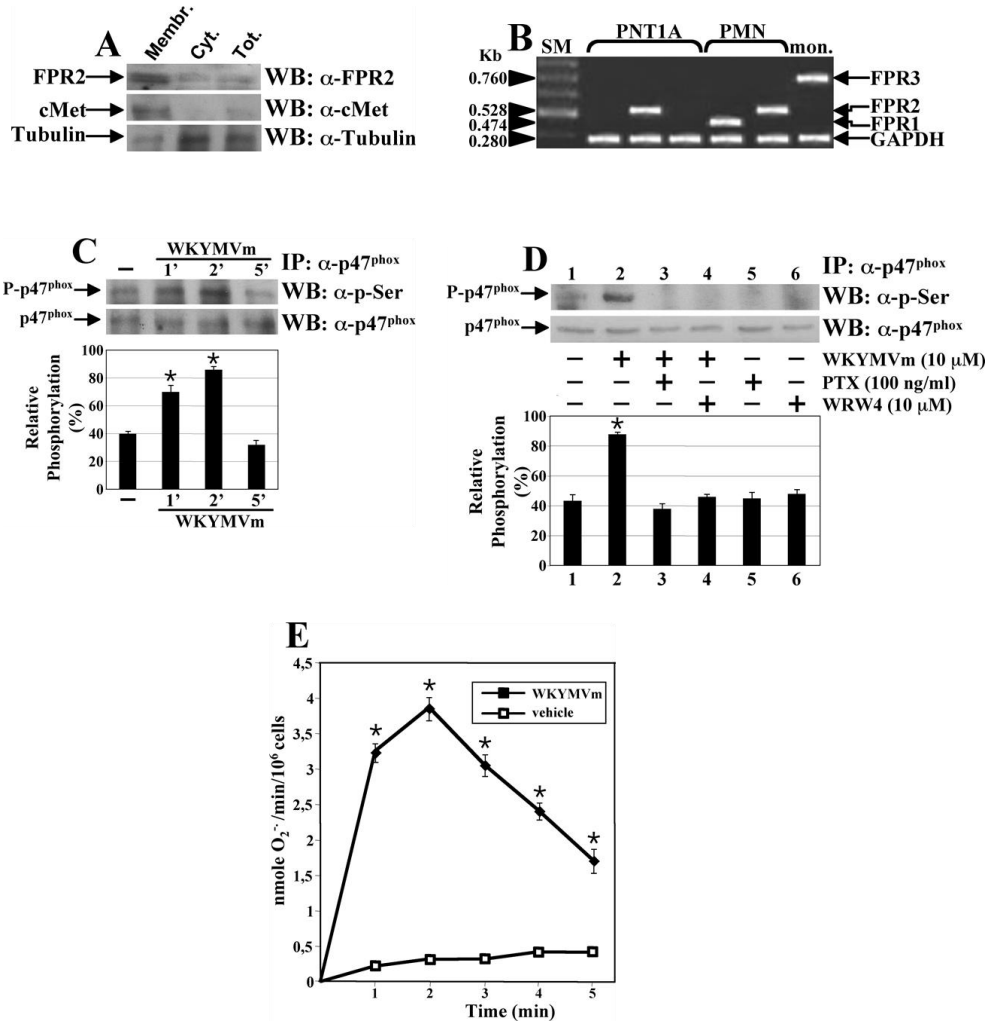
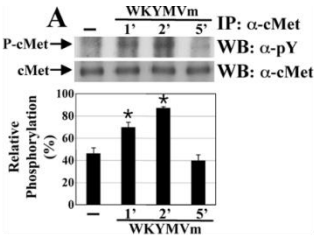
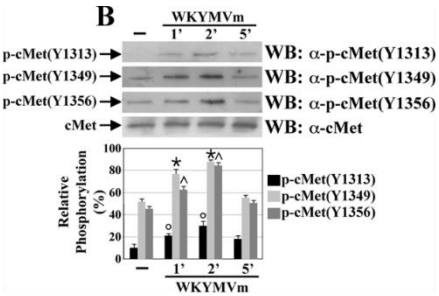


Fig.1. FPR2 is a functional receptor in PNT1A cells. (A) Membrane (Membr.), cytoplasmic (Cyt.) and whole (Tot.) lysates (50 μg) were analyzed by western blot with an α- FPR2 antibody. (B) cDNAs from PNT1A cells, PMN and monocytes (mon.) were coamplified by using FPRs and GAPDH primers; (C) Cell were exposed to WKYMVm for the indicated times, or (D) preincubated with PTX or WRW4 before stimulation. Whole lysates (1 mg) were immunoprecipitated with an a-p47phox antibody and p47phox serine phosphorylation was detected by using an α-p-Ser antibody. An α-p47phox antibody served as a control for protein loading. (E) Superoxide production was determined as the SOD-sensitive rate of reduction of cytochrome c.$P < 0.05$ compared with unstimulated cells.

3.2. FPR2 activation promotes the phosphorylation of Y1313/Y1349/ Y1356 residues of c-Met

Cross-talk between GPCRs and TKRs plays an instrumental role in orchestrating downstream signalling molecules. We analyzed the ability of FPR2 to transactivate c-Met and, in immunoblotexperiments, we observed that stimulation with WKYMVm induces the time-dependent phosphorylation of the HGF receptor (Fig. 2A). In particular, the FPR2 agonist promotes the phosphorylation of Y1313/Y1349/Y1356 residues of c-Met within the first 2 min which decreases after 5 min of stimulation, both in PNT1A (Fig. 2B) and primary prostate epithelial cells (Fig. 2C). Furthermore, preincubation of PNT1A cells with the c-Met inhibitor SU11274 or PTX or WRW4 (Fig. 2D), or with siRNAs against FPR2 or c-Met (Fig. 2E), before WKYMVm stimulation, results in a significant reduction in the phosphorylation level of these tyrosines. Oxidation and/or reduction of cysteine sulfhydryl groups of phosphotyrosine phosphatases (PTPases) tightly controls the activity of TKRs. Nox family is considered the main cytosolic source of ROS which can contribute to TKR transactivation by inhibiting a PTPase activity [50] and, in turn, shifting the equilibrium state of TKR from non-phosphorylated to phosphorylated. We preincubated cells with the NADPH-oxidase-specific inhibitor apocynin or with a siRNA against p22phox before WKYMVm stimulation, and we observed that blockade of NADPH oxidase function prevents FPR2-induced phosphorylation of Y1313/Y1349/Y1356 residues of c-Met (Fig. 2F). We also incubated cells with PMA, an oxidase inducer, and we observed that it promotes the phosphorylation of Y1349 and Y1356 residues, but not of Y1313 residue, only after 5 min and with a kinetic different from that induced by FPR2 stimulation(Fig. G). This suggests that FPR2-mediated ROS production plays a crucial role in the FPR2/HGF cross-talk mechanism.


A
WKYMVm
– 1' 2' 5'
IP: α-cMet
P-cMet
WB: α-pY
cMet
WB: α-cMet
Relative Phosphorylation (%)
100
80
60
40
20
0
*
*
– 1' 2' 5'
WKYMVm


B
WKYMVm
– 1' 2' 5'
p-cMet(Y1313)
WB: α-p-cMet(Y1313)
p-cMet(Y1349)
WB: α-p-cMet(Y1349)
p-cMet(Y1356)
WB: α-p-cMet(Y1356)
cMet
WB: α-cMet
Relative Phosphorylation (%)
100
80
60
40
20
0
*
*^
^
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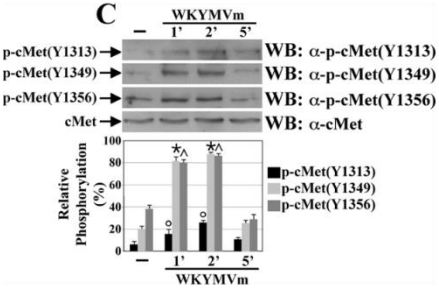
p-cMet(Y1313)
p-cMet(Y1349)
p-cMet(Y1356)
– 1' 2' 5'
WKYMVm


C
WKYMVm
– 1' 2' 5'
p-cMet(Y1313)
WB: α-p-cMet(Y1313)
p-cMet(Y1349)
WB: α-p-cMet(Y1349)
p-cMet(Y1356)
WB: α-p-cMet(Y1356)
cMet
WB: α-cMet
Relative Phosphorylation (%)
100
80
60
40
20
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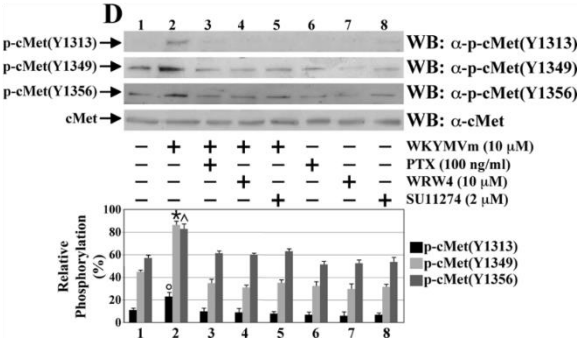
p-cMet(Y1313)
p-cMet(Y1349)
p-cMet(Y1356)
– 1' 2' 5'
WKYMVm


D
1 2 3 4 5 6 7 8
p-cMet(Y1313)
WB: α-p-cMet(Y1313)
p-cMet(Y1349)
WB: α-p-cMet(Y1349)
p-cMet(Y1356)
WB: α-p-cMet(Y1356)
cMet
WB: α-cMet
– + + + + – – – WKYMVm (10 μM)
– – + – – + – – PTX (100 ng/ml)
– – – + – – + – WRW4 (10 μM)
– – – – + – – + SU11274 (2 μM)
Relative Phosphorylation (%)
100
80
60
40
20
0
*^
°
p-cMet(Y1313)
p-cMet(Y1349)
p-cMet(Y1356)
1 2 3 4 5 6 7 8

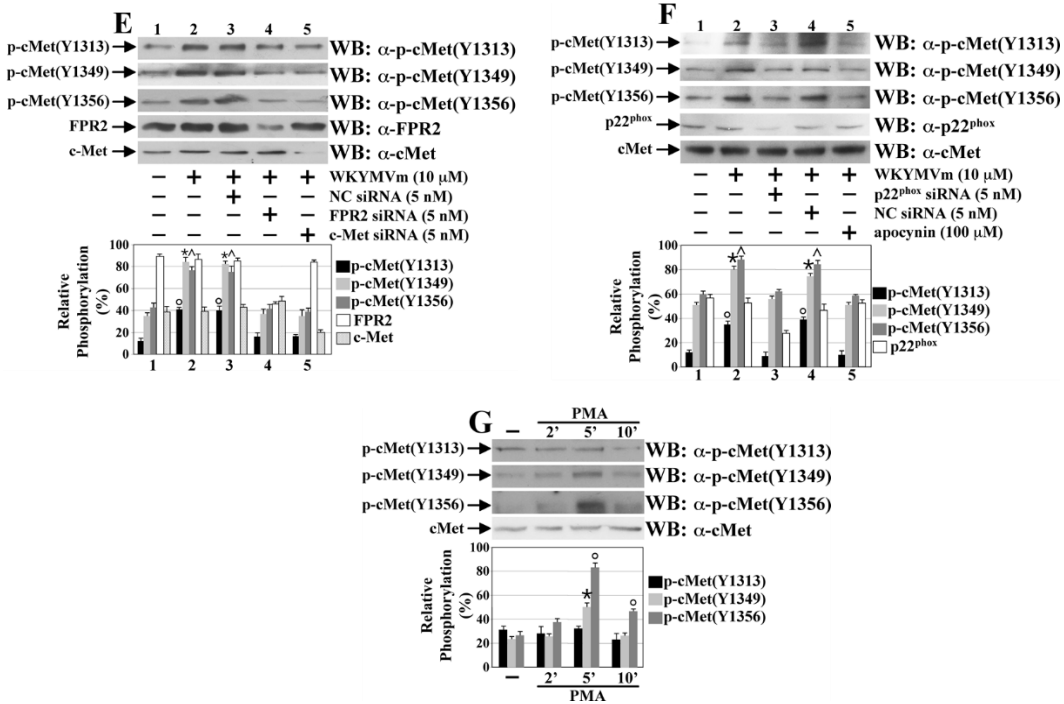


Fig.2. FPR2 activation results in c-Met trans-phosphorylation. (A) Whole lysates (800 μg) from PNT1A cells were immunoprecipitated with an α-cMet antibody and c-Met tyrosine phosphorylation level was detected with an α-pY antibody. PNT1A (B) or primary prostate epithelial cells (C) were stimulated with WKYMVm for the indicated times. (D) PNT1A cells were preincubated with PTX or WRW4 or SU11274, or (E) with siRNAs against FPR2 (FPR2 siRNA) or siRNA against c-Met (c-Met siRNA), or (F) withsiRNA against p22phox (p22phox siRNA) or with apocynin, before stimulation. A negative control siRNA (NC siRNA) was included in the experiments. (G) PNT1A cells were stimulated with PMA for the indicated times. Whole lysates (50 μg) were subjected to immunoblotting analysis and phosphorylation of Y1313/Y1349/Y1356 residues of c-Met was detected with α-p-cMet(1313), α-p-cMet(1349) or α-p-cMet(1356) antibodies. $P < 0.05$ compared with unstimulated cells.

3.3. FPR2/c-Met cross-talk triggers STAT3 pathway

The single multifunctional docking site located in the C-terminus of the HGF receptor contains the sequence Y1349VHVNATY1356- VNV, which provides binding sites for a variety of SH2-containingsignal transducers and effectors. STAT3 binds to the sequence following the phosphorylated Y1356 residue [54]and its association with c-Met results in the phosphorylation of a conserved tyrosine residue (Y705), which is required for promoting the dimerization of STAT3. Activated STAT3 is then translocated in

the nucleus where it acts as a transcriptional factor. Full transcriptional activityand DNA binding capacity are manifested only when the serine 727 residue of STAT3 is also phopshorylated. In time-dependent western blot experiments, we observed that WKYMVm induces the rapid phosphorylation of Y705 and S727 residues of STAT3 (Fig. 3A), as well as the nuclear translocation of activated STAT3 (Fig. 3B). We also preincubated cells with PTX or WRW4 or SU11274 (Fig. 3C), or with siRNAs against FPR2 or c-Met (Fig. 3D), and we observed that these treatments prevent the WKYMVm-induced activation of STAT3. Furthermore, we blockaded NADPH oxidase function by pretreating cells with apocynin or with a siRNA against p22phox and we observed that FPR2-induced phosphorylation of Y705 and S727 residues of STAT3 was prevented (Fig. 3E).

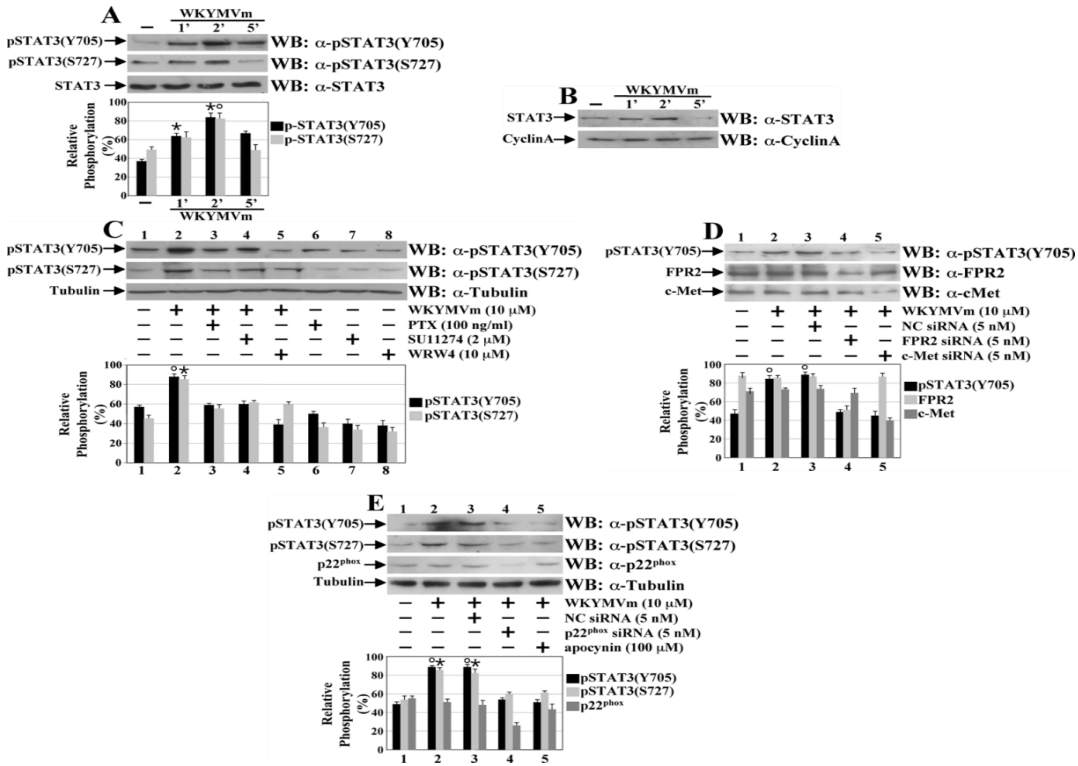


Fig.3. FPR2 activation triggers STAT3 pathway. (A) PNT1A cells were stimulated for the indicated times with WKYMVm and specific phosphorylation of STAT3 was detected with α-pSTAT3(Y705) or α-pSTAT3(S727) antibodies. An α-STAT3 antibody served as a control for protein loading. (B) STAT3 nuclear translocation was analyzed on nuclear extracts (50 μg) with an α-STAT3 antibody. The same filter was reprobed with an a-cyclin A antibody. (C) Cells were preincubated with PTX or SU11274 or WRW4, or (D) with FPR2 siRNA or c-Met siRNA, or (E) with a p22phox siRNA or apocynin, before WKYMVm stimulation. A NC siRNA was included in the experiments. Proteins (50 μg) were subjected to immunoblotting analysis with α-p-STAT3(Y705) or α-p-STAT3(S727) antibodies.$P < 0.05$ compared with unstimulated cells.

3.4. FPR2-induced c-Met trans-phosphorylation generates specific docking sites for PLC-cγ

Signal transducers that bind sequences surrounding tyrosines 1349 and 1356 residues in c-Met can interact with the receptor either directly or indirectly through the scaffolding protein Gab1, which represents the key coordinator of the cellular responses toc-Met [55]. After the interaction with the receptor, Gab1 becomes phosphorylated on several tyrosine residues that, in turn, recruit a number of

signalling effectors, including PLC-γ1 which can weakly bind c-Met also directly [55]. We analyzed PLC-γ1 activation in WKYMVm-stimulated PNT1A cells and we observed in time-course experiments that the FPR2 agonist induces PLC-γ1 activation with maximal phosphorylation of Y783 residue occuring at 2 min (Fig.4A). PLC-γ1(Y783) phosphorylation is prevented by preincubation with PTX or WRW4 or SU11274, before WKYMVm stimulation (Fig.4B). We also analyzed the PKC isoforms activated as a consequence of the hydrolysis of phosphatidylinositol-4-5-bisphosphate by PLC-γ1, by analyzing the cellular partitioning of PKC isozymes in growth-arrested and WKYMVm-stimulated cells. In response to the FPR2 agonist, of the seven PKC isoenzymes that we examined (data not shown) only PKCα translocates to the membrane fraction and a significant increase in the amount wasdetected within 2 min of exposure to WKYMVm (Fig.4C). In contrast, no translocation of PKCδ and PKCε was observed (Fig.4C). Furthermore, the Gi protein specific inhibition, or a specific FPR2 antagonist, or an inhibitor of c-Met kinase activity, completely prevents PKCα activation (Fig.4D), suggesting that it depends on FPR2-dependent c-Met phosphorylation.

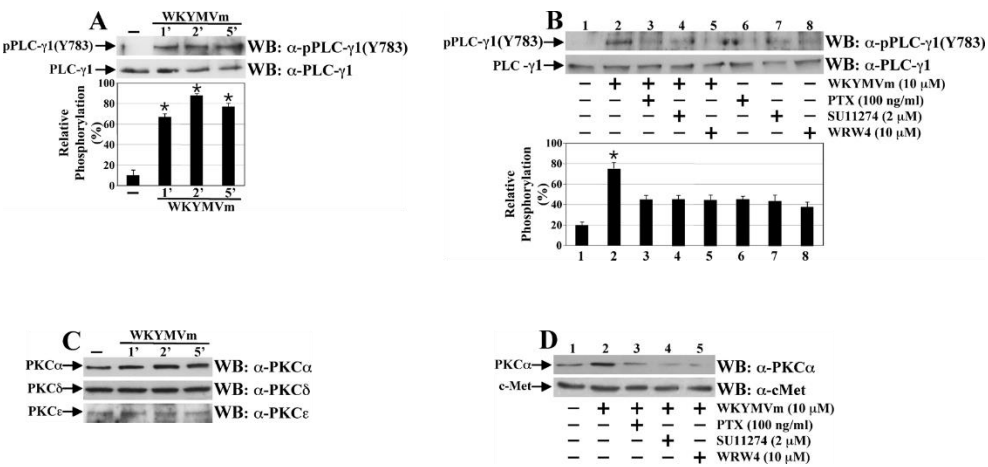


Fig.4. FPR2-induced c-Met transactivation generates specific docking sites for PLC-γ. (A) Proteins were purified from PNT1A cells exposed to WKYMVm for the indicated times, or (B) preincubated with PTX or SU11274 or WRW4, before Nstimulation. Fifty micrograms of lysates were analyzed with an α-

pPLC-γ(Y783) antibody. (C) Translocation of PKC isoforms was analyzed on membrane extracts (30 µg) with α-PKCα or α-PKCδ or α-PKCε antibodies. (D) Cells were preincubated with PTX, or SU11274 or WRW4 before stimulation and 30 µg of purified membrane proteins were analyzed with anα-PKCα antibody. The same filter was reprobed with an a-cMet antibody.

3.5. FPR2-induced activation of the multifunctional docking site of c- Met triggers PI3K/Akt pathway

Nine domains of Gab1 containing a single tyrosine are phosphorylated in vitro by HGF and three of these tyrosines (Y458/ Y473/Y590) bind the p85 regulatory subunit of PI3K. In HGF receptor there is also an other recognition motif (Y1313EVM) which can represent a potential binding site for PI3K. In immunoblotting experiments we observed that WKYMVm induces PI3K(p85) phosphorylation within 2 min of stimulation (Fig.5A). This is prevented by pretreating cells with PTX, or SU11274, or WRW4, or apocynin (Fig.5B). PI3K is the key component for the activation of Akt signalling. In the PI3K/Akt pathway, formation of 3-phosphoinositides by PI3K enables the activation of Akt by phosphoinositide-dependent protein kinases 1 and 2, which phosphorylate Akt at threonine 308 and serine 473 residues, respectively. We analyzed PI3K(p85) activity by analyzing Akt phosphorylation in response to FPR2 stimulation and the results showed that WKYMVm induces Akt(S473) phosphorylation within the same time frame as PI3K(p85) phosphorylation (Fig.5C). The preincubation of cells with highly selective PI3K inhibitors or PTX or WRW4 or SU11274 (Fig.5D), or with siRNAs against FPR2 or c-Met (Fig.5E), or with a siRNA against p22phox (Fig.5F), significantly prevents WKYMVm-induced Akt (S473) phosphorylation.

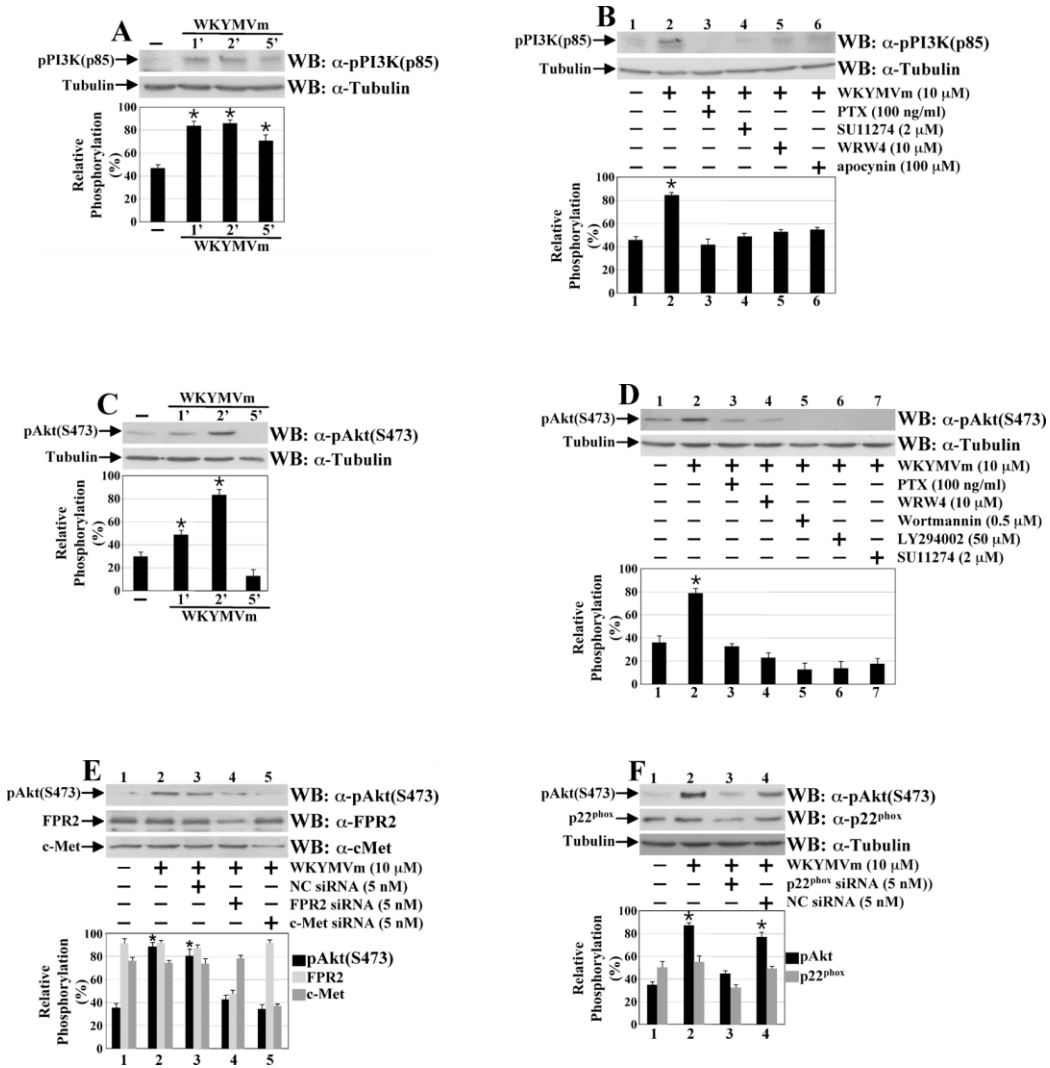


Fig.5. FPR2/c-Met cross-talk triggers the activation of PI3K/Akt pathway. (A and C) Cells were exposed with WKYMVm for the indicated times or (B) preincubated with PTX or SU11274 or WRW4 or apocynin, or (D) with PTX or WRW4 or Wortmannin or LY294002 or SU11274, or (E) with FPR2 siRNA or c-Met siRNA, or (F) with a p22phox siRNA, before stimulation. A NC siRNA was included in the experiments. Proteins (50 μg) were analyzed with (A and B) an α-pPI3K(p85) antibody, or (C, D, E and F) with an α-pAkt(S473) antibody. Anα-Tubulin antibody was used as a control of protein loading. $P < 0.05$ compared with unstimulated cells.

3.6.FPR1 is a biologically functional receptor in ECV cells

The Vascular endothelial growth factor (VEGFR2) is a cell surface receptor for vascular endothelial growth factor.We analyzed the expression of FPRs and NOX enzymes in ECV cells. By RT-PCR we detected FPR1 but not FPR2 and FPR3, and NOX2 and NOX4 isoforms of NADPH oxidase, providing the evidence of FPR1 and NADPH expression in endothelial cells (Fig.6).

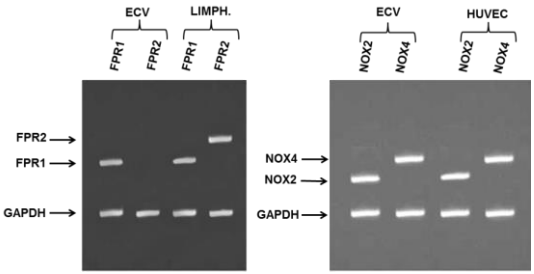


Fig.6. ECV cells express FPR1 on the plasma membrane. cDNA from ECV, limphatic and HUVEC cells were coamplified by using FPRs, NOXs and GAPDH primers.

We first analyzed the ability of FPR1 to activate NOXs. Phosphorylation and membrane translocation of the cytosolic oxidase subunits are considered key events in the assembly of phagocytic and nonphagocytic NADPH oxidase.Western blot analysis showed that NADPH oxidase regulatory subunit p47phox is rapidly phosphorylated on serine residues upon exposure to N-fMLP (Fig.7A) and that this is prevented by preincubation with PTX (Fig.7B).

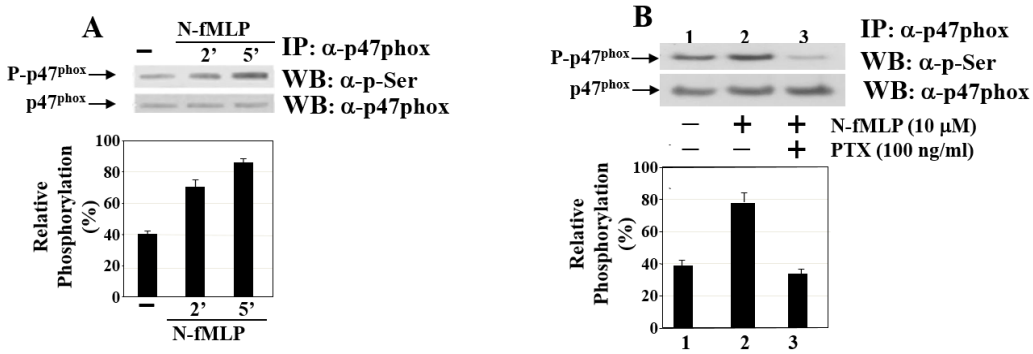


Fig.7. FPR1is a fuctional receptor in ECV cells. (A) Cell were exposed to N-fMLP for the indicated times, or (B) preincubated with PTX before stimulation. Whole lysate (1mg) were immunoprecipitated with an α-p47phox antobody and p47phox serine phosphorilation was detected by using an α-p-Ser antibody. An α-p47phox antibody served as a control for protein loading.P < 0.05 compared with unstimulated cells.

3.7. NADPH-oxidase-dependent superoxide generation is required for FPR1-induced VEGFR2 transactivation

Cross-communication between different signaling systems plays a key role to coordinate the plethora of extracellular stimuli to which a cell is subjected under several physiological or pathological conditions. Cell-surface receptors are the key components of these networks and the inter-receptor crosstalk acts as a general signaling mechanism connecting and diversifying signal transduction pathways. We analyzed the ability of FPR1 to transactivate VEGFR2. We observed that stimulation with the FPR1 agonist promotes the phosphorylation of Y996 residues of VEGFR2 in in time-dependent manner (Fig.8A). Furthermore, preincubation of ECV cells with PTX, before N-fMLP stimulation, results in a significant reduction in the phosphorylation level of this tyrosine (Fig.8B). NADPH oxidase is considered the main cytosolic source of ROS, which are able to inactivate phosphotyrosine-phosphatases (PTPs) by oxidation of a cysteine in the catalytic domain, unbalancing intracellular phosphorylation equilibrium. The enhanced activity of phosphotyrosine-kinases (PTKs) can mediate the trans-phosphorylation of tyrosines in the cytosolic region of RTKs. We preincubated cells with the NADPH-oxidase-specific inhibitor

apocynin or with a siRNA against p22phox before N-fMLP stimulation, and we observed that blockade of NADPH oxidase function prevents FPR1-induced phosphorylation of Y996 residues of VEGFR2 (Fig.8C).

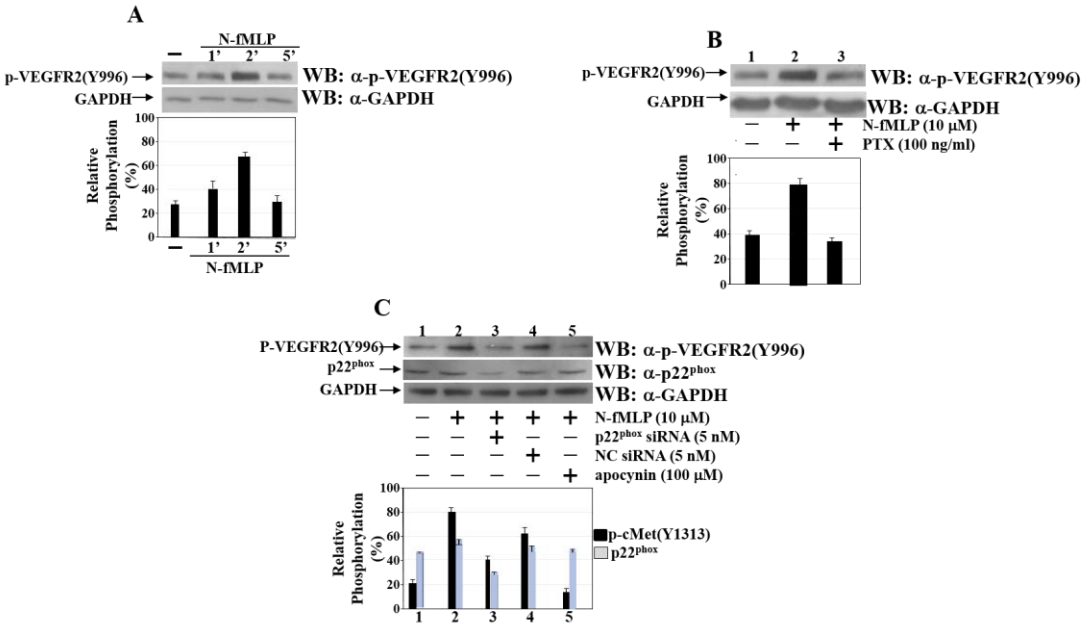


Fig.8. FPR1 activation results in VEGFR2 trans-phosphorilation. (A) ECV cells were stimulated for indicated times with N-fMLP and VEGFR2 tyrosine phosphorilation level was detected with an α-p-VEGFR2(Y996) antobody. (B) ECV were preincupated with PTX, or (C) with a siRNA against p22phox (p22phox siRNA) or with apocynin, before stimulation. A negative control siRNA (NC siRNA) was included in the expreriments. Proteins (50 μg) were subjected to immunoblotting analysis with an α p-VEGFR2(Y996) antibody. α-GAPDH antibody was used as a control of protein loading. $P < 0.05$ compared with unstimulated cells.

These results suggests that ROS production plays a crucial role in FPR1/VEGFR2 cross-talk mechanism.

3.8. FPR1/VEGFR2 cross-talk triggers STAT3 pathway

The trans-phosphorylation of tyrosines in the cytosolic region of VEGFR2 provide docking sites for assembly and activation of signaling complexes. Since VEGFR mediates angiogenesis via STAT3 pathway,we analized STAT3 activation by phosphorylation of

its conserved tyrosine residue (Y705) which is required for promoting the STAT3 dimerization. Activated STAT3 is then translocated in the nucleus where it acts as a transcriptional factor. Full transcriptional activity and DNA binding capacity are manifested only when the serine 727 residue of STAT3 is also phosphorylated. We observed that N-fMLP induces the phosphorylation of Y705 and Ser727 of STAT3 in time-dependent manner (Fig.9A). We also preincubated cells with PTX (Fig.9B) and we observed that this treatment prevents the N-fMLP induced activation of STAT3. Furthermore, we blockaded NADPH oxidase function by pretreating cells with apocynin or with a siRNA against p22phox and we observed that FPR1-induced phosphorylation of Y705 residue of STAT3 was prevented (Fig.9C,D). We observed also the nuclear translocation of activated STAT3 (Fig.9E).

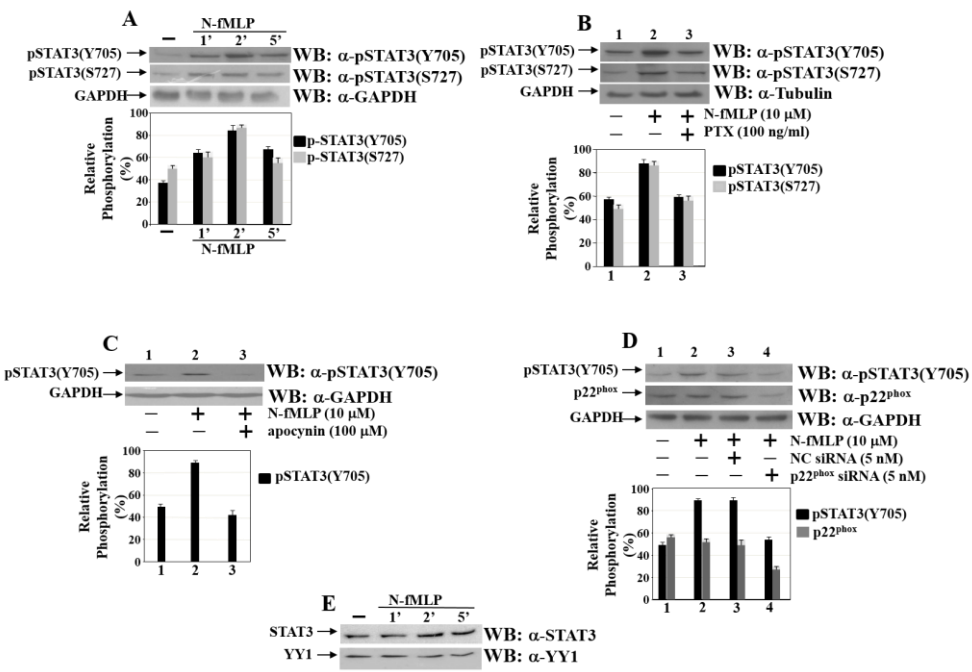


Fig.9. FPR1 activation triggers STAT3 pathway. (A) ECV cells were stimulated for the indicated times with N-fMLP and specific phosphorylation of STAT3 was detected with α-pSTAT3(Y705) or α-pSTAT3(S727) antibodies (B) Cells were preincubated with PTX (C,D) or apocynin or with a p22phox siRNA, before N-fMLP stimulation (C,D). A NC siRNA was included in the experiments. Proteins (50 μg) were subjected to immunoblotting analysis with α-p-STAT3(Y705) or α-p-STAT3(S727) antibodies. STAT3 nuclear translocation was analyzed on nuclear extracts (50 μg) with an a-STAT3 antibodyThe same filter was reprobed with an α-YY1 antibody. $P < 0.05$ and $P < 0.05$ compared with unstimulated cells.

3.9. FPR1-induced VEGFR trans-phosphorylation generates specific docking sites for PLC-Cγ

PLC is rapidly tyrosine-phosphorylated and associated with VEGFR2 both in endothelial cells and NIH3T3 cells [56]. We analyzed PLC-γ activation in N-fMLP stimulated ECV cells and we observed in time-course experiments that the FPR1 agonist induces PLC-γ activation with maximal phosphorylation occuring at 1 min. (Fig.10A). We also analyzed the PKC isoforms activated as a consequence of the hydrolysis of phosphatidylinositol-4-5-bisphosphate by PLC-γ. In response to the FPR1 agonist, PKC α and δ translocates to the membrane fraction and a significant increase in the amount was detected within 2' min of exposure to N-fMLP (Fig.10B).

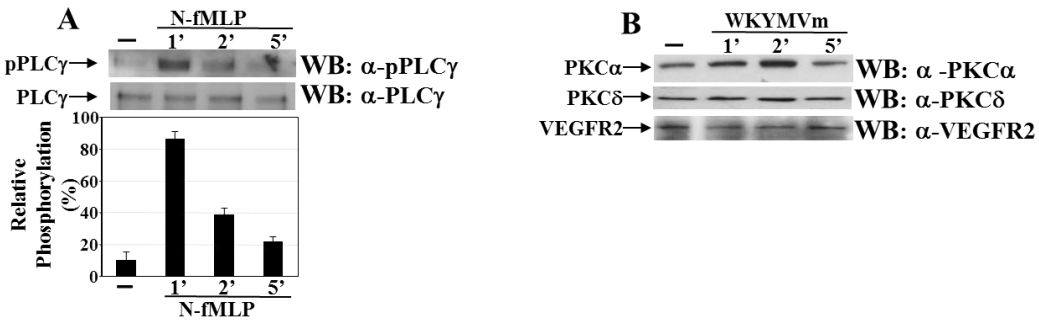


Fig.10.VEGFR2 transactivation generates specific docking site for PLCγ. (A) Proteins were purified from ECV cells exposed to N-fMLP for the indicated times. 80 μg of lysate were analyzed with anα-p-PLCγ antibody. (B) traslocation of PKC isoforms was analyzed on membrane extracts (30μg) with α-PKCα or α-PKC δ antibody. The same filter was reprobed with anα-VEGFR2 antibody.

3.10.FPR1-mediatedPI3K/AKT pathway requires VEGFR2 activation

We analyzed PI3K(p85) activity by analyzing Akt phosphorylation in response to FPR1 stimulation and the results showed that N-fMLP induces Akt(S473) phosphorylation (Fig.11A). The preincubation of ECVcells with PTX(Fig.11B) or apocynin or with a siRNA against p22phox (Fig.11C,D), significantly prevents N-fMLP-induced Akt(S473) phosphorylation. The preincubation with highly selective PI3K inhibitors also prevents Akt phosphorylation (Fig.11E).

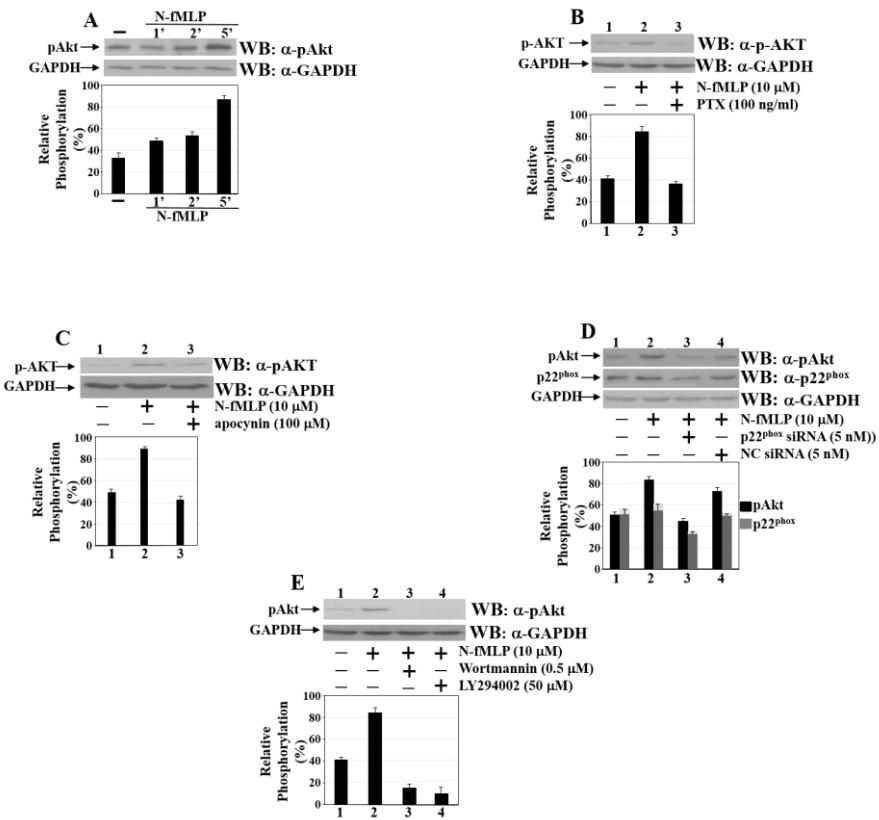


Fig.11. FPR1/VEGFR2 cross-talk triggers the activation of PI3K/Akt pathway. (A) ECV cells were exposed with N-fMLP for the indicated times or (B) preincubated with PTX (C) or apocynin, or (D) with a p22phox siRNA, or Wortmannin or LY294002 (E) before stimulation.. Proteins (50 μg) were analyzed

with an α p-AKT(S473) antibody.α-GAPDH antibody was used as a control of protein loading. $P < 0.05$ compared with unstimulated cells.

3.11. *FPR2 is expressed in nuclear fraction of Calu-6 and AGS cells*

The expression of several functional GPCRs is not exclusively restricted to the plasma membrane but is also extended to different compartments, including the nuclear membrane. Many of the observed nuclear effects elicited by these receptors are not prevented by classical inhibitors, that exclusively target cell surface GPCRs, presumably because of their structures, lipophilic properties, or affinity for nuclear receptors. Furthermore, these intracellular receptors may regulate signaling cascades that differ from those of their cellular membrane counterparts [29].We isolated membrane, cytosolic and nuclear fractions from CaLu-6 and AGS human cells and we examined FPRs subcellular distribution by western blot. The results show that an anti-FPR2 antibody, but not anti-FPR1 or anti-FPR3 antobodies (data not shown), detects the receptor in membrane and nuclearfractions of Calu6 (Fig.12A) and AGS (shCRT) cells (Fig.12B). In gastric cells (AGS) stably trasfected with a short hairpin (shRNA) that targets FPR2 (shFPR2) nuclear expression is prevented compared with AGS cells stably transfected with a shRNA control (AGSshCRT) (Fig.12C). The purified cellular components were validated by using anti-LAP2, anti-CD44 and anti-RAB7 and tubulin antibodies, for nuclear membrane, lysosome and cytosolic fractions, respectively. Confocal microscopy in Calu6 cells revealed a fluorescent signal for FPR2 (green) distributed intracellularly (Fig. 12D, Panel A) and, as expected, an intense fluorescent signal for LAP2 (Fig. 12D, Panel B) on the nuclear membrane (red). By using Imaris image analysis software, we demonstrated that FPR2 colocalizes with the nuclear LAP2 (yellow/orange) (Fig.12D, Panel C).

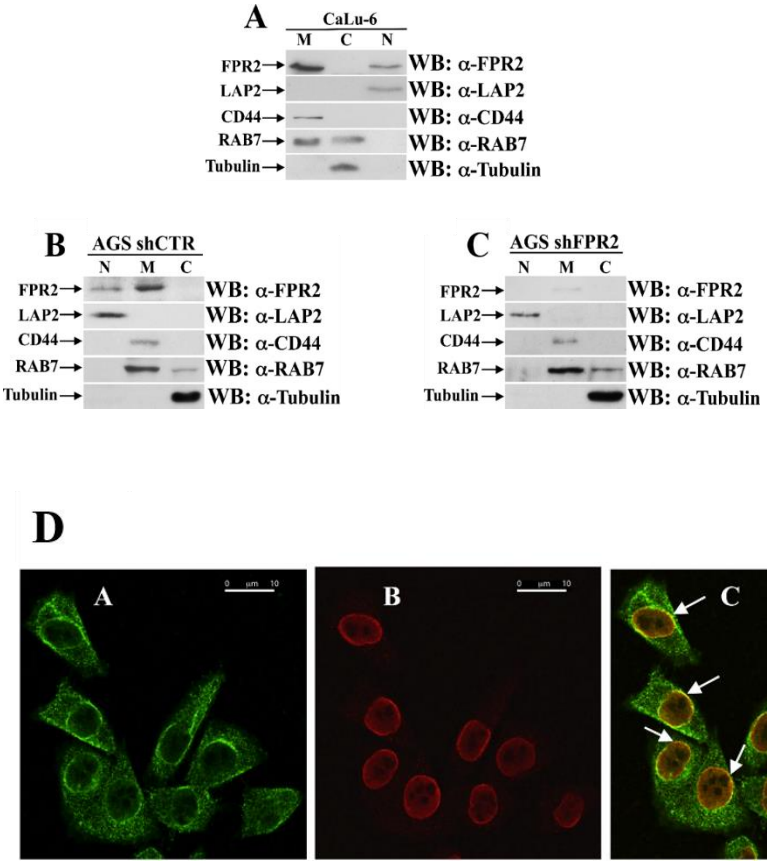


Fig.12. Nuclear FPR2 expression in CaLu-6 cells. (A-B-C) Membrane (M), cytosolic (C) and nuclear (N) fractions were purified from CaLu-6 and AGS cells. Protein extracts (30 μg) were resolved on 10% SDS-PAGE and immunoblots were probed with α-FPR2. α-EGFR, α-LAP2, α-CD44, α-RAB7 or α-Tubulin were used as markers for fractionation purity and as loading control. (D) Confocal immunofluorescence microscopy of CaLu-6 cells. Localization of FPR2 was detected by incubating cells with an α-FPR2 antibody. Cells were then washed in 1% BSA in PBS and incubated with goat anti-mouse Alexa Fluor 488-conjugated secondary antibody (Panel A; green). Nuclear protein LAP2 was detected by incubating cells with α-LAP2 antibody for 20 min and with secondary donkey anti-goat Alexa Fluor 680 antibody for 1 h (Panel B; red). Staining was performed by incubating cells with DAPI. Merged signals (Panel C; yellow/orange) show co-localization of FPR2 and LAP2 (arrows). Cells showed no staining with secondary antibody alone. Data are representative of three independent experiments.

Following stimulation with the appropriate agonist, several GPCRs have been detected in the perinuclear or nuclear regions, this

localization can be attributed to translocation of a GPCR from the cell surface or de novo synthesis of the receptor [30].

3.12. FPR2 amino acid sequence contains a NLS in the third intracellular loop

Nuclear localization of proteins requires a NLS characterized by a short stretch of basic amino acid residues. This is recognized by members of the importin family, which act as carriers to transport the substrate protein across the nuclear pore structure [50]. Nuclear GPCRs might be derived from the cell membrane and their transfer to the nucleus can be attributed to a nuclear localization motif. We analyzed FPR2 sequence for the search of NLS by using cNLS mapper program and the results identified a stretch of basic aminoacids in the third cytoplasmic loop of the receptor (Fig.13).

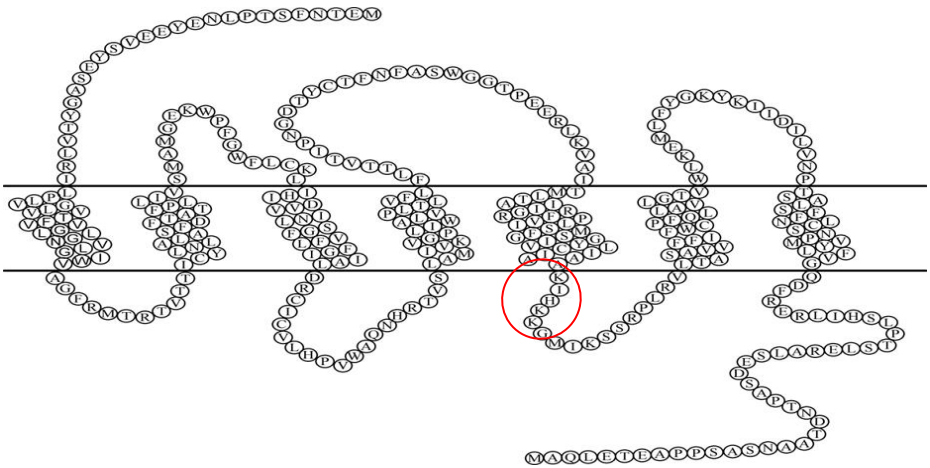


Fig.13. Schematic representation of the human FPR2. The putative NLS in the third intracellular loop, containing basic aminoacids,.

Apelin Receptor and Urotensin II receptor, which are members of the GPCR family, also show the presence of a NLS in the third cytoplasmic loop. However, a NLS could not be required for nuclear localization. In fact, endothelin receptor subtypes A and B, which have a perinuclear distribution, localize to the nuclear membrane via de novo synthesis and retrograde transport [40].

3.13. [I^{125}]WKYMVm specifically binds FPR2 in isolated nuclei

To determine whether nuclear FPR2 immunoreactivity was representive of the presence of a functional ligand-binding receptor, radioligand binding assays were performed on nuclei purified from CaLu-6 cells. The experiments, performed using [^{125}I]WKYMVm, revealed a binding that was specifically displaced by increasing concentrations of unlabeled ligand in a concentration-dependent manner (Fig14A). The saturability of the binding sites for [^{125}I]WKYMVm was tested by adding increasing concentrations of the radioligand, and receptor binding analysis performed with the Origin program predicted a Kd of 245 pM (Fig.14B).

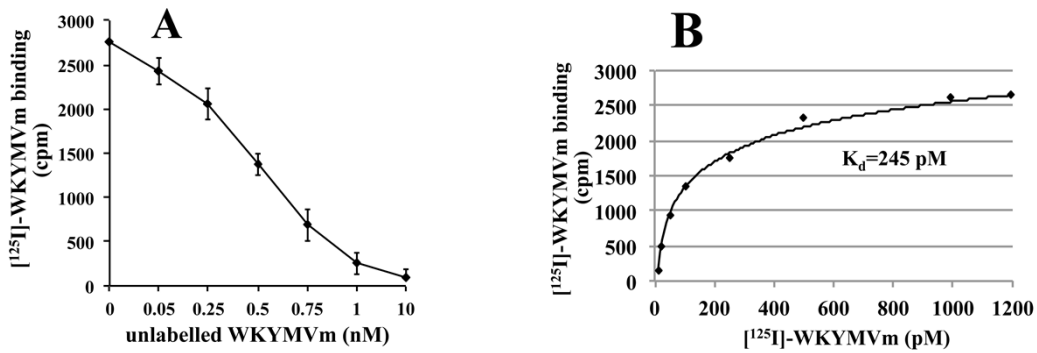


Fig.14 Specific binding of [^{125}I]WKYMVm to nuclear FPR2. (A) Purified nuclei from CaLu-6 cells were incubated for 90 min at 30 °C in binding buffer containing 0.5 nM [^{125}I]WKYMVm and increasing concentrations of unlabeled peptide, as indicated. (B) Nuclei were incubated with increasing concentrations of the radioligand in binding buffer supplemented (non-specific binding) or not (specific binding) with a 1000-fold molar excess of unlabeled WKYMVm, as indicated, for 90 min at 30 °C. Specific binding was determined by the difference between total and non-specific binding. Radioactivity was quantified using a β-counter. The experiments were performed in triplicate.

3.14. Nuclear-associated FPR2 is a functional receptor

The physiological functions of nuclear GPCRs are poorly understood. They can regulate signaling pathways that differ from those of their counterparts at the cell surface and the consequent biological responses could result from the integration of extracellular and intracellular signaling events [29].Therefore, to evaluate whether

nuclear-associated FPR2 was active, we co-immunoprecipated FPR2 and Gαi from purified nuclei stimulated or not stimulated with WKYMVm and we determined Gαi expression levels by western blot analysis in Calu6 and AGS cells. In untreated cells we observed a basal level in Gαi expression, which decreased upon stimulation with WKYMVm (Fig. 15A,B) in both cell lines. In AGS(shFPR2) cells where FPR2 expression is silenced with a specific shRNA we didn't observe Gαi nuclear expression compared to control (Fig15C). These results suggest that nuclear-associated FPR2 is a functional receptor and can respond to a specific agonist.

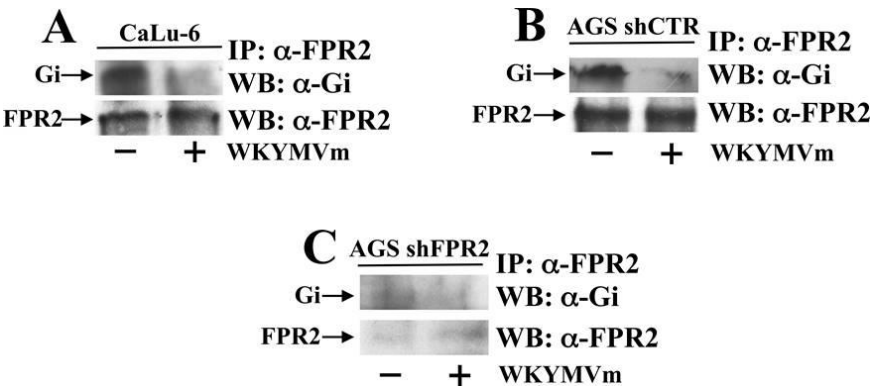


Fig.15.Nuclear FPR2 is a functional receptor. (A-B-C) Nuclei were isolated from CaLu-6 and AGS cells stimulated with 10 μM WKYMVm for 5 min. Nuclear lysates (400 μg) were immunoprecipitated with α-FPR2 and electrophoresed by SDS-PAGE. Immunocomplexes were probed with α-Gi or α-FPR2 antibodies.

Effectors associated with post G protein GPCR signaling, including mitogen-activated protein kinase (MAPK) pathway, is also present in the nucleus. Therefore, we tested the functionality of nuclear-associated FPR2 by assessing the ability of the receptor to activate ERKs. p42MAPK and p44MAPK are nuclear and cytoplasmic protein kinases and in both compartments MAPK signal transduction system is regulated by serine/threonine phosphorylation [57]. Isolated intact CaLu-6 and AGS (shCRT) nuclei were stimulated with WKYMVm for different times and nuclear lysated were incubated with an α-pERK antibody. The results showed a time-dependent

phosphorylation of nuclear p42MAPK, but not of p44MAPK (Fig.16 A,B), which was prevented by preincubation with the antagonist peptide WRWWWW (Fig. 16 C,D) in both cell lines.

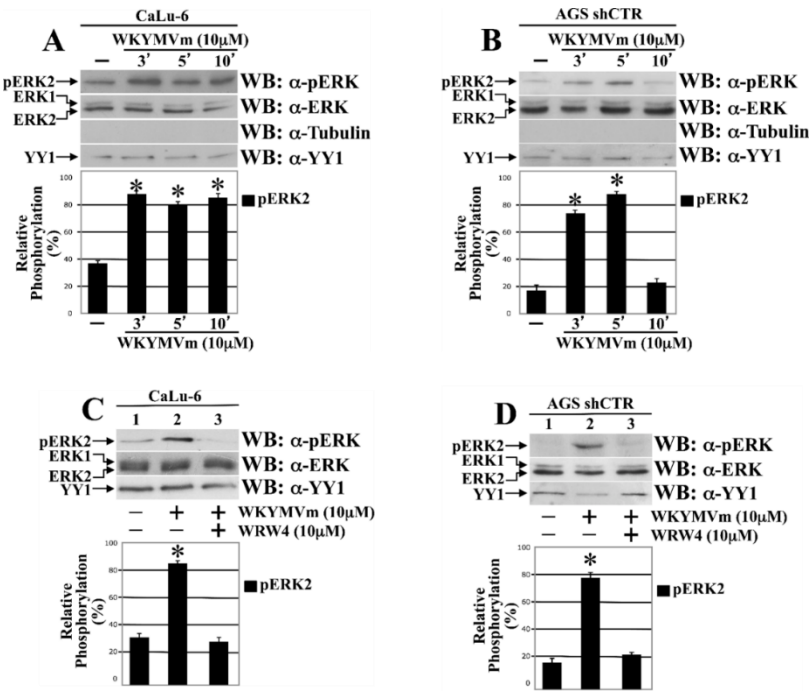


Fig.16.Nuclear FPR2 is a functional receptor. (A and B) Nuclei were purified from serum-deprived CaLu-6 and AGS(CRT) cells and stimulated with 10μM WKYMVm for the indicated times or (C and D) preincubated with the indicated concentration of WRWWWW (WRW4) before stimulation. Nuclear lysates (30 μg) were resolved on 10% SDS–PAGE. ERK phosphorylation was detected by western blot with an α-phospho-ERK antibody (α-pERK). The arrow indicates the phosphorylated form of $p42^{MAPK}$ (pERK2). An α-ERK antibody served as a control for protein loading. The arrows indicate the non-phosphorylated forms of ERK1 and ERK2. All the blots are representative of three separate experiments of identical design. Protein phosphorylation levels were quantitatively estimated by densitometry using a Discover Pharmacia scanner equipped with a Sun Spark Classic densitometric workstation. *p<0.05 and °p<0.05 compared with unstimulated nuclei.

Activated MAPK phosphorylate and activate several transcription factors, such as c-Jun and c-Myc, resulting in the modulation of genes associated with proliferation. Furthermore, the activation of FPR2 with WKYMVm effectively phosphorylates c-Jun N-terminal kinase (JNK) [58]. In order to activate gene expression, transcription factors

have to localize to the nucleus. Nuclear abundance of c-Jun correlates with target gene activity and excessive c-Jun activation can drive cells towards apoptosis or changes in differentiation, whereas decreased c-Jun function can reduce proliferation. c-Myc is located predominantly in the nucleus where it is found in a dispersed granular nucleoplasmic pattern and can be directly phosphorylated by ERKs. We analysed c-Jun and c-Myc activation in purified nuclei of Calu-6 and AGS(shCRT) cells stimulated with WKYMVm and we observed a time-dependent phosphorylation of the two transcriptional factors (Fig.17A,B), which was prevented by the preincubation with the FPR2 antagonist (Fig.17C,D).

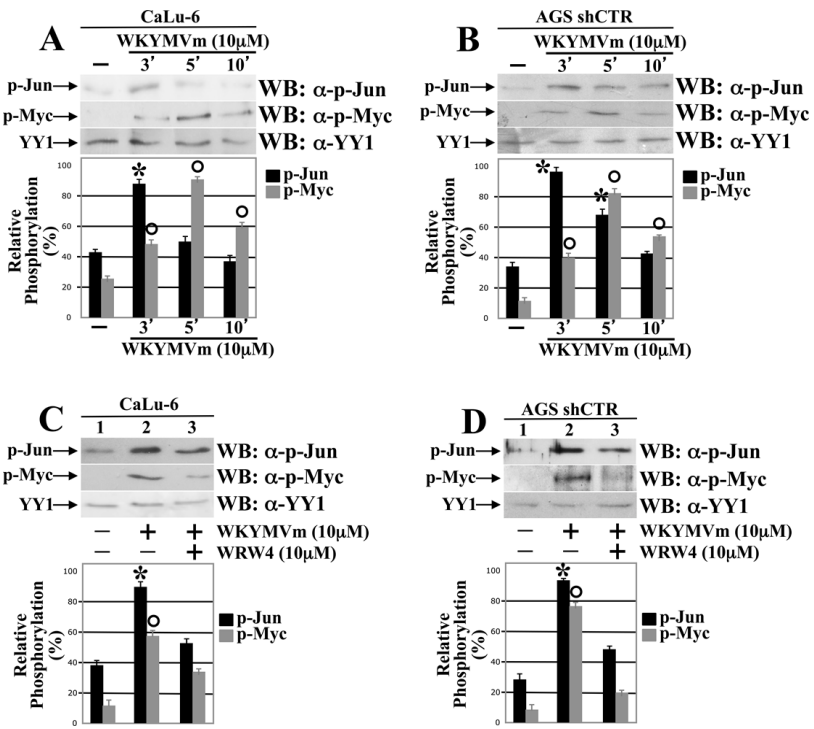


Fig.17.Nuclear FPR2 is a functional receptor. (A and B) Nuclei were purified from serum-deprived CaLu-6 and AGS cells and stimulated with 10μM WKYMVm for the indicated times or (C and D) preincubated with the indicated concentration of WRWWWW (WRW4) before stimulation. Nuclear lysates (30 μg) were resolved on 10% SDS–PAGE. c-Jun and c-Myc phosphorylation was detected with α-phospho-Jun (α-p-Jun) and α-phospho-Myc (α-p-Myc) antibodies, respectively. α-Tubulin and α-YY1 were used as markers for nuclear purity. All the blots are representative of three separate experiments of

identical design. Protein phosphorylation levels were quantitatively estimated by densitometry using a Discover Pharmacia scanner equipped with a Sun Spark Classic densitometric workstation. *p<0.05 and °p<0.05 compared with unstimulated nuclei.

These results indicate that nuclear (or perinuclear) FPR2 might play a more direct role in regulating gene expression and our in progress studies are aimed to identify which genes are regulated by nuclear FPR2.

3.15. NLS mutagenesis analysis.

To test whether the putative NLS located in the third intracellular loop of FPR2 was required for the nuclear localization of the receptor, we performed single and multiple mutagenesis in a Myc-tagFPR2 construct, substituting the basic residue K230 into Alanine (FPR2mut3) and H229, K230 and K231 into non-polar aminoacids Alanine, whithin the 227-KIHKK-231 sequence (FPR2mutBis) (Fig.18A). FPR2wt and the two mutated constructs were individually overexpressed in HEK293 cells and analysed by immunofluorescence. The results show that fluorescence was partially localized in the nucleus of cells transiently transfected with Myc-tagFPR2wt or Myc-tagFPR2mut3 (K230A point mutation) (Fig. 18B, Panels B and C). In contrast, nuclear localization of FPR2 was abolished in cells transfected with Myc-tagFPR2mutBis (H229A/K230A/K231A triple mutant) (Fig. 18B, Panel D).

A

FPR2 w.t.	5'-GCA GCC AAG ATC CAC AAA AAG GGC ATG ATT AA-3' **A A K I H K K G M I K** 3'-CGT CGG TTC TAG GTG TTT TTC CCG TAC TAA TT-5'
FPR2 mut3	5'-GCA GCC AAG ATC CAC GCA AAG GGC ATG ATT AA-3' **A A K I H A K G M I K** 3'-CGT CGG TTC TAG GTG CGT TTC CCG TAC TAA TT-5'
FPR2 mutBis	5'-GCA GCC AAG ATC GCC GCA AAG GGC ATG ATT AAA TC-5' **A A K I A A A G M I K S** 3'-CGT CGG TTC TAG CGG CGT TTC CCG TAC TAA TTT AG-3'

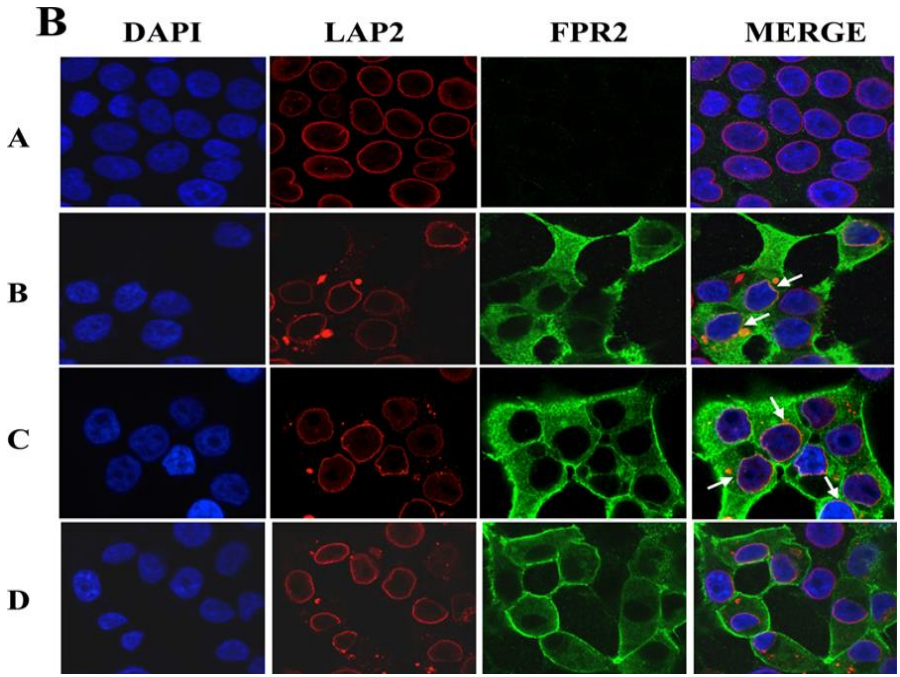


Fig.18. FPR2 aminoacid sequence contains a NLS. (A) The aminoacid sequence of NLS in the third intracellular loop of FPR2 is boxed in red. The mutated aminoacids are shown in white in the indicated mutant constructs. (B) HEK293 cells were transfected with FPR2wt or FPR2mut3 or FPR2mutBis. Immunofluorescence was performed as described in Materials and Methods. Localization of FPR2 and LAP2 was detected by incubating cells with α-FPR2 and α-LAP2 antibodies, respectively. Staining was performed by incubating cells with DAPI. Panel A, cells were transfected with empty vector. Panel B, cells were transfected with FPR2wt. Panel C, cells were transfected with FPR2mut3 (K230A). Panel D, cells were transfected with FPR2mutBis (H229A/K230A/K231A). Merge (yellow/orange) show co-localization of DAPI, FPR2 and LAP2 (arrows). Cells showed no staining with secondary antibody alone. Data are representative of three independent experiments.

Western blot analysis, performed on nuclear proteins purified from HEK293 cells transfected with the three constructs, was in agreement with immunofluorescence results. Cells transfected with FPR2wt showed nuclear localization of the receptor (Fig.19C, lane B), which was partially prevented when cells were transfected with FPR2mut3 (Fig.19C, lane C). FPR2 nuclear localization was completely abolished in cells transfected with the triple mutant FPR2mutBis

(Fig.19C, lane D). Taken together these data indicate that H229 and K231 residues in the KIHKK sequence play a key role in the nuclear localization or translocation of FPR2. Accordingly, we did not observe nuclear expression of FPR1 and FPR3, where K231 is replaced by the polar aminoacids Q and N, respectively.

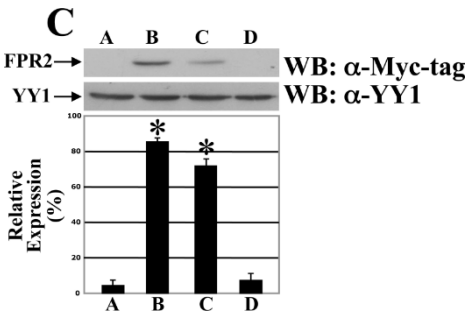


Fig.19. FPR2 aminoacid sequence contains a NLS (C) Nuclear lysates were purified from HEK293 cells transfected with empty vector (lane A), FPR2wt (lane B), FPR2mut3 (lane C) or FPR2mutBis (lane D). Proteins (30 μg) were resolved on 10% SDS-PAGE and FPR2 was detected by western blot with an α-Myc-tag antibody. α-YY1 was used as a marker for nuclear purity and as loading control. Relative expression was quantitatively estimated by densitometry using a Discover Pharmacia scanner equipped with a Sun Spark Classic densitometric workstation. *$p<0.05$ compared with cells transfected with the empty vector. The blots are representative of three separate experiments of identical design.

4. Discussion

The results obtained during my Ph.D. show that the Formyl-peptide Receptors are functionally expressed in several cell types unrelatedto polymorphonuclear compartment where they were initially identified. These receptors are promiscuous for the heterogeneity of intracellular signaling cascades activated, for their tissue specific expression or to the nature of theligands with which the receptors interact.

We demonstrate that in serum-deprived PNT1A and ECV cells, stimulation of FPR2 by a specific agonist results in NADPH-oxidase-dependent superoxide generation and RTKs transactivation. Furthermore, we show that ROS play a key role in bridging the signals from FPR2 to RTKs as demonstrated by the effects of apocynin, and a siRNA against p22phox on thetransactivation. Our results also indicate that, as a consequence of the trans-phosphorylation process the phosphotyrosine residues of RTKs provide docking sites for recruitmentand triggering of the STAT3 pathway. Several GPCRs can transactivate RTKs and the cross talk between the two classes of receptors is a crucial signaling mechanism that serves to expand the cellular communication network, thereby assisting the transmission of growth signals. In glioblastoma cells EGFR transactivation is mediated by the binding of N-fMLP to FPR1 and inhibition of EGFR phosphorylation significantly reduces FPR agonist-induced tumor cell chemotaxis and proliferation. Thus, FPR expressed in glioblastoma cells can exploit the EGFR capacity to amplify tumor growth [59]. A molecular mechanism that can contribute to RTKs transactivation by GPCR ligands is the generation of ROS, which in turn inactivate PTPases that tightly control the activity of RTK. In fact, oxidation and reduction of protein cysteine sulfhydryl groups of PTPases may act as a molecular switch to start or to stop signaling. Plasma membrane-associated Nox enzymes catalyze the deliberate and regulated generation of ROS. In contrast to the cytotoxic amounts of superoxide generated by phagocytes, the non-phagocytic Nox family members are recognized as producers of low levels of ROS that play critical roles in maintaining normal physiologic processes and that stimulate intracellular signaling cascades via activation ofkinases and inhibition

of PTPases. Under physiological conditions, the intracellular production of ROS does not alter the redox state of cells, which have large reserves of reducing agents. This reducing intracellular environment allows agonist-induced increases in ROS to function as second messengers by limiting their effect in time and space. A major attribute of non-phagocytic NADPH oxidases is that not only are they constitutively active but their activity is sensitively influenced by a wide variety of (patho)physiological stimuli. Several pathological conditions are associated with overproduction of ROS by Nox enzymes. They include chronic diseases that tend to appear late in life, such as Alzheimer disease, atherosclerosis, hypertension, diabetic nephropathy, lung fibrosis, and cancer. In many of these diseases overproduction of ROS also results from increased expression of Nox enzymes and/or of their regulatory subunits [27].

STAT3 is a member of the STAT family of cytoplasmic transcription factors. It requires extrinsic tyrosine phosphorylation to become activated and this event is induced by RTKs, cytoplasmic c-Src tyrosine kinases, and components of the JAK family. Specific formyl peptide receptor agonists also activate STAT3. We found that in PNT1A and ECV cells exposure to agonists induces FPR1/2-dependent phosphorylation of STAT3 at the Y705 and S727 residues,suggesting that STAT3 activation is also a part of the FPR1/2-dependent signaling cascade. We also observed MEK-dependent ERK phosphorylation in WKYMVm-stimulated cells. It hasbeen shown that the MAPK pathway plays an important role in the regulation of STAT3 signaling and that ERK are known to phosphorylate STAT3 at the S727 residue. FPR2 was initially known as a low-affinity receptor for N-fMLP. In the past few years several ligands have been identified, making FPR2 the most promiscuous in the FPR family with respect to agonist selectivity. Interestingly, most of the newly identified agonists for FPR2 do not share substantial sequence homology; thus, FPR2 behaves as a “pattern recognition” receptor that can be activated by a wide variety of unrelated ligands [16]. There have been ongoing efforts in several laboratories to study the ligand and FPR2 interaction, in part because of the potential for FPR2 to become a therapeutic target.It has been shown that both

agonists and antagonists for FPR2 have therapeutic value. In fact, WKYMVm increases neutrophil bactericidal activity in chemotherapy-treated cancer patients and enhances endogenous expression of TRAIL, a novel potential anticancer agent, in human monocytes and neutrophils. Moreover, the administration of WKYMVm protects against death by enhanced bactericidal activity and inhibition of vital organ inflammation and immune cell apoptosis in a cecal ligation and puncture sepsis mouse model. WKYMVm, activating FPR2, also potently inhibits HIV-1 Env-mediated fusion and viral infection through heterologous desensitization of the chemokine receptors CCR5 and CXCR4, suggesting a novel approach to the development of anti-HIV-1 reagents.The use of FPR2 by SAA, Aβ42, and human prion peptide suggests that this receptor may play a crucial role in proinflammatory aspects of systemic amyloidosis, Alzheimer disease, and prion diseases. This observation prompted studies in search of antagonists, which areimportant for delineating the signal transduction cascade associated with receptor activation and as a basis for developing anti-inflammatory therapeutic agents [21]. Several antagonists for FPR2 have been identified. These include the chemotaxis inhibitory protein of Staphylococcus aureus, the FPR2 inhibitory protein FLIpr, the bile acids deoxycholic and chenodeoxycholic, and Quinc-7, a synthetic nonpeptide developed through chemical modification of Quinc-1[60]. Moreover, W-rich peptides, such as WRW4, exert an antagonistic effect on WKYMVm-induced FPR2 signaling, suggesting their use for the treatment of several diseases in which FPR2 isknown to play a role.

It should be also noted that because FPR is overexpressed in human glioblastoma cells, at least some members of the FPR family might also be valuable biomarkers for cancer diagnosis, therefore increasing the number of available biomarkers for cancer diagnosis and staging.

The transactivation of RTKs by FPR2 in human cells may have important pathophysiological implications. The expression of FPR2 in these cells could render these cells responsivenot only to WKYMVm, but also to agonists contained in the environment of necrotic tumor cells. As a result of these interactions, FPR2 could activate intracellular signaling molecules such as ROS and ERKs that, in turn,

could trigger RTKs transactivation, the STAT3 pathway, and cell growth. This suggests that clarification of the resulting signaling cascades may open the way to new drugs that interfere with the FPR2 signaling pathway and that targeting bothFPR2 and RTKs may yield superior therapeutic effects compared with targeting either receptor separately.

The results also show for the first time that FPR2 localizes on the nuclear membrane of Calu-6 and AGS cells. Studies of intracellular trafficking of a few different GPCRs, evoked by exposure to ligand or by cellular activation through ligand-independent mechanisms, have shown that GPCRs may be inserted into nuclear membranes for prolonged periods. Nuclear GPCRs compose distinctive signaling units there, and respond to specific intracellular ligand by transducing nuclear transcriptional signals that differ from those sent by their plasma membrane complexes [39]. The physiological functions and mechanisms mediated by GPCRs on the nuclear membrane are not yet completely understood. A full complement of downstream signal trasduction components is present on the nuclear membrane including G proteins and enzyme effectors. Therefore, the resulting biological effects might result from the intregation of extracellular and intracellular signaling pathways. The nuclear (or perinuclear) localization can be attributed to translocation of a GPCR from the cell surface or *de novo* synthesis of the receptor. Nuclear GPCRs can be constitutively activated or activated by specific ligands. Further confirm of nuclear localization of GPCR constitutively expressed, but also internalized in a ligand depend or indipendent manner, is supported by the recognition of a nuclear localization sequence (NLS). Searches in data bank revealed 17 GPCRs with a clearly recognizable NLS motif in the eighth helix including adenosine, angiotensin, bradykinin, and endothelin receptors. However, a putative NLS motif is located at the carboxyl-terminal region of the third intracellular loop in other receptors such as apelin receptor [50]. The nuclear distribution of FPR2 was assessed by western blot and immunofluorescence experiments using antibodies against the isoform of interst.Nuclear GPCRs might be derived from the cell membrane and their transfer to the nucleus can be attributed to a nuclear

localization motif which is recognized by members of the importin family, which act as carriers to transport the substrate protein across the nuclear pore [50]. The demonstration that FPR2 is expressed at the nuclear level raises questions about its function at this location. In general, nuclear-localized receptors may regulate distinct signaling pathways, suggesting that biological responses mediated by FPR2 are not only initiated at the cell surface but might result from the integration of extracellular and intracellular signaling pathways. Therefore, an important focus is to target single pathways associated with a plasma membrane or nuclear GPCR. This pathway-selective strategy might be based on targeting the assembly or trafficking of signaling complexes to distinct subcellular destinations through biased ligands, thus providing a selective set of discriminatory modulators. Pharmaceutical retention of FPR2 at the plasma membrane could be a novel strategy for inhibiting nuclear activities of FPR2 in pathological processes that involve this receptor. The access of ligands to nuclear receptors is another open question. Such ligands must be biosynthesized inside the cells or to cross the cellular membrane. FPR2 agonists that do not permeate cells have access only to receptors expressed on the plasma membrane. This observation provides a new concept for bioavailability of a drug, which should be defined also in terms of access of an agonist to intracellular domains of a receptor, and not only in the current terms of systemic absorption and tissue distribution. Taken together, our data indicate that FPRs play new unexepected roles in cell signaling and function. The promiscuity of these receptors in binding different ligands, coupled with their presence in different cells and tissues, indicates a diverse role in multiple biological settings. A better understanding of these fundamental functions could lead to the identification of new therapeutic targets for drug development.

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WKYMVm-induced cross-talk between FPR2 and HGF receptor in human prostate epithelial cell line PNT1A

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ABSTRACT

Cross-communication between GPCRs and TKRs represents a mechanism to amplify the information exchange throughout the cell. We show that WKYMVm, an FPR2 agonist, induces the phosphorylation of Y1313/Y1349/Y1356 residues of c-Met and triggers some of the molecular responses elicited by c-Met/HGF binding, such as STAT3, PLC-γ1/PKCα and PI3K/Akt pathways. The critical role of NADPH oxidase-dependent superoxide generation in this cross-talk mechanism is supported by the finding that blockade of NADPH oxidase function prevents c-Met trans-phosphorylation and the downstream signalling cascade. These results highlight the function of FPR2 to activate a interconnected signalling network and suggest novel possibilities for therapeutic interventions.

1. Introduction

Eukaryotic cells have developed highly efficient mechanisms of receptor-mediated cell communication to coordinate and integrate extracellular signals. The formyl-peptide receptors FPR1, FPR2 and FPR3 belong to the G protein-coupled receptors (GPCR) super-family and are coupled to pertussis toxin (PTX)-sensitive G_i proteins [1]. FPR1 is efficiently activated by *N*-formyl-methionyl-leucyl-phenylalanine, whereas FPR2 shows an higher binding efficiency for WKYMVm [2]. The expression of these receptors has been demonstrated in several cell types [3] and their important biological functions are supported by the identification of high affinity host-derived agonists. [4–6].

Despite GPCRs lack intrinsic tyrosine kinase activity, tyrosine phosphorylation of a tyrosine kinase receptor (TKR) occurs in response to binding of specific agonists of several such receptors. GPCR ligands increase tyrosine phosphorylation of TKRs either by increasing the kinase activity or by inhibiting an associated protein tyrosine phosphatase [7]. These events are mediated by reactive oxygen species (ROS) whose concentration increases transiently in cells stimulated with GPCR agonists. Several evidence support the role of ROS in TKR transactivation. For instance, in human lung cancer cells stimulation of FPR2 by WKYMVm induces ROS-dependent EGFR tyrosine phosphorylation [8] and in human carcinoma cells ROS mediate c-Met transactivation by GPCRs [9]. The deliberated and regulated generation of ROS is catalyzed by enzymes that belong to NADPH oxidase (Nox) family, constituted by membrane and cytosolic components. Phosphorylation of the regulatory cytosolic protein $p47^{phox}$ on several serine residues is associated with oxidase activation [10].

The c-Met tyrosine kinase receptor (c-Met) is a cell surface receptor for hepatocyte growth factor (HGF) [11]. Following HGF binding, the tyrosine kinase domain of c-Met undergoes trans-phosphorylation on the Y1234 and Y1235 residues in the activation loop [12]. Kinase activation by autophosphorylation leads to the subsequent phosphorylation of Y1349 and Y1356 residues in the C-terminal multifunctional docking site, resulting in the activation of c-Met signalling [12]. Previously, we demonstrated that PNT1A cells express a functional c-Met and cell exposure to NK1, a splice variant of HGF, induces the phosphorylation of Y1313/Y1349/Y1356 residues of c-Met which provide docking sites for the activation of intracellular signalling pathways [13].

The aims of this study were to analyze the cross-talk between FPR2 and c-Met in PNT1A cells and to identify intracellular signalling cascades triggered by the WKYMVM-mediated activation of HGF receptor. We show that stimulation of FPR2 by its agonist results in the phosphorylation of tyrosine 1313/1349/1356 residues of c-Met and promotes some of the molecular responses elicited by the binding of HGF to its receptor.

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Review

Distinct Signaling Cascades Elicited by Different Formyl Peptide Receptor 2 (FPR2) Agonists

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Abstract: The formyl peptide receptor 2 (FPR2) is a remarkably versatile transmembrane protein belonging to the G-protein coupled receptor (GPCR) family. FPR2 is activated by an array of ligands, which include structurally unrelated lipids and peptide/proteins agonists, resulting in different intracellular responses in a ligand-specific fashion. In addition to the anti-inflammatory lipid, lipoxin A4, several other endogenous agonists also bind FPR2, including serum amyloid A, glucocorticoid-induced annexin 1, urokinase and its receptor, suggesting that the activation of FPR2 may result in potent pro- or anti-inflammatory responses. Other endogenous ligands, also present in biological samples, include resolvins, amyloidogenic proteins, such as beta amyloid (Aβ)-42 and prion protein $(Prp)_{106–126}$, the neuroprotective peptide, humanin, antibacterial peptides, annexin 1-derived peptides, chemokine variants, the neuropeptides, vasoactive intestinal peptide (VIP) and pituitary adenylate cyclase activating polypeptide (PACAP)-27, and mitochondrial peptides. Upon activation, intracellular domains of FPR2 mediate signaling to G-proteins, which trigger several agonist-dependent signal transduction pathways, including activation of phospholipase C (PLC), protein kinase C (PKC) isoforms, the phosphoinositide 3-kinase (PI3K)/protein kinase B (Akt) pathway, the mitogen-activated protein kinase (MAPK) pathway, p38MAPK, as well as the phosphorylation of cytosolic tyrosine kinases, tyrosine kinase receptor transactivation, phosphorylation and nuclear translocation of regulatory transcriptional factors, release of calcium and production of oxidants. FPR2 is an attractive therapeutic target, because of its involvement in a range of normal physiological processes and pathological diseases. Here, we review and discuss the most significant findings on the

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Review

Cell-Surface Receptors Transactivation Mediated by G Protein-Coupled Receptors

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Abstract: G protein-coupled receptors (GPCRs) are seven transmembrane-spanning proteins belonging to a large family of cell-surface receptors involved in many intracellular signaling cascades. Despite GPCRs lack intrinsic tyrosine kinase activity, tyrosine phosphorylation of a tyrosine kinase receptor (RTK) occurs in response to binding of specific agonists of several such receptors, triggering intracellular mitogenic cascades. This suggests that the notion that GPCRs are associated with the regulation of post-mitotic cell functions is no longer believable. Crosstalk between GPCR and RTK may occur by different molecular mechanism such as the activation of metalloproteases, which can induce the metalloprotease-dependent release of RTK ligands, or in a ligand-independent manner involving membrane associated non-receptor tyrosine kinases, such as c-Src. Reactive oxygen species (ROS) are also implicated as signaling

Expression of Formyl-peptide Receptors in Human Lung Carcinoma

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Abstract. *Background/Aim: Formyl-peptide receptors (FPRs) are expressed in several tissues and cell types. The identification of markers involved in cell growth may further allow for molecular profiling of lung cancer. We investigated the possible role of FPRs as molecular markers in several types of lung carcinomas which is the main cause of cancer death worldwide. Materials and Methods: Tumor tissue samples were collected from six patients affected by lung cancer. Biopsies were analyzed for expression of FPR isoforms both in tumoral and peritumoral tissue by real-time polymerase chain reaction (PCR), western blot and immunofluorescence. Results: Real-time PCR, western blot and immunofluorescence analyses showed that FPR expression is lower in types of human lung cancer tissues when compared to the surrounding peritumoral tissues. Conclusion: The study of the mechanistic basis for the control of FPR expression in normal peritumoral versus tumoral tissues could provide the basis for new diagnostic and therapeutic interventions.*

The human formyl-peptide receptors FPR1, FPR2 and FPR3 belong to the G-protein-coupled receptor (GPCR) family (1) and were first detected in phagocytic leukocytes and in monocytes. Their expression has also been demonstrated in several tissues and cell types at the protein or mRNA levels (2, 3) and their relevant biological functions have emerged through identification of high affinity host-derived agonists. Such ligands trigger intracellular signaling cascades involving phosphatidylinositol 3-kinase, protein kinase C, mitogen-activated protein kinases, signal transducer and activator of transcription, cellular Src kinase, as well as several transcription factors (4-6).

Despite FPRs lack of intrinsic tyrosine kinase activity, tyrosine phosphorylation of a tyrosine kinase receptor (RTK) occurs in response to binding of agonists to such receptors, triggering mitogenic pathways. FPR-mediated RTK transactivation may occur by different molecular mechanisms, which include the activation of metalloproteases or the activation of membrane associated non-receptor tyrosine kinases (7). Further evidence supports the role of reactive oxygen species (ROS) in FPR-mediated RTK transactivation. For instance, in human lung cancer cells, FPR2 stimulation induces ROS-dependent Epidermal growth factor receptor (EGFR) tyrosine phosphorylation (8) and in human prostate cells, ROS mediate hepatocyte growth factor receptor transactivation by FPR2 (9).

Lung cancer remains the leading cause of cancer-related death worldwide. Tobacco smoking and air pollution exposure are mainly implicated in lung cancer development (10-13). Cytotoxic chemotherapy offers modest prolongation in survival, although the additional gain in terms of response rate and survival with combinations of chemotherapy and monoclonal antibodies have reached a plateau (14-16). The development of molecular profiling technologies to assess DNA, RNA, protein and metabolites have provided

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