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Original Contribution

NADPH-oxidase-dependent reactive oxygen species mediate EGFR transactivation by FPRL1 in WKYMVm-stimulated human lung cancer cells

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ABSTRACT

Cross talk between unrelated cell surface receptors, such as G-protein-coupled receptors (GPCR) and receptor tyrosine kinases (RTK), is a crucial signaling mechanism to expand the cellular communication network. We investigated the ability of the GPCR formyl peptide receptor-like 1 (FPRL1) to transactivate the RTK epidermal growth factor receptor (EGFR) in CaLu-6 cells. We observed that stimulation with WKYMVm, an FPRL1 agonist isolated by screening synthetic peptide libraries, induces EGFR tyrosine phosphorylation, $p47^{phox}$ phosphorylation, NADPH-oxidase-dependent superoxide generation, and c-Src kinase activity. As a result of EGFR transactivation, phosphotyrosine residues provide docking sites for recruitment and triggering of the STAT3 pathway. WKYMVm-induced EGFR transactivation is prevented by the FPRL1-selective antagonist WRWWWW, by pertussis toxin (PTX), and by the c-Src inhibitor PP2. The critical role of NADPH-oxidase-dependent superoxide generation in this cross-talk mechanism is corroborated by the finding that apocynin or a siRNA against $p22^{phox}$ prevents EGFR transactivation and c-Src kinase activity. In addition, WKYMVm promotes CaLu-6 cell growth, which is prevented by PTX and by WRWWWW. These results highlight the role of FPRL1 as a potential target of new drugs and suggest that targeting both FPRL1 and EGFR may yield superior therapeutic effects compared with targeting either receptor separately.

The human formyl peptide receptor (FPR) and its variants, FPR-like 1 (FPRL1) and FPR-like 2 (FPRL2), belong to the G-protein-coupled seven-transmembrane receptor (GPCR) family [1]. They are all coupled to the G_i family of G proteins, as indicated by the total loss of cell response to their agonists upon exposure to pertussis toxin (PTX) [2,3]. FPR and FPRL1 were first detected in phagocytic leukocytes, and FPRL2 was found in monocytes and in dendritic cells [4]. The three receptors were subsequently identified in other cell types and tissues at the protein and/or mRNA level [2,3,5–7], suggesting that these receptors have functions in addition to those exerted in polymorphonuclear cells (PMN).

FPRL1 is less efficiently activated by *N*-formylmethionylleucylphenylalanine (N-fMLP) than FPR, as also shown by its higher binding efficiency for WKYMVm, a modified peptide isolated by screening synthetic peptide libraries [8]. A variety of other agonists also bind FPRL1 in several cell types with high affinity. These include lipoxin A_4 eicosanoid, annexin 1, uPAR, the V3 region of the HIV-1 envelope glycoprotein gp120, the acute-phase protein SAA, the 42-amino-acid form of β-amyloid, the human prion peptide, and the cathelicidin LL-37 [3,9,10]. Binding of various agonists to FPRL1 triggers the activation of intracellular signaling molecules including calcium, PKC isoforms, phospholipases A2 and D, and mitogen-activated protein kinases (MAPK), including p38MAPK [9,11]. In several cell types, PKC, ERK, and p38MAPK are involved in the phosphorylation, on multiple serine residues, of the cytosolic regulatory subunit $p47^{phox}$ of NADPH oxidase in vitro and in vivo and, in turn, in NADPH-oxidase-dependent superoxide generation [7,12–14]. In nonphagocytic cells, the deliberate and regulated generation of superoxide plays a key role in a variety of essential biological processes and is catalyzed by enzymes that belong to the NADPH oxidase (Nox) family [15]. This includes Nox1, abundant in colon, brain, and vascular cells; Nox2/$gp91^{phox}$, the classic phagocyte catalytic component of the respiratory burst oxidase, which has important roles in other tissues as well; Nox3, located in the inner ear; Nox4, a widely distributed Nox abundant in the kidney, bone, and vascular cells; Nox5, a calcium-regulated enzyme mainly expressed in lymphoid tissues and testis; and Duox1 and Duox2, dual oxidases that also contain a peroxidase-like domain [16]. Nox2, Nox3, Nox4, Duox1, and Duox2 are also expressed in a number of lung cell types, such as airway/epithelial and mesenchymal cells [17].

Abbreviations: ROS, reactive oxygen species; FPRL1, formyl peptide receptor-like 1; ERK, extracellular-signal-regulated kinase; PTX, pertussis toxin; EGFR, epidermal growth factor receptor; STAT, signal transducer and activator of transcription.

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Furthermore, homologues of $p47^{phox}$ and $p67^{phox}$, denominated NOXO1 (NOX organizing protein 1) and NOXA1 (NOX activating protein 1), respectively, have been identified [18].

Nox1 is constitutively active in unstimulated cells and this is explained by the absence of regulatory phosphorylation sites on NOXO1 and by the ability of NOXO1 to localize to the resting cell membrane [19]. However, Nox1 may be further activated by platelet-derived growth factor (PDGF) and angiotensin II in vascular smooth muscle cells [20,21] and by phorbol ester in HEK293 and COS7 cells in a cell-type-specific manner [22]. Nox2/$gp91^{phox}$ is dormant in resting cells and is stimulated by several agonists, such as N-fMLP, which induces $p47^{phox}$ phosphorylation by either proline-directed kinases or PKC [12,23]. The phosphorylated serine residues of $p47^{phox}$ expose an SH3 binding site that interacts with the proline-rich region of $p22^{phox}$ and facilitates translocation to the membrane. $p67^{phox}$ then binds to the translocated $p47^{phox}$, providing a binding site for activated Rac and forming the functional enzyme. Nox3 functions together with $p22^{phox}$ as an enzyme constitutively producing superoxide, which can be regulated by the combinatorial use of the organizers and activators [24]. Nox4 requires only the membrane subunit $p22^{phox}$ for reactive oxygen species (ROS) generation and seems to be constitutively active [23]. However, a further Nox4 activation is observed in lipopolysaccharide-stimulated HEK293 cells, insulin-stimulated adipocytes, and angiotensin II-stimulated mesangial cells [25–27]. Nox5, Duox1, and Duox2 are regulated by increased intracellular calcium levels due to the presence of calcium-binding EF-hands [15,23].

We previously showed that, in serum-deprived IMR90 human fibroblasts, exposure to growth factors stimulates a nonphagocytic NADPH oxidase, and treatment with NADPH oxidase inhibitors results in the impairment of the serum-induced signaling cascade [28]. We also demonstrated that these cells express FPRL1 and that stimulation with WKYMVm induces MEK- and PKC-dependent $p47^{phox}$ phosphorylation and NADPH-oxidase-dependent superoxide generation [7,29].

Receptor tyrosine kinases (RTK) are an important subclass of transmembrane proteins. The epidermal growth factor receptor (EGFR) is the most important member of this family, being implicated in growth stimulation in a wide variety of malignant tumors. Stimulation of the receptor by EGF results in dimerization and subsequent autophosphorylation on tyrosine residues, thereby generating phosphotyrosine docking sites that activate intracellular signaling cascades. EGFR activation can also be induced by GPCR, which are a large group of cell-surface receptors that exert a wide variety of biological functions [30]. GPCR agonists increase tyrosine phosphorylation of EGFR either by increasing the kinase activity or by inhibiting an associated phosphatase activity that is mediated by oxidants [31]. The observation that GPCR stimulation induces EGFR activation serves as a paradigm for interreceptor cross talk, because it combines the broad diversity of GPCR with the potent signaling capacities of EGFR.

One of the best known downstream targets of activated EGFR is the signal transducer and activator of transcription (STAT) 3 [32,33]. STAT3 is a latent cytoplasmic transcription factor that transduces signals from cell membrane to the nucleus and is involved in the regulation of many genes in various cell types [34]. The activity of STAT3 is associated with the phosphorylation of the Tyr705 residue that is required for STAT3 dimerization, as well as with nuclear translocation and DNA binding. The full transcriptional activity is manifested only when the Ser727 residue, in the transactivation domain, is also phosphorylated [35]. Although activation of STAT3 has generally been associated with cytokines and mitogenic growth factor signaling, several ligands for GPCR also activate STAT3 in several cell types. These include angiotensin II in vascular smooth muscle cells [36], α-melanocyte-stimulating hormone in B lymphocytes [37], WKYMVm in RBL-2H3 cells [38], and Orphanin FQ and N-fMLP in hematopoietic cells [39].

In this study, we analyzed the intracellular signaling cascade triggered by WKYMVm in the human lung cancer CaLu-6 cell line. These cells express EGFR at high levels [40] and show change in the metabolism or in the generation of superoxide in response to various stimuli [41,42]. The results show that: (i) these cells express a biologically functional FPRL1 receptor, (ii) stimulation of FPRL1 by WKYMVm induces G_i protein- and NADPH-oxidase-dependent c-Src activation and EGFR tyrosine phosphorylation, (iii) FPRL1-dependent EGFR transactivation triggers activation of the STAT3 pathway, and (iv) as a consequence of the FPRL1-induced signaling, WKYMVm promotes cell proliferation in CaLu-6 cells.

Materials and methods

Reagents and cell culture treatments

The WKYMVm and WRWWWW (WRW4) peptides were synthesized and HPLC-purified by PRIMM (Milan, Italy). SDS–PAGE reagents were from Bio-Rad (Hercules, CA, USA). Protein A/G Plus agarose, anti-active phosphorylated ERK1/2, anti-tubulin, anti-c-Src, anti-$p47^{phox}$, anti-FPRL1, anti-EGFR, anti-STAT3, anti-cyclin A, anti-p-Y, and anti-rabbit antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Anti-p-c-Src(Tyr416), anti-p-STAT3(Tyr705), and anti-p-STAT3 (Ser727) were from Cell Signaling Technology (Danvers, MA, USA). Protein A–horseradish peroxidase and anti-mouse Ig–horseradish peroxidase were from Amersham Pharmacia Biotech (Little Chalfont, Buckinghamshire, UK). PD098059, AG1478, PP2, PP3, and genistein were purchased from Calbiochem (La Jolla, CA, USA). PTX, apocynin, anti-p-Ser antibody and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) were from Sigma (St. Louis, MO, USA).

$p22^{phox}$ siRNA (SI03078523) and negative control siRNA (SI03650318) were purchased from Qiagen (Hiden, Germany).

CaLu-6 cells 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. Cells were cultured until they reached 80% confluence, starved in serum-free DMEM for 24 h, and successively stimulated with WKYMVm peptide at the final concentration of 10 μM for various times, as indicated in the figures. In other experiments, serum-deprived cells were preincubated with 50 μM PD098059 for 90 min, 100 ng/ml PTX for 16 h, 10 μM PP2 for 45 min, 10 μM PP3 for 45 min, 60 μM genistein for 1 h, 2 μM AG1478 for 1 h, 100 μM apocynin for 2 h, or 10 μM WRW4 for 15 min before stimulation with 10 μM WKYMVm for 2 min.

In silencing experiments 4×10^5 cells were incubated for 12 h with 5 nM siRNAs in DMEM containing 10% FBS in the presence of 20 ml HiPerFect (Qiagen). Cells were then serum-deprived for 24 h before stimulation with 10 μM WKYMVm for 2 min.

RNA preparation and RT-PCR analysis

Total RNA was extracted from CaLu-6 cells and PMN with the RNAeasy Mini kit (Qiagen) according to the manufacturer's instructions, and 0.1 μg of RNA was used as template for RT-PCR experiments. To amplify FPRL1 we designed the sequences of sense oligonucleotide 5′-AATTCACATCGTGGTGGACA-3′ and antisense primer 5′-GAGGCAGCTGTTGAAGAAGG-3′, according to the sequence of the human FPRL1 coding region. These primers generate a 688-bp fragment. For human FPR, sense primer 5′-CTCCAGTTGGACTAGCC-3′ and antisense primer 5′-CCATCACCCAGGGCCCA-3′ were used to yield a 500-bp product. For human GAPDH, sense primer 5′-CCATGGAGAAGGCTGGG-3′ and antisense primer 5′-CGCCACAGTTTCCCGGA-3′ amplified a 280-bp fragment.

Western blot and immunoprecipitation analysis

Growth-arrested cells were stimulated with 10 μM WKYMVm for various times in the presence or absence of the appropriate amount of

specific inhibitors. CaLu-6 cells were rinsed with cold phosphate-buffered saline (PBS), lysed with 0.5 ml RIPA buffer (50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 1% NP-40, 1 mM EDTA, 0.25% sodium deoxycholate, 1 mM NaF, 10 μM Na_3VO_4, 1 mM phenylmethylsulfonyl fluoride, 10 μg/ml aprotinin, 10 μg/ml pepstatin, 10 μg/ml leupeptin), and incubated at 4 °C for 45 min. Protein concentration was determined using a Bio-Rad protein assay. Western blot analysis was performed as previously described [10,28,29]. Antigen–antibody complexes were detected with the ECL chemiluminescence reagent kit (Amersham Pharmacia Biotech). Nuclear protein extracts were prepared with a Qproteome nuclear protein kit (Qiagen) according to the manufacturer's instructions. In immunoprecipitation experiments, cell lysates containing equal amounts of proteins were incubated with 3 μg of either anti-EGFR or anti-$p47^{phox}$ antibody overnight at 4 °C. Immunocomplexes were mixed with 30 μl of Protein A/G Plus agarose and rotated for 45 min at 4 °C. The immunoprecipitates were then washed three times with cold PBS, resuspended in 40 μl of Laemmli buffer, boiled for 5 min, pelleted by short centrifugation, and separated by 10% SDS–PAGE. Phosphorylated protein levels were quantitatively estimated by densitometry using a Discover Pharmacia scanner equipped with a Sun Spark Classic densitometric workstation.

Assay of superoxide production

The method used to determine $O_2^{\bullet-}$ is described elsewhere [10]. Membranes and cytosol were isolated from serum-starved human lung cancer cells stimulated with 10 μM WKYMVm for various times. NADPH-dependent superoxide generation was determined as the superoxide dismutase-sensitive rate of reduction of cytochrome *c*. Briefly, combinations of 10 μg of membrane and 200 μg of cytosol proteins in PBS were incubated at room temperature in the presence of 15 μM GTPγ-S, 100 μM cytochrome *c*, and 10 μM FAD in a total volume of 1 ml. NADPH (100 μM) was then added and the production of superoxide was monitored at 550 nm. The specificity of cytochrome *c* reduction was controlled by the addition to control samples of 200 U/ml superoxide dismutase. Rates of $O_2^{\bullet-}$ production were calculated from the linear segment of the increase in absorbance at 550 nm and translated into nanomoles of $O_2^{\bullet-}$ by the extinction coefficient of cytochrome *c*, $\Delta E_{550}/\Delta t = 21.1\ mM^{-1}\ cm^{-1}$, considering that 1 mol of $O_2^{\bullet-}$ reduces 1 mol of cytochrome *c*. The Student *t* test was used to compare individual treatments with their respective control value and $p<0.05$ versus values obtained with growth-arrested CaLu-6 cells was considered significant.

In vitro kinase assay

The in vitro kinase assay for c-Src activity was performed using denatured rabbit muscle enolase (Sigma) as an exogenous substrate. c-Src was immunoprecipitated by 5 μg of anti-c-Src antibody and protein A/G–Sepharose beads overnight at 4 °C. The immunoprecipitates were washed three times with 1% Nonidet P-40 lysis buffer and twice with 25 mM Tris–HCl, pH 7.4, and 10 mM $MnCl_2$. The beads were then incubated with 20 μl of kinase buffer (50 mM Tris–HCl, pH 7.4, 10 mM $MnCl_2$, 1 mM dithiothreitol) containing 2 μg of acid-denatured enolase and 10 mCi of [γ-^{32}P]ATP (PerkinElmer Life Sciences, Waltham, MA, USA) per reaction for 10 min at 37 °C. Reactions were stopped by adding Laemmli sample buffer and resolved by SDS–PAGE followed by autoradiography. Rabbit muscle enolase was denatured with 25 mM acetic acid at 30 °C for 10 min and then added to 1/10 of the total kinase reaction volume.

Statistical analysis

All data presented are expressed as means ± SD 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 $p<0.05$.

Cell viability

CaLu-6 cells were plated at 4×10^4 cells per well in 200 μl of complete culture medium containing 10 μM WKYMVm in 96-well plates (Corning USA) in the presence or absence of 100 ng/ml PTX or 10 μM WRW4 and incubated at 37 °C for 12, 24, and 36 h. MTT (5 mg/ml in PBS) was then added to each well and incubated for 4 h. After careful removal of the medium, 200 μl of dimethyl sulfoxide (DMSO) was added to each well. The absorbance of the resulting formazan salts was recorded on a microplate reader at the wavelength of 540 nm. The effect of WKYMVm on cell growth was assessed as percentage cell viability. Four independent experiments were performed.

Results

FPRL1 is a biologically functional receptor in CaLu-6 cells

We first analyzed the expression of FPR and FPRL1 in the CaLu-6 cell line by RT-PCR analysis, performed with specific primers for the coding sequences of the two receptors, and by Western blot analysis using an α-FPRL1 antibody. We observed that FPRL1 mRNA, but not FPR, is expressed in these cells (Fig. 1A) and we detected the presence of the band corresponding to FPRL1 protein at the expected molecular size in immunoblot experiments (Fig. 1B). The α-FPRL1 antibody recognized the same band in cellular lysates purified from IMR90 human fibroblasts, which express FPRL1 [7] (Fig. 1B). We previously demonstrated that, in growth-arrested IMR90 human fibroblasts, activation of FPRL1 by WKYMVm induces ERK phosphorylation, $p47^{phox}$ membrane translocation, and NADPH oxidase activation [7]. Therefore, we next evaluated the ability of WKYMVm to activate the MAPK cascade, by analyzing the effect of cell exposure to the FPRL1 agonist on the phosphorylation state of ERK. We observed in time-response experiments that this treatment induces a rapid activation of ERK, which is sustained after 10 min of exposure to WKYMVm (Fig. 2A). Furthermore, the preincubation of CaLu-6 cells either with PD098059, a selective inhibitor of MEK, or with PTX, which blocks G_i proteins in their inactive form, before WKYMVm stimulation, prevents ERK phosphorylation (Fig. 2B).

Phosphorylation and membrane translocation of the cytosolic oxidase subunits are considered key events in the assembly of phagocytic and nonphagocytic NADPH oxidase [7,43]. In IMR90 cells, $p47^{phox}$ is a substrate for ERK kinase activity, and p44MAPK/p42MAPK phosphorylation is considered a prerequisite for NADPH oxidase activation [7]. Therefore, we investigated the molecular mechanisms

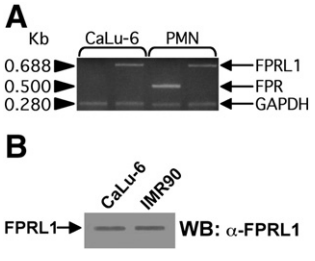

Fig. 1. Expression of FPRL1 in CaLu-6 cells. (A) Total RNAs were purified from CaLu-6 cells and PMN as a control. cDNAs were coamplified using FPR and GAPDH primers and FPRL1 and GAPDH oligonucleotides designed for human FPR, FPRL1, and GAPDH coding sequences. PCR products were separated on a 1.5% agarose gel and stained with ethidium bromide. (B) Cell lysates were purified from CaLu-6 cells and IMR90 cells as a control. A 10% SDS–PAGE gel was loaded with 50 μg of proteins and FPRL1 was detected by Western blot using a specific anti-FPRL1 antibody (α-FPRL1). The experiments were performed in triplicate.

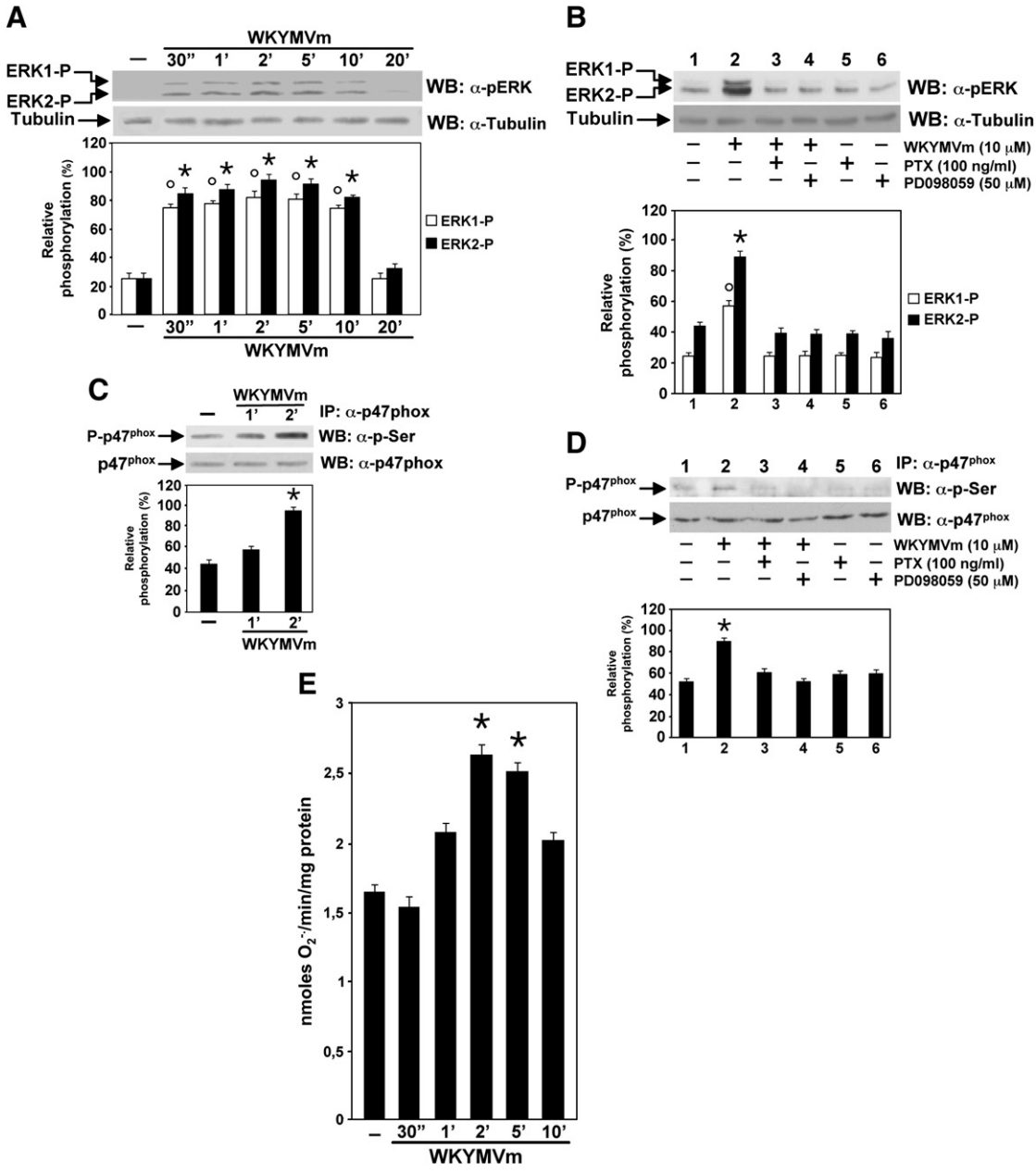

Fig. 2. ERK activation, $p47^{phox}$ phosphorylation, and NADPH-oxidase-dependent ROS generation in WKYMVm-stimulated CaLu-6 cells. (A) Cell lysates were purified from growth-arrested CaLu-6 cells stimulated for various times with 10 μM WKYMVm or (B) preincubated with the indicated concentrations of PTX or PD098059 before stimulation. Proteins (20 μg) were resolved on 10% SDS–PAGE and ERK phosphorylation was detected by Western blot with an anti-phospho-ERK antibody (α-p-ERK). An anti-tubulin antibody (α-tubulin) served as a control for protein loading. The arrows indicate the phosphorylated forms, ERK1-P and ERK2-P, of $p44^{MAPK}$ and $p42^{MAPK}$, respectively. (C) Serum-deprived CaLu-6 cells were stimulated for the indicated times with 10 μM WKYMVm or (D) preincubated with PTX or PD098059 before stimulation. One milligram of cell lysates was immunoprecipitated with an anti-$p47^{phox}$ antibody (α-$p47^{phox}$), and $p47^{phox}$ phosphorylation (P-$p47^{phox}$) was detected using an anti-phospho-serine antibody (α-p-Ser). An α-$p47^{phox}$ antibody served as a control for protein loading. All the blots are representative of at least three separate experiments of identical design. Protein expression 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 cells. (E) Ten micrograms of membrane and 200 μg of cytosolic proteins were purified from CaLu-6 cells grown in serum-free medium and stimulated with WKYMVm for the indicated times. Proteins were incubated in a NADPH oxidase activity assay and the specificity of cytochrome *c* reduction was monitored at 550 nm by using the SOD-inhibitable reduction of cytochrome *c*, as described under Materials and methods. *$p<0.05$ compared with serum-starved cells (—). The experiments were performed in triplicate.

underlying FPRL1-mediated NADPH-oxidase-dependent superoxide generation in CaLu-6 cells. Western blot analysis showed that the NADPH oxidase regulatory subunit $p47^{phox}$ is rapidly phosphorylated on serine residues upon exposure to WKYMVm (Fig. 2C) and that preincubation with PTX or PD098059 significantly prevents $p47^{phox}$ phosphorylation (Fig. 2D). Furthermore, time-response experiments showed that, consequent to activation of the NADPH oxidase regulatory subunit, stimulation of FPRL1 by WKYMVm induces NADPH-dependent superoxide generation, with maximal production occurring at 2 min (Fig. 2E). Taken together these results indicate that in CaLu-6 cells FPRL1 is a biologically functional receptor.

NADPH-oxidase-dependent superoxide generation is required for FPRL1-induced EGFR transactivation

It has been shown that in glioblastoma cells the activation of FPR by N-fMLP results in EGFR transactivation, suggesting that the two receptors synergistically cooperate to exacerbate the malignant

behavior of these tumor cells [44,45] and to produce angiogenic factors [46]. Because EGFR is expressed at high levels in CaLu-6 cells [40], we examined the ability of FPRL1 to transactivate EGFR. Fig. 3A shows that stimulation of CaLu-6 cells with 10 μM WKYMVm results in a time-dependent EGFR phosphorylation, with maximal phosphorylation of tyrosine residues occurring at 2 min. Furthermore, preincubation of cells with PTX, before stimulation with the FPRL1 agonist, results in a significant reduction in the phosphorylation level of tyrosines of EGFR, consequent to G_i-protein-specific inhibition (Fig. 3B).

A large body of evidence indicates that ROS are signaling intermediates in RTK activation [47–50]. ROS-mediated inhibition of phosphotyrosine phosphatase (PTPase) activity results in an equilibrium shift from the nonphosphorylated to the phosphorylated state of RTK. To investigate the role of NADPH-oxidase-dependent ROS generation in FPRL1-induced EGFR transactivation, we preincubated cells with the NADPH-oxidase-specific inhibitor apocynin or with a siRNA against $p22^{phox}$ before WKYMVm stimulation. We observed in immunoblot experiments that blockade of NADPH oxidase function prevents FPRL1-induced EGFR tyrosine phosphorylation (Figs. 3C and D), suggesting that NADPH oxidase activity is indeed required for EGFR transactivation.

c-Src activity is required for FPRL1-induced EGFR transactivation

Several cytosolic signal transduction proteins are implicated in the EGFR transactivation process. It has been suggested that Src-family tyrosine kinases function as both upstream and downstream mediators in GPCR-induced EGFR transactivation [51]. Inhibitor studies indicate the presence of c-Src upstream of EGFR in immortalized hypothalamic neurons [52], in LPA-stimulated COS-7 cells [53], and in vascular smooth muscle cells [54]. In contrast, in other experimental systems EGFR tyrosine phosphorylation has been reported to be independent of c-Src activity [55]. We investigated the role of c-Src in FPRL1-induced EGFR transactivation and we observed that in serum-deprived CaLu-6 cells exposed to WKYMVm, preincubation with genistein, a general tyrosine kinase inhibitor; or with the tyrphostin AG1478; or with PP2, an inhibitor of c-Src tyrosine kinase, prevents EGFR tyrosine phosphorylation (Figs. 4A and B). These results suggest that c-Src plays a key role in bridging the signals from FPRL1 to EGFR in these cells.

It has been shown that in U87 and FPRL1/CHO cells the stimulation of FPRL1 by WKYMVm increases c-Src kinase activity [56]. Therefore, we evaluated the ability of the FPRL1 agonist to activate c-Src in CaLu-6 cells in an in vitro kinase assay using enolase and [^{32}P]ATP as substrates. As shown in Fig. 5A, c-Src kinase activity was increased in cells exposed to WKYMVm for 2 min (lane 2) and was completely prevented by preincubation with PTX (lane 3).

c-Src activity is regulated by phosphorylation at two distinct tyrosine residues. Autophosphorylation of the Tyr416 residue in the kinase domain activates c-Src, whereas phosphorylation of the Tyr527 residue in the C-terminal tail by the C-terminal Src kinase blocks c-Src activity. We analyzed c-SrcTyr416 phosphorylation levels in growth-arrested CaLu-6 cells stimulated for various times with WKYMVm by using a phospho-specific antibody directed toward the phosphorylated Y416 residue of c-Src. Western blotting analysis showed that the c-SrcTyr416 phosphorylation level was regulated in a time-dependent manner, with maximal phosphorylation occurring at 2 min (Fig. 5B).

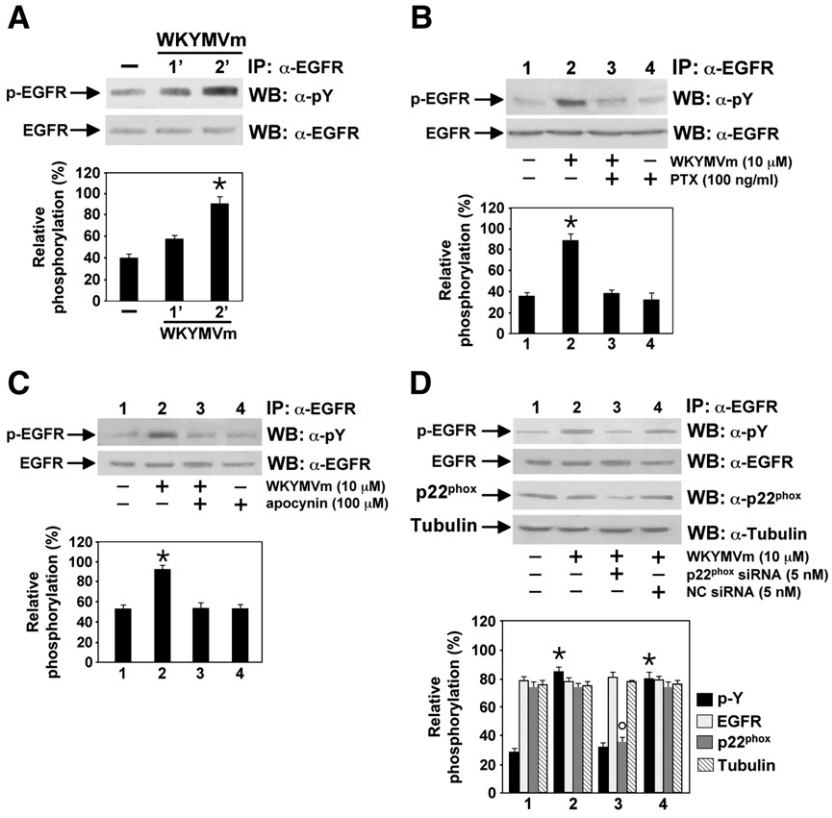

Fig. 3. FPRL1-induced EGFR transactivation depends on NADPH oxidase activation. (A) Growth-arrested CaLu-6 cells were incubated with 10 μM WKYMVm for the indicated times or preincubated with (B) 100 ng/ml PTX or (C) 100 μM apocynin before stimulation. (D) Serum-deprived cells were incubated for 12 h with 5 nM siRNA against $p22^{phox}$ ($p22^{phox}$ siRNA) or a negative control siRNA (NC siRNA) in DMEM containing 10% FBS in the presence of 20 ml HiPerFect. Cells were then serum-deprived for 24 h before stimulation with 10 μM WKYMVm for 2 min. Cell lysates containing 800 μg of proteins were incubated with 3 μg of anti-EGFR (α-EGFR) and immunocomplexes were mixed with 30 μl of Protein A/G Plus agarose. The immunoprecipitates were loaded on 10% SDS–PAGE gels and EGFR phosphorylation (p-EGFR) was detected with an anti-phospho-tyrosine antibody (α-p-Y). An α-EGFR and an α-tubulin antibody served as controls for protein loading. An α-$p22^{phox}$ antibody served as a control for interference. All the blots are representative of at least three separate experiments of identical design. Protein expression levels were quantitatively estimated by densitometry. °$p<0.05$ and *$p<0.05$ compared with unstimulated cells.

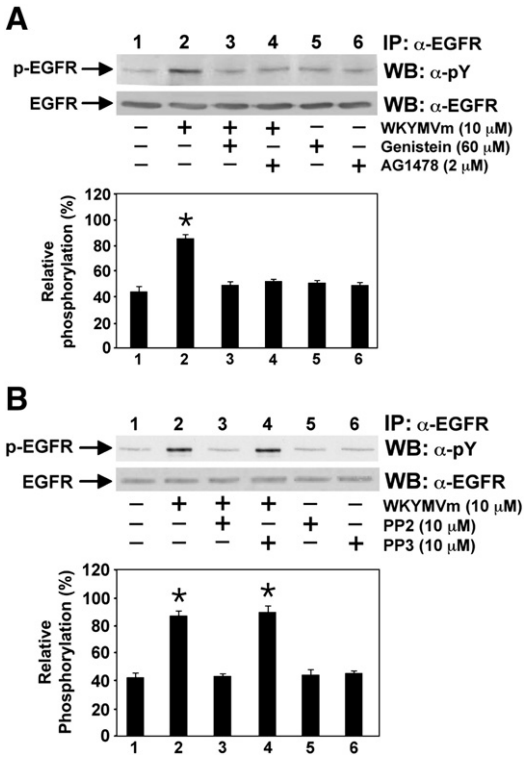

Fig. 4. General and specific tyrosine kinase inhibitors prevent FPRL1-induced EGFR phosphorylation. (A) Serum-deprived CaLu-6 cells were preincubated with genistein or AG1478 or (B) PP2 or PP3 at the indicated concentrations, before stimulation with 10 μM WKYMVm. Cell lysates (800 mg) were purified and EGFR was immunoprecipitated with an anti-EGFR antibody (α-EGFR). Proteins were resolved on 10% SDS–PAGE and EGFR phosphorylation (p-EGFR) was detected with an anti-phospho-tyrosine antibody (α-p-Y). An α-EGFR antibody served as a control for protein loading. The experiments were performed in triplicate. Phosphorylated protein levels were measured by densitometry. $^{*}p<0.05$ compared with unstimulated cells.

We also pretreated cells with PTX before WKYMVm stimulation and we found that the G_i-protein-specific inhibition significantly prevents c-SrcTyr416 phosphorylation (Fig. 5C).

c-Src is sensitive to intracellular redox conditions as demonstrated by the ROS-dependent inactivation of the PTPases that control its phosphorylation status [57]. Therefore, we investigated the role of NADPH-oxidase-dependent ROS generation in FPRL1-induced c-Src activation. We observed that pretreatment with the NADPH oxidase inhibitor apocynin or preincubation with a siRNA against $p22^{phox}$, before stimulation with WKYMVm for 2 min, prevents c-SrcTyr416 phosphorylation (Figs. 5D and E), suggesting that FPRL1-induced c-Src kinase activity requires NADPH oxidase activation.

WRW4 prevents the FPRL1-induced downstream signal transduction cascade

The peptide WRW4 antagonizes the binding of the specific FPRL1 ligand WKYMVm, thereby inhibiting intracellular calcium increase, FPRL1-induced neutrophil activation [58], and ERK phosphorylation [10]. We exposed CaLu-6 cells to WRW4 to further investigate the role of FPRL1 in the downstream signaling cascade and we observed, in dose–response experiments, that preincubation with WRW4, before WKYMVm stimulation, prevents ERK activation (Fig. 6A) and c-SrcTyr416 residue phosphorylation (Fig. 6B), with the maximal effect occurring at a concentration of 10 μM. Preincubation with 10 μM WRW4 also inhibits FPRL1-dependent EGFR transactivation (Fig. 6C) and $p47^{phox}$ phosphorylation (Fig. 6D).

FPRL1-induced EGFR transactivation triggers STAT3 activation

STAT proteins are activated as a consequence of ligand binding to cytokine and growth factor receptors such as EGFR and PDGF-R. STAT 2, 4, and 6 are activated by a small subset of cytokines, whereas STAT 1, 3, 5a, and 5b are activated also by growth factors [59]. Binding of EGF to its cognate receptor results in EGFR dimerization and phosphorylation and in the activation of receptor-associated Janus kinases (JAK). Recruitment, phosphorylation of STAT3 on the Tyr705 residue, and dimerization of STAT3 represent the trigger of the JAK/STAT3 cascade. Activated STAT3 is then translocated to the nucleus to activate target genes. Full transcriptional activity and DNA binding capacity are manifested only when the STAT3 Ser727 residue is also phosphorylated. In addition to cytokine and growth factor receptors, a number of GPCR agonists also activate STAT3 [36–39]. In glioblastoma cells N-fMLP induces a rapid and transient phosphorylation of STAT3 at the Tyr705 and Ser727 residues by binding to FPR [45] and, in RBL-2H3 cells, the Ser727 residue of STAT3 is phosphorylated consequent to the binding of WKYMVm to FPRL1 [38]. Furthermore, as a result of transmembrane signaling, uPAR activates the JAK/STAT pathway mediated by FPRL1 [60]. Therefore, we analyzed the FPRL1-induced STAT3 activation in growth-arrested CaLu-6 cells. We observed that, in time-dependent Western blot experiments, stimulation with 10 μM WKYMm rapidly induces phosphorylation of the STAT3 Tyr705 residue (Fig. 7A), the nuclear translocation of activated STAT3 (Fig. 7B), and the phosphorylation of the STAT3 Ser727 residue (Fig. 7C). Taken together the above results indicate that activation, dimerization, and nuclear translocation of STAT3 are a part of the FPRL1-dependent signaling cascade.

We next examined the molecular mechanisms involved in FPRL1-induced STAT3 activation by pretreating CaLu-6 cells with PTX, AG1478, and genistein. These experiments show that phosphorylation of the STAT3 Tyr705 residue greatly depends on FPRL1 and EGFR activation, being prevented by PTX and by general and EGFR-specific tyrosine kinase inhibitors (Fig. 8A). Furthermore, the phosphorylation of Tyr705 of STAT3 is c-Src-dependent, because it is prevented by the preincubation of CaLu-6 cells with PP2 before WKYMVm stimulation, and it is MEK-independent, as observed by the lack of effect of the MEK inhibitor PD098059 (Fig. 8B). In addition, preincubation of serum-deprived CaLu-6 cells with PTX before agonist stimulation completely prevents STAT3 Ser727 phosphorylation (Fig. 8C). The MAPK pathway plays an important role in the regulation of STAT3 signaling and, in particular, ERK were demonstrated to phosphorylate STAT3 at the Ser727 residue [61]. In CaLu-6 cells preincubation with PD098059, before WKYMVm stimulation, completely prevents the phosphorylation of STAT3 at Ser727, whereas, as expected, the general tyrosine kinase inhibitor genistein has no effect on serine phosphorylation (Fig. 8C). Consequently, ERK activity seems to be required for FPRL1-induced STAT3 Ser727 phosphorylation.

FPRL1-induced signaling cascade promotes cell proliferation

We evaluated the cellular consequences of FPRL1-induced signaling by analyzing the effects of exposure to WKYMVm on CaLu-6 cell growth. We observed that the addition of 10 μM WKMVm to the cells results in a time-dependent growth response, with the maximum level of CaLu-6 growth occurring after 24 h of exposure (Fig. 9A). Furthermore, inhibition of G_i proteins by PTX or preincubation with the FPRL1 antagonist WRW4 significantly prevents cell proliferation (Fig. 9B). This indicates that the FPRL1-dependent intracellular signaling cascades triggered by WKYMVm result in an increased growth rate of the human lung cancer CaLu-6 cell line, which can be prevented by blockade of FPRL1, highlighting the role of this receptor as a potential target of new drugs.

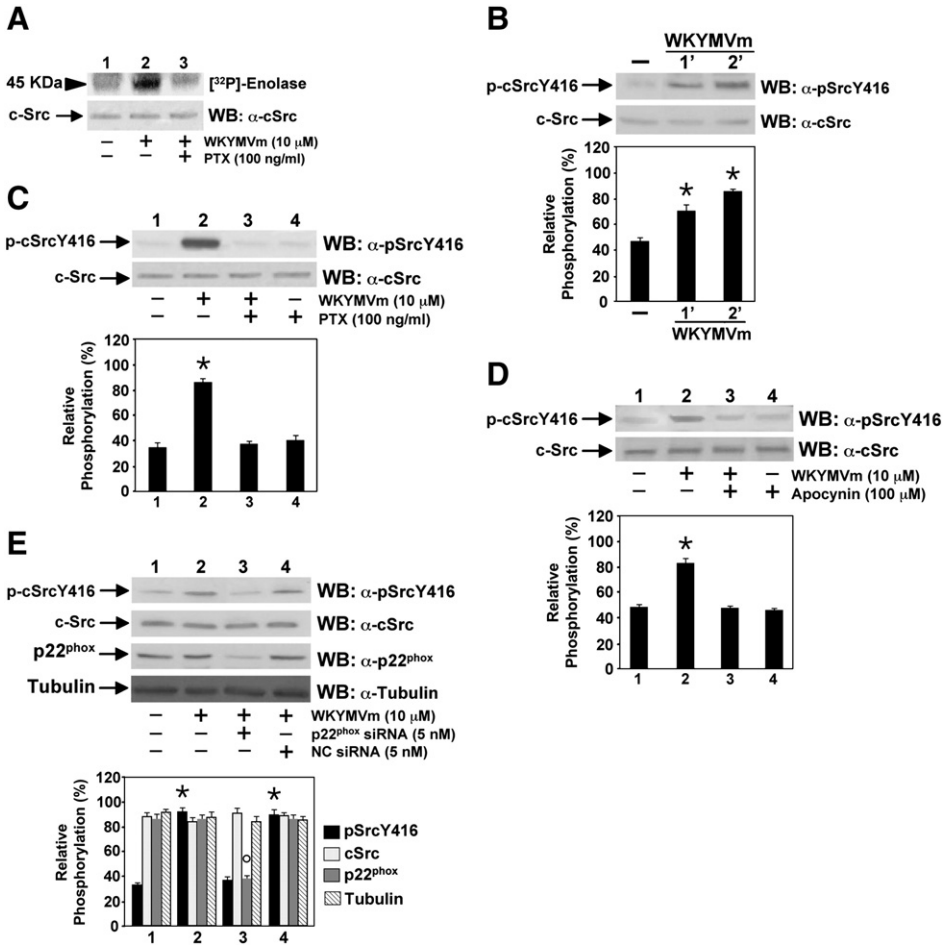

Fig. 5. FPRL1-dependent c-Src activation depends on NADPH oxidase activity. (A) The cellular activity of c-Src was detected by in vitro kinase assay. CaLu-6 cells were exposed to 10 μM WKYMVm in the presence or absence of 100 ng/ml PTX. Cell lysates were immunoprecipitated with an anti-c-Src antibody and cellular c-Src kinase activity was directly measured on protein A/G agarose beads carrying a c-Src immunocomplex in the presence of the in vitro substrate enolase and [γ-^{32}P]ATP. Samples were resolved by 10% SDS–PAGE and autoradiography. (B) Cells were exposed to 10 μM WKYMVm for the indicated times or preincubated with (C) 100 ng/ml PTX or (D) 100 μM apocynin before stimulation. Fifty micrograms of protein was loaded on 10% SDS–PAGE and c-Src Y416 phosphorylation (p-c-SrcY416) was detected with an anti-phospho-Src Y416 antibody (α-p-SrcY416). An α-c-Src antibody served as a control for protein loading. (E) Serum-deprived cells were incubated for 12 h with 5 nM siRNA against p22^{phox} (p22^{phox} siRNA) or a negative control siRNA (NC siRNA) in the presence of 20 ml HiPerFect. Cells were serum-deprived for 24 h and then exposed to 10 μM WKYMVm for 2 min. Cell lysates containing 50 μg of proteins were loaded on 10% SDS–PAGE gels and c-Src Y416 phosphorylation was detected with an α-p-SrcY416 antibody. An α-c-Src and an α-tubulin antibody served as controls for protein loading. An anti-p22^{phox} antibody (α-p22^{phox}) served as a control for interference. All the blots are representative of at least three separate experiments of identical design. Protein expression levels were quantitatively estimated by densitometry. °$p<0.05$ and *$p<0.05$ compared with unstimulated cells.

Discussion

We demonstrate that in serum-deprived CaLu-6 cells, stimulation of FPRL1 by a specific agonist results in NADPH-oxidase-dependent superoxide generation and EGFR transactivation. Furthermore, we show that ROS play a key role in bridging the signals from FPRL1 to EGFR by modulating c-Src kinase activity, as demonstrated by the effects of PP2, apocynin, and a siRNA against p22^{phox} on c-Src Y416 phosphorylation and EGFR transactivation. Our results also indicate that, as a consequence of the transphosphorylation process the phosphotyrosine residues of EGFR provide docking sites for recruitment and triggering of the STAT3 pathway. Finally, the FPRL1-induced signaling promotes an increased growth rate of CaLu-6 cells.

EGFR is a cell-surface receptor and is a member of the c-erb-B family of tyrosine kinases, known to be overexpressed in a variety of human malignant tumors and cells, including lung carcinoma and the CaLu-6 cell line [40]. In addition to activation by EGF, its cognate ligand, several GPCR can also transactivate EGFR and the cross talk between the two receptors is a crucial signaling mechanism that serves to expand the cellular communication network. In fact, the receptors for angiotensin [62], LPA [63], CXCL12 [64], bombesin [65], thrombin [66], and endothelin-1 [67] also transactivate EGFR, thereby assisting the transmission of growth signals. In glioblastoma cells EGFR transactivation is mediated by the binding of N-fMLP to FPR [44] and inhibition of EGFR phosphorylation significantly reduces FPR agonist-induced tumor cell chemotaxis and proliferation [44,68]. Thus, FPR expressed in glioblastoma cells can exploit the EGFR capacity to amplify tumor growth. In line with these results we show that, as a consequence of FPRL1-induced signaling, WKYMVm promotes an increased growth rate of CaLu-6 cells, which is prevented by blockade of FPRL1.

Much evidence suggests that GPCR-induced EGFR transactivation may involve the EGFR ligand-dependent pathway through the metalloprotease-dependent release of EGF-like ligands [69], or the EGFR ligand-independent pathway, which can involve nonreceptor tyrosine kinases such as c-Src [70,71]. In glioblastoma cells N-fMLP-induced EGFR phosphorylation requires the presence of FPR and of G_i proteins associated with the receptor and is controlled by c-Src tyrosine kinase [44]. Furthermore, stimulation of FPRL1 by WKYMVm in both U87 and FPRL1/CHO cells increases c-Src kinase activity [56]. In line with these results our study shows that FPRL1-induced signaling induces an increase in c-Src kinase activity and that c-Src plays a key role in the control of EGFR transactivation.

Another molecular mechanism that can contribute to RTK transactivation by GPCR ligands is the generation of ROS, which in

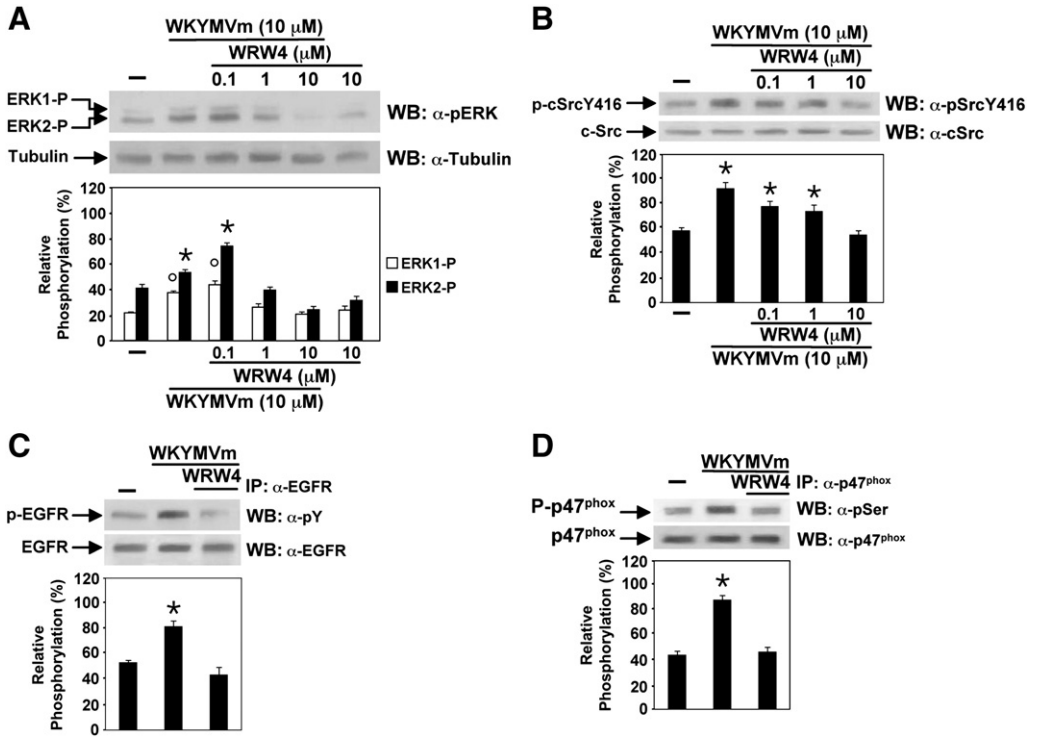

Fig. 6. WRW4 prevents the FPRL1-induced signal transduction cascade. Cell lysates were obtained from serum-deprived CaLu-6 cells exposed to 10 μM WKYMVm for 2 min in the presence or absence of increasing concentrations of WRWWWW (WRW4), as indicated. (A) Twenty micrograms of protein was resolved by 10% SDS–PAGE and subjected to immunoblotting analysis with an anti-phospho-ERK antibody (α-p-ERK). An anti-tubulin antibody (α-tubulin) served as a control for protein loading. The arrows indicate the phosphorylated forms, ERK1-P and ERK2-P, of $p44^{MAPK}$ and $p42^{MAPK}$, respectively. (B) Fifty micrograms of protein was subjected to 10% SDS–PAGE and subsequent immunoblotting by using an anti-phospho-Src Y416 antibody (α-p-SrcY416). The same filter was reprobed with an anti-c-Src antibody (α-c-Src). (C) Growth-arrested CaLu-6 cells were exposed to 10 μM WKYMVm for 2 min or preincubated with 10 μM WRW4 for 15 min before stimulation. Proteins (800 μg) were immunoprecipitated with an anti-EGFR antibody (α-EGFR) and resolved on 10% SDS–PAGE. Tyrosine phosphorylation of EGFR was detected with an anti-phospho-tyrosine (α-p-Y) antibody. An α-EGFR antibody served as a control for protein loading. (D) Cell lysates were purified from CaLu-6 cells stimulated with 10 μM WKYMVm for 2 min or preincubated with 10 μM WRW4 for 15 min before stimulation. One milligram of protein was immunoprecipitated with an anti-$p47^{phox}$ antibody (α-$p47^{phox}$) and resolved on 10% SDS–PAGE. $p47^{phox}$ phosphorylation (P-$p47^{phox}$) was detected using an anti-phospho-serine antibody (α-p-Ser). An α-$p47^{phox}$ antibody served as a control for protein loading. All the blots are representative of three separate experiments. Phosphorylated protein levels were quantitatively estimated by densitometry. $^{\circ}p<0.05$ and $^{*}p<0.05$ compared with unstimulated cells.

turn inactivate PTPases that tightly control the activity of RTK [49,50,72–75]. 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 nonphagocytic 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 of kinases and inhibition of PTPases [16]. 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 [76]. A major attribute of nonphagocytic 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 [77].

ROS also influence c-Src at several levels, both directly by modulating Src kinase activity and indirectly by modulating factors that regulate c-Src kinase activity [78]. We show that FPRL1-induced signaling triggers NADPH-oxidase-dependent superoxide generation, which plays a crucial role in EGFR transactivation by modulating c-Src tyrosine kinase activity, although a concomitant ligand-dependent transactivation of EGFR cannot be ruled out. On the other hand, several studies show that c-Src kinase activity influences NADPH-oxidase-dependent ROS generation at several levels by facilitating activation of the NADPH oxidase cofactors required to form the complex [78,79].

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 RTK such as EGFR, cytoplasmic c-Src tyrosine kinases, and components of the JAK family. Specific formyl peptide receptor agonists also activate STAT3 [38,45]. We found that in CaLu-6 cells exposure to WKYMVm induces FPRL1-dependent phosphorylation of STAT3 at the Y705 and S727 residues, suggesting that STAT3 activation is also a part of the FPRL1-dependent signaling cascade. We also observed MEK-dependent ERK phosphorylation in WKYMVm-stimulated CaLu-6 cells, which is consistent with our previous results obtained in IMR90 human fibroblasts [7]. It has been 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 [61]. We demonstrate that S727 phosphorylation of STAT3 is prevented by the MEK1 inhibitor PD098059, suggesting that ERK phosphorylation is crucial for WKYMVm-induced STAT3 activation in CaLu-6 cells.

FPRL1 was initially known as a low-affinity receptor for N-fMLP. In the past few years several ligands have been identified, making FPRL1 the most promiscuous in the FPR family with respect to agonist selectivity. Interestingly, most of the newly identified agonists for FPRL1 do not share substantial sequence homology; thus, FPRL1 behaves as a “pattern recognition” receptor that can be activated by a wide variety of unrelated ligands.

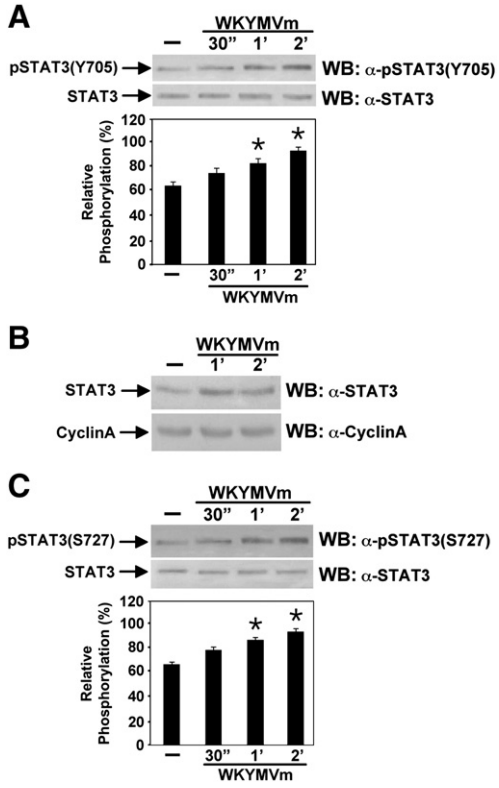

Fig. 7. FPRL1-induced EGFR transactivation triggers the STAT3 pathway. Total and nuclear cell lysates were purified from serum-starved CaLu-6 cells exposed to 10 μM WKYMVm for various times, as indicated. (A) Fifty micrograms of total protein was resolved by 10% SDS–PAGE and specific phosphorylations of STAT3 were detected with anti-phospho-STAT3 Y705 (α-p-STAT3(Y705)) or (C) anti-phospho-STAT3 S727 (α-p-STAT3(S727)) antibodies. An anti-STAT3 antibody (α-STAT3) served as a control for protein loading. (B) A 10% SDS–PAGE gel was loaded with 50 μg of nuclear extracts and STAT3 nuclear translocation was determined by Western blot with an α-STAT3 antibody. The same filter was reprobed with an anti-cyclin A antibody (α-CyclinA). All the experiments were performed in triplicate. Phosphorylated protein levels of STAT3 Y705 and STAT3 S727 were quantitatively estimated by densitometry. $^{*}p<0.05$ compared with unstimulated cells.

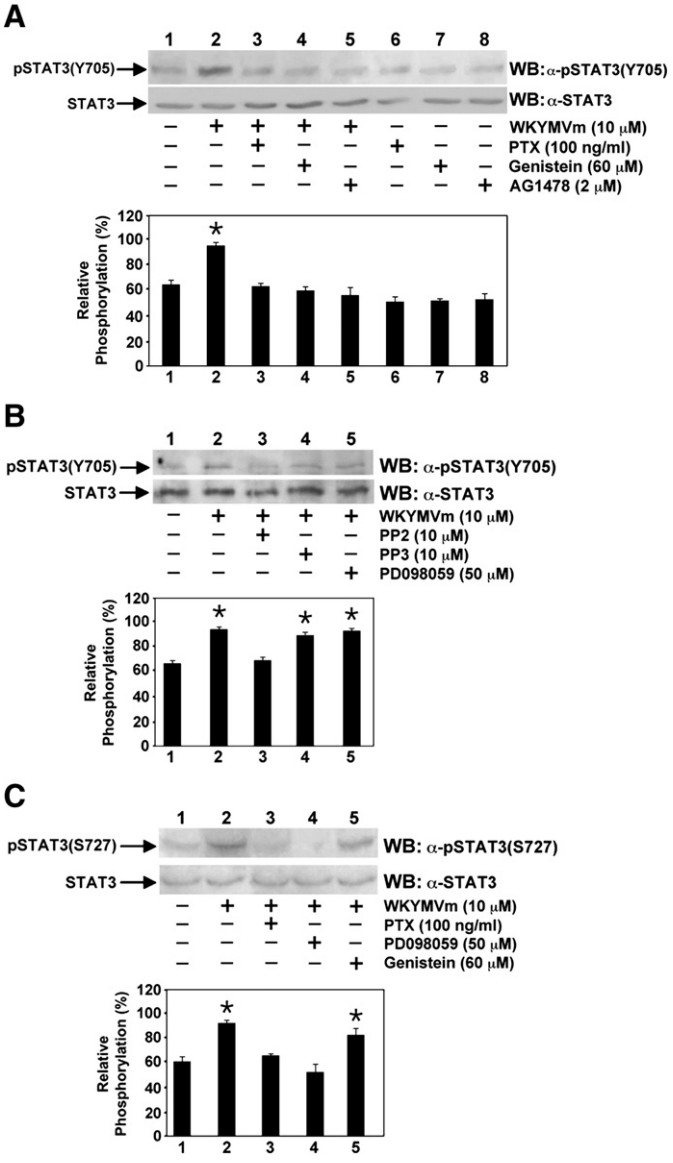

Fig. 8. FPRL1-induced STAT3 signaling requires activation of EGFR, c-Src, and MEK1. Serum-deprived CaLu-6 cells were exposed to 10 μM WKYMVm for 2 min or preincubated with the appropriate concentrations of inhibitors before stimulation, as described under Materials and methods. Total proteins (50 mg) were electrophoresed and transferred to Immobilon P membranes. (A) Cells were preincubated for 16 h with PTX, or for 1 h with genistein, or for 1 h with AG1478 at the indicated concentrations, before stimulation. The blot was incubated with a primary antibody against STAT3 phosphorylated at the Tyr705 residue (α-p-STAT3(Y705)). (B) Growth-arrested CaLu-6 cells were pretreated for 45 min with PP2, or for 45 min with PP3, or for 90 min with PD098059 at the indicated concentrations, before exposure to WKYMVm. An α-p-STAT3(Y705) antibody was used to detect STAT3 Y705 phosphorylation. (C) Human lung cancer cells were preincubated with PTX, PD098059, or genistein at the indicated concentrations, before stimulation with the FPRL1 agonist. The blot was incubated with an α-p-STAT3(S727) antibody to detect the phosphorylated form of STAT3 Ser727. Total STAT3 was detected with an antibody against nonphosphorylated STAT3 (α-STAT3). All the blots are representative of at least three separate experiments of identical design. Phosphorylated protein levels were quantitatively measured by densitometry. $^{*}p<0.05$ compared with unstimulated cells.

There have been ongoing efforts in several laboratories to study the ligand and FPRL1 interaction, in part because of the potential for FPRL1 to become a therapeutic target.

It has been shown that both agonists and antagonists for FPRL1 have therapeutic value. In fact, WKYMVm increases neutrophil bactericidal activity in chemotherapy-treated cancer patients [80] and enhances endogenous expression of TRAIL, a novel potential anticancer agent, in human monocytes and neutrophils [81]. 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 [82]. WKYMVm, activating FPRL1, 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 [83].

The use of FPRL1 by SAA, Ab42, 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 are important for delineating the signal transduction cascade associated with receptor activation and as a basis for developing anti-inflammatory therapeutic agents. Several antagonists for FPRL1 have been identified. These include the chemotaxis inhibitory protein of *Staphylococcus aureus,* the FPRL1 inhibitory protein FLIpr, the bile acids deoxycholic and chenodeoxycholic, and Quin-c7, a synthetic nonpeptide developed through chemical modification of QuinC-7 [84–86]. Moreover, W-rich peptides, such as WRW4, exert an antagonistic effect on WKYMVm-induced FPRL1 signaling, suggesting their use for the treatment of several diseases in which FPRL1 is known to play a role [58].

It should be also noted that because FPR is overexpressed in human glioblastoma cells [87], at least some members of the FPR family might

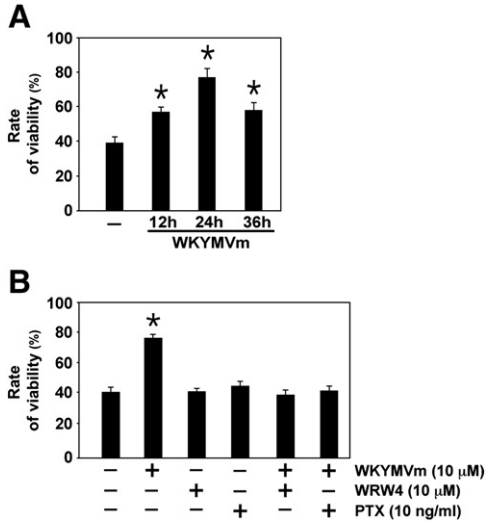

Fig. 9. FPRL1-induced signaling promotes cell growth in CaLu-6 cells. (A) CaLu-6 cells were plated at 4×10^4 cells per well in 200 μl of culture medium containing 10 μM WKYMVm in 96-well plates and incubated at 37 °C for 12, 24, and 36 h. (B) Cells were incubated for 24 h with the FPRL1 agonist in the presence or absence of PTX or WRW4 at the indicated concentrations. MTT (5 mg/ml in PBS) was added to each well and incubated for 4 h. After removal of the medium, 200 μl of DMSO was added to each well. The absorbance of the resulting formazan salts was recorded on a microplate reader at the wavelength of 540 nm. The effect of WKYMVm on cell growth was assessed as percentage of cell viability. Four independent experiments were performed. $^*p<0.05$ compared with unstimulated cells.

also be valuable biomarkers for cancer diagnosis, therefore increasing the number of available biomarkers for cancer diagnosis and staging.

The transactivation of EGFR by FPRL1 in human lung carcinoma cells may have important pathophysiological implications. The expression of FPRL1 in CaLu-6 cells could render these cells responsive not only to WKYMVm, but also to agonists contained in the environment of necrotic tumor cells. As a result of these interactions, FPRL1 could activate intracellular signaling molecules such as ROS, ERK, and c-Src that, in turn, could trigger EGFR 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 FPRL1 signaling pathway and that targeting both FPRL1 and EGFR may yield superior therapeutic effects compared with targeting either receptor separately.

Acknowledgments

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