A novel role for farnesyl pyrophosphate synthase in fibroblast growth factor-mediated signal transduction

John F. REILLY[1], Shawndra D. MARTINEZ, Gregory MICKEY and Pamela A. MAHER[2]
Department of Cell Biology, The Scripps Research Institute, 10550 N. Torrey Pines Road, La Jolla, CA 92037, U.S.A.

Farnesyl pyrophosphate synthase (FPPS) catalyses the formation of a key cellular intermediate in isoprenoid metabolic pathways. Here we describe a novel role for this enzyme in fibroblast growth factor (FGF)-mediated signalling. We demonstrate the binding of FPPS to FGF receptors (FGFRs) using the yeast two-hybrid assay, pull-down assays and co-immunoprecipitation. The interaction between FPPS and FGFR is regulated by the cellular metabolic state and by treatment with FGF-2. Overexpression of FPPS inhibits FGF-2-induced cell proliferation, accompanied by a failure of the FGF-2-mediated induction of cyclins D1 and E. Overexpression of FPPS in fibroblasts also promotes increased farnesylation of Ras, and temporally extends FGF-2-stimulated activation of the Ras/ERK (extracellular-signal-regulated kinase) cascade. These data suggest that, in addition to its role in isoprenoid biosynthesis, FPPS may function as a modulator of the cellular response to FGF treatment.

Key words: cell proliferation, cyclin, ERK, farnesylation, Ras.

INTRODUCTION

The fibroblast growth factor (FGF) family comprises a rapidly expanding group of polypeptides with mitogenic and morphogenic effects on a wide variety of cell types [1]. The action of FGFs on cells is mediated by high-affinity cell surface FGF receptors (FGFRs). To date, four FGFR genes have been cloned, each coding for a transmembrane protein with a cytoplasmic tyrosine kinase domain [2]. The diversity of the receptor family is increased by alternative splicing, which has been demonstrated for all four FGFRs [2,3]. The binding of FGFs to cells results in receptor dimerization, which leads to activation of the cytoplasmic kinase domain. Following these events, intracellular proteins are recruited to the receptor, and carry out the downstream signal transduction events.

A limited number of cytosolic proteins that bind to FGFRs have been identified. These include phospholipase Cγ, FGF receptor substrate 2 (FRS2), Shc and Grb14 [4–7]. Activation of FGFR and the subsequent signal transduction events are involved in the regulation of a wide variety of cellular processes, and it is likely that other cytoplasmic proteins participate in these responses. With more than 20 FGFs identified to date, and four receptors with multiple molecular forms, FGF-mediated signal transduction represents a complex set of events. The present study was undertaken to identify additional components of the FGFR signal transduction pathway.

The cytoplasmic domain of FGFR1 (CD-R1) was used as bait in a two-hybrid screen of a mouse fibroblast cDNA library. One of the proteins identified in this screen was farnesyl pyrophosphate synthase (FPPS; EC 2.5.1.10; also referred to as farnesyl diphosphate synthase). FPPS catalyses the sequential condensation of dimethylallyl pyrophosphate with two molecules of isopentenyl pyrophosphate to form farnesyl pyrophosphate (FPP), a key cellular intermediate for the biosynthesis of sterol and non-sterol isoprenoids [8]. FPP is a biosynthetic precursor of cholesterol and steroid hormones, dolichols, haem A and ubiquinone. In addition, the 15-carbon FPP and its 20-carbon derivative geranylgeranyl pyrophosphate are involved in prenylation, a post-translational modification found on a variety of cellular proteins that is required for their proper cellular localization and biological function [9]. Proteins modified by farnesylation include Ras GTPases, nuclear lamins A and B, the γ subunit of transducin, and rhodopsin kinase. Geranylgeranylated proteins are much more common than farnesylated proteins [10]; these include the γ subunit of most heterotrimeric G-proteins and members of the Rab, Rac, Rap and Rho subfamilies of small GTPases. Prenylation is a stable modification that confers membrane localization and promotes specific protein–protein interactions, and thus plays a critical role in intracellular trafficking and signal transduction pathways. The present study characterizes the binding of FPPS to FGFR, and describes the role of this interaction in FGF-2-mediated signal transduction.

EXPERIMENTAL

Yeast two-hybrid screen and cloning of mouse FPPS

Experiments used the Matchmaker GAL4 yeast two-hybrid system and an NIH 3T3 cell cDNA library (Clontech, Palo Alto, CA, U.S.A.), and were carried out as previously described [7]. Several positive clones from the yeast two-hybrid screen coded for an in-frame murine homologue of FPPS that included a short 5′ and the entire 3′ untranslated regions. These sequence data

Abbreviations used: CD-R1, cytoplasmic domain of FGFR1; CKI, cyclin-dependent kinase inhibitor; DMEM, Dulbecco's modified Eagle's medium; ECL, enhanced chemiluminescence; EGF, epidermal growth factor; ERK, extracellular-signal-regulated kinase; FGF, fibroblast growth factor; FGFR, fibroblast growth factor receptor; FPP, farnesyl pyrophosphate; FPPS, farnesyl pyrophosphate synthase; FRS2, FGF receptor substrate 2; FTase, farnesyl transferase; GAP, GTPase-activating protein; GST, glutathione S-transferase; MAP kinase, mitogen-activated protein kinase; MEK, MAP kinase/ERK kinase; PDGF, platelet-derived growth factor; RBD, Ras-binding domain.

[1] Present address: Neurome, Inc., 11149 N. Torrey Pines Road, La Jolla, CA 92037, U.S.A.
[2] To whom correspondence should be addressed (e-mail pmaher@scripps.edu).

Nucleotide sequence data have been submitted to the GenBank®, DDBJ, EMBL and GSDB Nucleotide Sequence Databases under accession number AF309598.

have been submitted to the GenBank database (accession no. AF309598). The coding region was amplified by PCR from the library and subcloned into pACT2 for use in the yeast two-hybrid assay, or into pcDNA3.1/HisA for transfection of mammalian cells. All constructs were verified by DNA sequencing.

Cell culture, transfection and preparation of lysates

NIH 3T3 cells were maintained in Dulbecco's modified Eagle's medium (DMEM; Gibco, Gaithersburg, MD, U.S.A.) with 10% (v/v) calf serum (Hyclone, Ogden, UT, U.S.A.). Prior to treatment, confluent cells were starved for 48 h in DMEM with 0.5% (v/v) serum. Cells were treated with the specified concentrations of recombinant human FGF-2 or 10% (v/v) serum for the indicated times. 293T cells were maintained in DMEM with 10% (v/v) fetal bovine serum (Hyclone). PC12 cells were maintained in DMEM with 10% (v/v) horse serum and 5% (v/v) fetal calf serum. Cells were treated with FGF-2 in N2 medium (Gibco) as described previously [11].

For transient transfections, NIH 3T3 cells were seeded at 4×10^5 cells/cm^2 and grown for 18 h in DMEM with 10% (v/v) serum. Cells were transfected with pcDNA3.1/HisA or pcDNA3.1/HisA/FPPS using LIPOFECTAMINE 2000™ (Gibco) according to the manufacturer's protocol. Cells were maintained in DMEM with 10% (v/v) serum for 24 h, then starved and treated with FGF-2 as described above. 293T cells were seeded at 1×10^5 cells/cm^2 and grown for 18 h in DMEM with 10% (v/v) serum. Cells were co-transfected with pcDNA3.1 constructs encoding full-length FGFR1, FGFR2, FGFR3 or FGFR4 and pcDNA3.1/HisA or pcDNA3.1/HisA/FPPS using LIPOFECTAMINE 2000™. Cells were maintained in DMEM with 10% (v/v) serum for 48 h.

For stable transfections, NIH 3T3 cells were seeded at 1.8×10^4 cells/cm^2 and grown for 18 h in DMEM with 10% (v/v) serum. Cells were changed to Opti-MEM and transfected with pcDNA3.1/HisA or pcDNA3.1/HisA/FPPS for 5 h using LIPOFECTAMINE™ according to the manufacturer's protocol. Cells were maintained in DMEM with 10% (v/v) serum for 48 h, then subcultured into DMEM with 10% (v/v) serum and 800 μg/ml G418 (Gibco). Stably transfected cells were isolated and cloned according to standard protocols, maintained under selection, and starved and treated as described above. Stable transfection of PC12 cells was carried out similarly, except that LIPOFECTAMINE 2000™ was used for the transfections and the PC12 cells were cultured in their normal growth medium. A light microscope (Inverted Microscope Diaphot-TMD; Nikon) equipped with a phase contrast condenser (Phase contrast-2 ELWD 0.3; Nikon), a 10× objective lens and a digital camera (Coolpix 990; Nikon) was used to capture images of the cells with the manual setting.

Following treatment, cells were washed twice in cold PBS, then scraped into lysis buffer containing 50 mM Hepes, pH 7.4, 150 mM NaCl, 50 mM NaF, 1.5 mM $MgCl_2$, 1 mM EGTA, 10% glycerol, 1% Triton X-100, 10 mM sodium pyrophosphate, 1 mM Na_3VO_4, 1 mM PMSF, 15 μg/ml aprotinin, 1 μg/ml pepstatin and 5 μg/ml leupeptin. Lysates were mixed at 4 °C for 30 min, then cleared by centrifugation at 16000 ***g*** for 10 min. Protein concentrations were determined using the BCA protein assay (Pierce, Rockford, IL, U.S.A.).

In vivo interaction studies

For immunoprecipitation, 200–500 μg of each cell lysate was incubated with 2 μg of a polyclonal antibody against FGFR1 (sc121), FGFR2 (sc122), FGFR3 (sc123) or FGFR4 (sc124; all from Santa Cruz Biotechnology, Santa Cruz, CA, U.S.A.) overnight at 4 °C, and complexes were collected on Protein A–Sepharose (Amersham-Pharmacia Biotech, Piscataway, NJ, U.S.A.). Complexes were washed twice in 20 mM Hepes, 150 mM NaCl, 0.1% Triton X-100 and 10% glycerol, then once in PBS. Bound proteins were eluted by boiling in SDS sample buffer, separated by SDS/PAGE, and transferred to nitrocellulose. Membranes were blotted with a monoclonal antibody against FGFR (mAb6) generated in this laboratory, a monoclonal antibody against the pcDNA3.1/HisA Xpress tag (sc7270; Santa Cruz Biotechnology), or a polyclonal antibody against rat FPPS (generously provided by P. Edwards, Department of Biological Chemistry and Medicine, UCLA, Los Angeles, CA, U.S.A.), followed by appropriate secondary antibodies. Immunoreactive bands were visualized with enhanced chemiluminescence (ECL) using SuperSignal substrate (Pierce).

In vitro binding assays

DNA coding for wild-type CD-R1 or the kinase-inactive CD-R1 K514M was subcloned into pGEX-5X-1 (Amersham-Pharmacia Biotech), and glutathione S-transferase (GST) fusion proteins were expressed in *Escherichia coli* strain BL21 and purified using glutathione–Sepharose. All constructs were verified by DNA sequencing, and fusion proteins were verified by SDS/PAGE and Western blotting with antibodies against GST (Amersham-Pharmacia Biotech) and FGFR1 (sc121).

Purified GST fusion proteins or GST alone (5 μg) were immobilized on glutathione–Sepharose and mixed overnight at 4 °C with 1.0 mg of each cell lysate. Precipitates were washed extensively in PBS, and bound proteins were eluted by boiling in SDS sample buffer, separated by SDS/PAGE and transferred to nitrocellulose. Membranes were blotted with a monoclonal antibody against the Xpress epitope or a polyclonal antibody against rat FPPS, followed by appropriate secondary antibodies. Immunoreactive bands were visualized with ECL using SuperSignal substrate.

FPPS activity assay

FPPS was assayed as previously described [12], with minor modifications. Vector and FPPS stable cell lines were grown to confluence, then washed and scraped into FPPS assay buffer containing 25 mM Hepes, pH 7.0, 5 mM KF, 2 mM $MgCl_2$, 1 mM dithiothreitol, 1% n-octyl β-D-glucoside, 1 mM PMSF, 15 μg/ml aprotinin, 1 μg/ml pepstatin and 5 μg/ml leupeptin. Lysates were cleared by centrifugation at 16000 ***g*** for 10 min. Reactions were carried out in a 150 μl final volume of assay buffer containing 18 μM geranyl pyrophosphate (Sigma), 13.3 μM [1-^{14}C]isopentenyl pyrophosphate (55 mCi/mmol; Sigma) and 10 μg of cell lysate protein. Reactions were incubated at 37 °C for 45 min, then stopped by addition of 150 μl of 2.5 M HCl in 80% (v/v) ethanol with 100 μg/ml farnesol as a carrier. Samples were incubated at 37 °C for 30 min to hydrolyse the FPP to farnesol, and then neutralized with 150 μl of 10% NaOH. Farnesol was extracted into 1 ml of n-hexane, and 200 μl of the organic phase was removed for liquid-scintillation counting. Unit activity is defined as synthesis of 1 pmol of FPP per min. Significant differences were identified using Student's *t* test.

Measurement of DNA synthesis

Vector and FPPS stable cell lines expressing low and high levels of recombinant FPPS were grown to confluence in 96-well dishes in DMEM with 10% (v/v) serum and 800 μg/ml G418, washed

in serum-free DMEM, then starved for 48 h in DMEM with 0.5 % (v/v) serum. Cells were treated with various concentrations of FGF-2 or with 10 % (v/v) serum for 24 h, followed by the addition of 0.2 μCi/well [*methyl*-^{3}H]thymidine (50 Ci/mmol; ICN) for 5 h. Additional cultures were treated with 5 ng/ml FGF-2 for various times up to 48 h, prior to the addition of [^{3}H]thymidine. Cultures were processed for scintillation counting as previously described [13].

Ras assays

Activation of Ras was measured using a previously described non-radioactive assay [14]. The Ras-binding domain (RBD) of Raf-1 fused to GST (GST–RBD) was expressed in *E. coli* strain BL21 from pGEX-RBD (kindly provided by D. Shalloway, Department of Molecular Biology and Genetics, Cornell University, New York, U.S.A.), and purified using glutathione–Sepharose. Vector and FPPS stable cell lines were scraped into lysis buffer and cleared by centrifugation. Lysates (250 μg) were combined with 10 μg of GST–RBD bound to glutathione–Sepharose and mixed overnight at 4 °C. Precipitates were washed three times in lysis buffer, and bound proteins were eluted by boiling in SDS sample buffer, separated by SDS/PAGE and transferred to nitrocellulose. Membranes were blotted with a monoclonal antibody against Ras (R02120; Transduction Labs) followed by an appropriate secondary antibody. Immunoreactive bands were visualized with ECL using SuperSignal substrate.

Farnesylation of Ras was assayed using a previously described method [15] with minor modifications. Vector and FPPS stable cell lines were scraped into a buffer containing 50 mM Hepes, pH 7.5, 150 mM NaCl, 5 mM $MgCl_2$, 1 mM $NaH_2PO_4 \cdot H_2O$, 1 % Triton X-100, 0.05 % SDS, 1 mM Na_3VO_4, 1 mM dithiothreitol, 1 mM PMSF, 15 μg/ml aprotinin, 1 μg/ml pepstatin and 5 μg/ml leupeptin, disrupted by sonication, cleared by centrifugation, and adjusted to 1 mg/ml. Equal volumes of lysate (0.5 mg of protein) and 4 % Triton X-114 were combined, vortexed, and incubated at 37 °C for 4 min and then at room temperature until the phases had separated completely. Ras was immunoprecipitated from the aqueous and detergent phases using 3 μg of monoclonal antibody Y13-259 (sc35; Santa Cruz Biotechnology). Complexes were collected on Protein A–Sepharose with 30 μg of rabbit anti-(rat IgG) (Jackson ImmunoResearch, West Grove, PA, U.S.A.), washed twice in 20 mM Hepes, 150 mM NaCl, 0.1 % Triton X-100 and 10 % glycerol, and then once in PBS. Bound proteins were eluted by boiling in SDS sample buffer, separated by SDS/PAGE and transferred to nitrocellulose, and Ras was detected as above. The percentage of Ras that was farnesylated was determined by densitometry of the blots, and significant differences were identified using Student's *t* test.

Raf-1 activity assay

Raf-1 kinase activity was measured by an immune complex kinase assay using purified GST–MEK1 [where MEK is mitogen-activated protein kinase (MAP kinase)/ERK (extracellular-signal-regulated kinase) kinase] as a substrate. MEK1 was subcloned into pGEX-5X-1 and purified from *E. coli* BL21 using glutathione–Sepharose. Vector and FPPS stable cell lines were scraped into lysis buffer and cleared by centrifugation. Lysates (250 μg) were pre-cleared with Protein A–Sepharose, and Raf-1 was immunoprecipitated using 1 μg of a polyclonal antibody (sc133; Santa Cruz Biotechnology). Complexes were collected on Protein A–Sepharose, washed twice in lysis buffer and twice in kinase assay buffer (25 mM Hepes, pH 7.5, 1 mM dithiothreitol, 1 mM EGTA, 25 mM β-glycerophosphate and 1 mM Na_3VO_4), and then resuspended in 25 μl of kinase assay buffer supplemented with 20 μM ATP, 20 mM $MgCl_2$ and 0.5 μg of GST–MEK1. Reactions were allowed to proceed for 20 min at 30 °C, and were stopped by boiling in SDS sample buffer. The products were separated by SDS/PAGE, transferred to nitrocellulose, and phosphorylated GST–MEK1 was detected using a phospho-specific antibody (New England Biolabs). Similar results were obtained using purified recombinant MEK1 (0.5 μg; Santa Cruz Biotechnology) and [γ-^{32}P]ATP (10 μCi, 4500 Ci/mmol; ICN) as substrates [16], followed by autoradiographic detection.

Analysis of FGF-induced signalling pathways and cell cycle proteins

Vector and FPPS stable cell lines were scraped into lysis buffer supplemented with SDS to a final concentration of 1 %, and disrupted by sonication. Lysates (10 μg) were separated by SDS/PAGE and transferred to nitrocellulose. Membranes were blotted with antibodies against the following: phospho-specific MEK1/2 (9421), phospho-specific ERK1/2 (9101), phospho-specific p70^{s6k} (9204), phospho-specific Akt (9271), Akt (9272) and phospho-specific p38 (9211) (all from New England Biolabs); Raf-1 (sc133), MEK2 (sc524), ERK (sc94), p70^{s6k} (sc230), p38 (sc728), cyclin E (sc481) and p21^{Cip1} (sc397) (all from Santa Cruz Biotechnology); and cyclin D1 (MS-210) and p27^{Kip1} (MS-256) (both from NeoMarkers, Fremont, CA, U.S.A.). After incubation with appropriate secondary antibodies, immunoreactive bands were visualized with ECL using SuperSignal substrate.

RESULTS

Identification of FPPS by interaction with FGFR1 in a yeast two-hybrid screen

An NIH 3T3 cell cDNA library was screened using CD-R1 as bait. The DNA sequence of one of the positive clones was 85 % and 92 % identical with those of human and rat FPPS respectively, and represents the murine homologue. The predicted amino acid sequence of murine FPPS is 84 % and 93 % identical with those of human and rat respectively, and key residues within the active site [17,18] are conserved among all three species.

The interaction between FGFR1 and FPPS was further characterized using the yeast two-hybrid assay. The CD-R1 construct is a constitutively active tyrosine kinase, as determined by anti-phosphotyrosine immunoblotting of a bacterially expressed GST fusion protein construct (results not shown). A point mutation that inactivates the kinase (K514M) did not affect binding of CD-R1 to FPPS. This indicates that kinase activity is dispensable for binding of FPPS, although the active kinase is required for the binding of other proteins [4,7].

In vivo binding of FPPS to FGFR

To confirm that the interaction between FGFR1 and FPPS detected in the yeast two-hybrid assay also occurs in mammalian cells, NIH 3T3 cells were transiently transfected with a construct encoding epitope-tagged FPPS (Figure 1A). Immunoprecipitation of native FGFR1 from these cells showed only weak co-precipitation of FPPS. However, starvation of the transfected cells in low-serum media increased the binding of FPPS to FGFR1, and treatment of the cells with FGF-2 for 24 h further enhanced the co-immunoprecipitation of FPPS with FGFR1. Short-duration treatment with FGF-2 (10–60 min) had no effect on the binding of FPPS to the receptor, and no

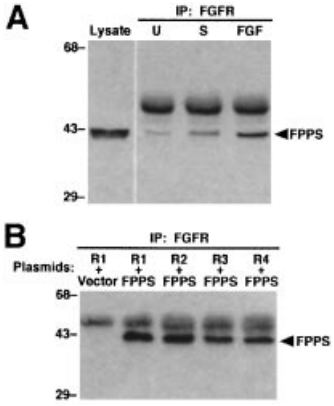

Figure 1 *In vivo* binding of FPPS to FGFR

(**A**) NIH 3T3 cells were transiently transfected with a construct expressing FPPS with an Xpress epitope tag. FGFR1 was immunoprecipitated (IP) from lysates of untreated cells (U), cells starved for 48 h in 0.5% (v/v) serum (S), or cells starved and then treated for 24 h with FGF-2 (FGF). Following washing, immune complexes were separated by SDS/PAGE, transferred to nitrocellulose, and blotted with a monoclonal antibody against the Xpress epitope tag. Similar results were obtained in three independent experiments. (**B**) 293T cells were transiently co-transfected with empty vector or with a construct encoding FPPS with an Xpress epitope tag and constructs encoding full-length FGFR1 (R1), FGFR2 (R2), FGFR3 (R3) or FGFR4 (R4). Receptors were immunoprecipitated (IP) using isoform-specific antibodies against FGFR, and analysed as above. Similar results were obtained in three independent experiments. Positions of molecular mass markers (kDa) are indicated on the left.

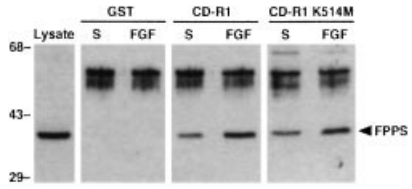

Figure 2 Receptor autophosphorylation is not required for binding of FPPS

Bacterially expressed GST–CD-R1 or GST–CD-R1 (K514M) was used to pull down endogenous FPPS from lysates of NIH 3T3 cells starved for 48 h in 0.5% serum (S) or cells starved and then treated for 24 h with FGF-2 (FGF). Lysates were incubated with GST alone or the indicated GST fusion protein immobilized on glutathione–Sepharose. Following washing, bound proteins were separated by SDS/PAGE, transferred to nitrocellulose and blotted with a polyclonal antibody against FPPS. Similar results were obtained in three independent experiments. Positions of molecular mass markers (kDa) are indicated on the left. Note that endogenous FPPS is $\sim$ 4 kDa smaller than epitope-tagged FPPS, and migrates as an $\sim$ 39 kDa band.

precipitation of FPPS was seen in control immunoprecipitations using normal rabbit IgG (results not shown). These data confirm an *in vivo* interaction between FGFR1 and FPPS in mammalian cells. Furthermore, they suggest that this interaction is regulated by the metabolic state of the cells and by FGF-2 treatment.

To examine the interaction between FPPS and FGFR2–4, we transiently co-transfected cells with constructs encoding epitope-tagged FPPS and each of the four FGFRs. Since FGFRs can heterodimerize [19], there is a possibility that an exogenously expressed receptor isoform could co-immunoprecipitate FPPS bound to native FGFR1. Thus we chose 293T cells, which express very little endogenous FGFR and respond very weakly to FGF-2 (results not shown). Immunoprecipitation of FGFR from co-transfected 293T cells demonstrated strong binding of FPPS to FGFR1 and FGFR2, and moderate binding to FGFR3 and FGFR4 (Figure 1B). An equivalent amount of each receptor isoform was immunoprecipitated, as determined by immunoblotting of complexes with an antibody that recognizes an epitope common to all four receptor isoforms (results not shown). FPPS did not co-immunoprecipitate with epidermal growth factor (EGF) receptor from co-transfected 293T cells (results not shown). These data indicate that FPPS binds specifically to all four FGFRs, and thus has the potential to affect FGF signalling in a wide variety of cell types.

The absence of an effect of short-term treatment with FGF-2 on the binding of FPPS to FGFR1 suggests that receptor autophosphorylation is not required for the *in vivo* interaction, as indicated by the experiments using the yeast two-hybrid assay. This was tested further in a mammalian system using pull-down assays with GST fusion proteins and lysates of untransfected NIH 3T3 cells (Figure 2). GST–CD-R1 bound to endogenous FPPS in lysates from starved cells, and an increase in binding was observed with 24 h of FGF-2 treatment. The kinase-inactive GST–CD-R1 K514M mutant also bound to endogenous FPPS in lysates from starved and FGF-2-treated cells in a manner similar to that observed with wild-type GST–CD-R1. Binding to GST alone was not detected. These data confirm that receptor autophosphorylation is not required for the interaction of FGFR1 with FPPS. However, in co-immunoprecipitation and GST pull-down experiments, starvation and FGF-2 treatment of cells resulted in a progressive increase in the binding of FPPS to FGFR1, suggesting that the interaction is regulated by FGFR activation, despite the fact that receptor autophosphorylation is not required for the physical interaction of the two proteins. Instead, it is possible that the interaction is regulated by an FGF-2-induced modification of FPPS, the nature of which remains to be determined.

Effects of FPPS on FGF-2-induced cell proliferation

To examine the biological role of the interaction between FGFR1 and FPPS, NIH 3T3 cells were stably transfected with a construct encoding epitope-tagged FPPS, or with the vector alone. Clones expressing low or high levels of exogenous FPPS were selected for analysis (Figure 3A). Cells expressing high levels of exogenous FPPS showed a 33% increase in FPPS enzymic activity in whole-cell lysates (Table 1), which is consistent with the increased level of FPPS expression in these cells, as determined by Western blotting. The enzyme activity of the cloned mouse FPPS was also verified using a bacterially expressed, purified maltose-binding-protein–FPPS fusion protein. Treatment of untransfected or stably transfected cells with FGF-2 had no effect on the expression of endogenous or epitope-tagged FPPS (results not shown).

Cell lines expressing FPPS showed reduced proliferation in response to FGF-2 compared with vector controls, as measured by [^{3}H]thymidine incorporation (Figure 3B). This effect was dependent upon the level of FPPS expression, with substantial inhibition of FGF-2-induced proliferation in cells expressing low levels of exogenous FPPS, and complete inhibition in cells expressing higher levels. This inhibition was not due to decreased expression or increased degradation of FGFR1 (results not shown). Treatment of all three cell lines with 10% (v/v) serum resulted in robust proliferation, indicating that the inhibition of proliferation seen in the FPPS-expressing cell lines in response to FGF-2 treatment does not represent a generalized effect on cell proliferation. To verify that the observed inhibition of proliferation was not simply the result of a delayed entry into S phase, cells were treated with FGF-2 for up to 48 h prior to the addition of [^{3}H]thymidine (Figure 3C). No major peak of incorporation was detected in the FPPS-transfected cells.

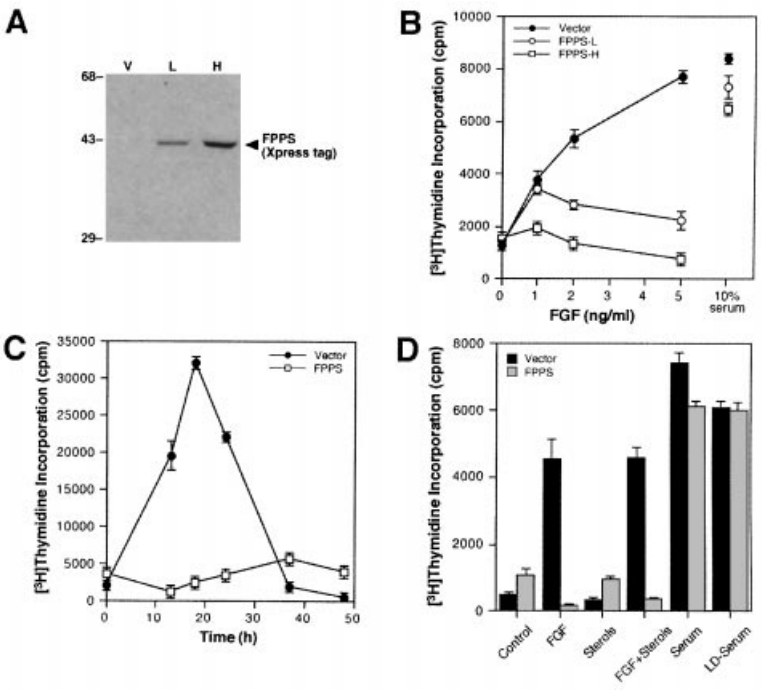

Figure 3 FPPS inhibits FGF-2-induced DNA synthesis

(**A**) Equal amounts of protein from stable NIH 3T3 vector control cells (V) or lines expressing either low levels (L) or high levels (H) of exogenous FPPS were separated by SDS/PAGE, transferred to nitrocellulose, and blotted with a monoclonal antibody against the Xpress epitope tag. Positions of molecular mass markers (kDa) are indicated on the left. (**B**) Quiescent cultures of stable NIH 3T3 vector control cells (●) or lines expressing either low levels (○) or high levels (□) of exogenous FPPS were treated for 24 h with the indicated concentrations of FGF-2 or with 10% (v/v) serum. Cells were then labelled for 5 h with [^{3}H]thymidine and analysed by liquid-scintillation counting. Values are expressed as incorporated radioactivity (c.p.m.) and represent means ± S.E.M. of quadruplicate measurements. Similar results were obtained in three independent experiments. (**C**) Cultures of vector control cells or cells expressing high levels of FPPS were treated with FGF-2 (5 ng/ml) for the indicated times then analysed as in (**B**). Symbols are as in (**B**). Values are expressed as incorporated radioactivity (c.p.m.) and represent means ± S.E.M. of quadruplicate measurements. Similar results were obtained in three independent experiments. (**D**) Cultures of vector control cells (solid bars) or cells expressing high levels of FPPS (shaded bars) were treated with FGF-2 (5 ng/ml), sterols (10 μg/ml cholesterol and 1 μg/ml 25-hydroxycholesterol), FGF plus sterols, 10% serum or 10% lipoprotein-depleted (LD) serum, and then analysed as in (**B**). Values are expressed as incorporated radioactivity (c.p.m.) and represent means ± S.E.M. of quadruplicate measurements. Similar results were obtained in two independent experiments.

Table 1 FPPS activity in transfected cells

NIH 3T3 cells were stably transfected with the indicated construct. MBP, maltose-binding protein. Unit activity is defined as synthesis of 1 pmol of FPP per min. Results are presented as means ± S.E.M. of four independent experiments. *Significance of difference: $P < 0.01$ compared with vector.

	Activity (units/mg of protein)	
Cells	Vector	FPPS
E. coli (MBP fusion)	6.0 ± 8.5	215.1 ± 1.6
NIH 3T3	85.3 ± 11.3	113.5 ± 12.0*

Since cholesterol is required for cell proliferation [20], one possible explanation for the inhibitory effects of FPPS on FGF-2-induced proliferation is perturbation of cholesterol metabolism in the transfected cells. However, treatment of FPPS-transfected cells with sterols (cholesterol and 25-hydroxycholesterol) alone or in combination with FGF-2 failed to induce proliferation (Figure 3D). In addition, the serum-induced proliferation of the FPPS-transfected cells was not simply due to the presence of cholesterol in the serum, since cells treated with lipoprotein-depleted serum showed a strong proliferative response (Figure 3D). These data indicate that the effects of FPPS overexpression on FGF-2-induced cell proliferation are not secondary to effects on cholesterol metabolism.

Effects of FPPS on FGF-2-mediated signal transduction pathways

The specific inhibition of FGF-2-induced proliferation in cells transfected with FPPS suggests an effect on FGF-2-mediated signal transduction. Activation of the FGFR induces a variety of intracellular signalling pathways, including the Ras/ERK cascade [21,22], the phosphatidylinositol 3′-kinase/Akt pathway [23,24], and the p38 MAP kinase pathway [11]. The FGF-2-induced activation of ERK involves the Shc- or FRS2-mediated coupling of FGFR to the Grb2–Sos complex [5,25], which stimulates the exchange of GTP for GDP on Ras, resulting in active, GTP-bound Ras. Since farnesylation of Ras is required for its membrane localization and subsequent activation by nucleotide exchange, the Ras/ERK cascade represents a potential target of the interaction between FPPS and FGFR1.

To investigate this possibility, we examined FGF-2-induced Ras activation in stable NIH 3T3 vector control cells and in the cell line expressing high levels of exogenous FPPS. Activation of Ras was determined by its binding to the RBD of Raf-1, which interacts with Ras only in the active, GTP-bound form [14]. In the vector-transfected cells, Ras was strongly activated following 1 h of FGF-2 treatment, and GTP-bound Ras decreased to control levels by 12 h (Figure 4A). However, in FPPS-transfected cells, FGF-induced activation of Ras was prolonged to at least

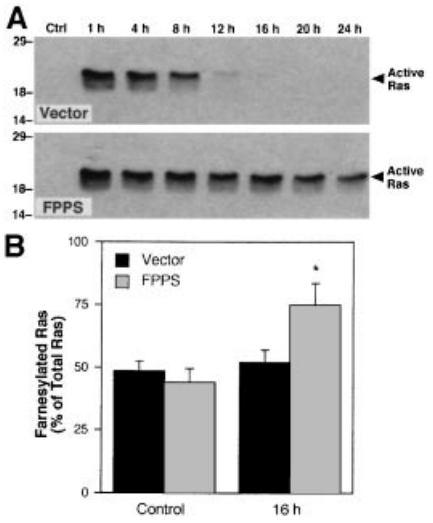

Figure 4 FPPS prolongs FGF-2-stimulated Ras activation

(**A**) GST–RBD was used to pull down active, GTP-bound Ras from lysates of stable NIH 3T3 vector control cells or cells expressing high levels of exogenous FPPS treated for the indicated times with FGF-2 (5 ng/ml). Following washing, bound proteins were separated by SDS/PAGE, transferred to nitrocellulose and blotted with a monoclonal antibody against Ras. Similar results were obtained in three independent experiments. Positions of molecular mass markers (kDa) are indicated on the left. (**B**) Stable NIH 3T3 cells (vector and FPPS-expressing) were treated for 16 h with FGF-2 (5 ng/ml), then farnesylated and unfarnesylated Ras from the lysates were separated by partitioning into Triton X-114. Ras was immunoprecipitated from the detergent and aqueous phases, and relative levels were quantified by the densitometry of immunoblots. Values are expressed as the percentage of total Ras partitioning into the detergent phase, and represent means ± S.E.M. of four independent experiments. $^{*}P < 0.01$ compared with untreated cells.

24 h. Total levels of cellular Ras were unchanged, as determined by immunoblotting of whole-cell lysates (results not shown). The amount of farnesylated Ras was determined by partitioning into Triton X-114 [26], with the hydrophobic farnesylated Ras recovered from the detergent phase and the unfarnesylated Ras recovered from the aqueous phase by immunoprecipitation [15]. In the stable vector line, approx. 50 % of total cellular Ras was farnesylated, and FGF-2-treatment for 16 h had no effect on Ras farnesylation (Figure 4B). In untreated, stable FPPS cells, the percentage of Ras that was farnesylated was similar to that in the vector line. However, treatment of stable FPPS cells with FGF-2 for 16 h significantly enhanced the percentage of Ras that was farnesylated, supporting a role for Ras farnesylation in the prolonged FGF-2-induced Ras activation seen in FPPS-transfected cells.

To determine if the effects of FPPS on FGF-2-stimulated Ras activation are transmitted through the entire Ras/Raf/ERK cascade, we examined the FGF-2-induced activation of Raf-1, MEK and ERK in the stable vector and FPPS cell lines. FGF-2-stimulated Raf-1 kinase activity was prolonged in the stable FPPS cell line compared with the vector control (Figure 5A). FGF-2-stimulated hyperphosphorylation of Raf-1 was also prolonged (Figure 5B), consistent with prolonged Ras activation that continues to activate Raf. Activated Raf phosphorylates and activates MEKs 1 and 2, which in turn phosphorylate and activate ERKs 1 and 2, the cytosolic and nuclear effectors of the cascade. Consistent with prolonged Raf activation in FPPS-transfected cells, activation of MEKs 1 and 2 and ERKs 1 and 2 was prolonged compared with vector-transfected controls (Figures 5C and 5D). Autophosphorylation of FGFR1 and FGF-2-induced activation of other signalling proteins, including p38 MAP kinase, Akt and $p70^{S6K}$, were similar in vector- and FPPS-transfected cells (results not shown). EGF-induced ERK activation was not prolonged in FPPS-transfected cells (Figure 5E), and the time courses of EGF- and platelet-derived growth factor (PDGF)-induced ERK activation in the vector and FPPS cell lines were similar (results not shown). These data indicate that the observed effects of FPPS overexpression are specific to the FGF-2-induced activation of the Ras/ERK cascade.

Effects of FPPS on cell cycle regulatory proteins

The specific inhibition of FGF-2-induced proliferation in FPPS-transfected cells suggests an effect on cell cycle regulatory proteins, which is likely to be mediated by the prolonged activation of ERK observed in these cells. To identify the point at which the cell cycle is affected, we examined the expression of the primary G_1-phase cyclins, D1 and E, in the stable vector- and FPPS-transfected cell lines (Figure 6). In the vector control cells, FGF-2 induced a broad increase in expression of cyclin D1, extending from 8 to 24 h, and peaking at 12 h. Cyclin E, which is expressed at the G_1/S phase transition, showed a smaller FGF-2-induced increase in vector control cells, peaking at 16 h. However, in the stable FPPS cell line, FGF-2-induced increases in cyclins D1 and E did not occur. Treatment with 10 % (v/v) serum induced the expression of cyclins D1 and E in the stable FPPS lines (results not shown), consistent with the serum-induced proliferation observed in these cells. In addition to the induction of cyclins D1 and E, degradation of the cyclin-dependent kinase inhibitor (CKI) $p27^{Kip1}$ is required for mitogen-induced G_1 progression [27]. In both vector and FPPS stable cell lines, FGF-2 treatment induced the degradation of $p27^{Kip1}$ over a similar time course (Figure 6), and no significant differences in the expression of another major CKI, $p21^{Cip1}$, were observed (results not shown). These data demonstrate a CKI-independent G_1 arrest in FGF-2-treated, FPPS-overexpressing cells, possibly resulting from the prolonged FGF-2-induced activation of the Ras/ERK cascade.

Effects of FPPS on FGF-2-induced PC12 cell differentiation

In contrast with its effects on fibroblasts, FGF-2 induces the differentiation of PC12 cells, which is accompanied by a prolonged increase in ERK activity [28]. Thus, if the main effect of FPPS overexpression on FGF-2 signalling is to promote the prolonged activation of the Ras/ERK cascade, then stable overexpression of FPPS in PC12 cells should not inhibit FGF-2-induced differentiation. To test this hypothesis, PC12 cells were stably transfected with the construct encoding epitope-tagged FPPS, or with the vector alone, and clones expressing moderate to high levels of exogenous FPPS were selected for analysis. As shown in Figure 7(A), the FPPS-expressing cells were flatter than the vector-transfected cells and had a tendency to clump together. However, treatment of FPPS-expressing PC12 cells with FGF-2 resulted in robust differentiation, which was similar to or more pronounced than that seen in the vector-transfected cells treated with FGF-2 (compare Figure 7B with Figure 7D). These data support the hypothesis that FPPS overexpression primarily affects FGF-2 signalling through its actions on the Ras/ERK cascade.

DISCUSSION

In the present study, we describe a novel interaction between FPPS and FGFR. FPPS is an enzyme that is required for the

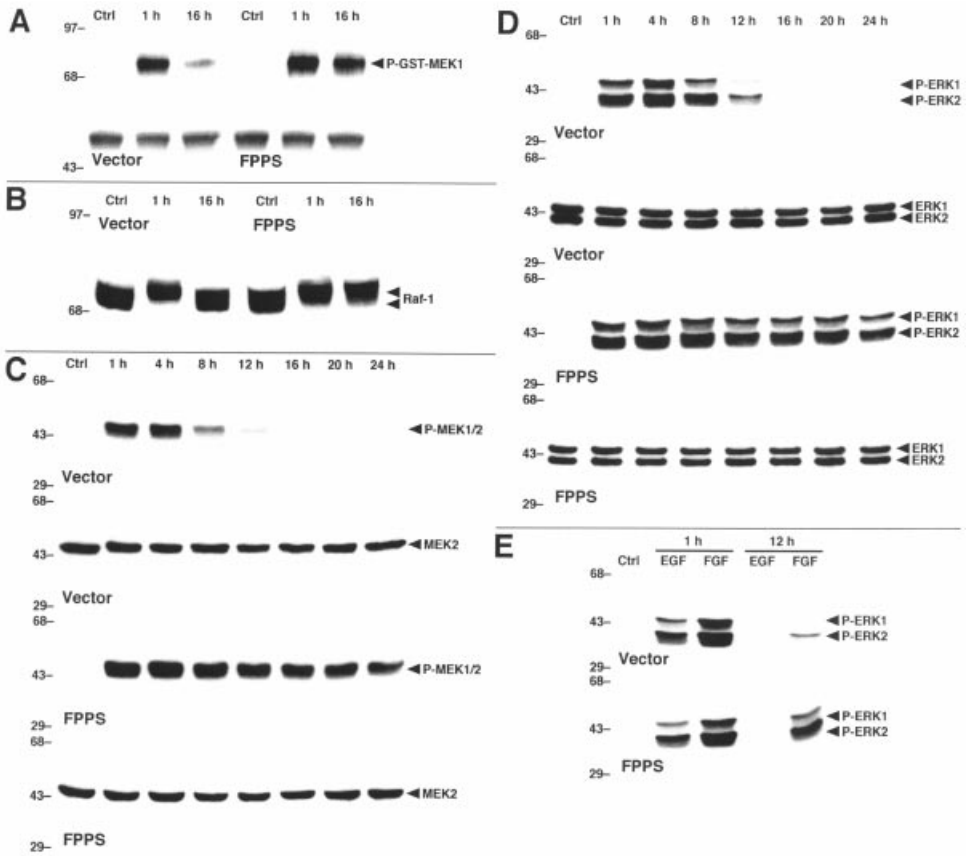

Figure 5 FPPS prolongs FGF-2-induced activation of the Ras/ERK cascade

(**A**) Stable NIH 3T3 vector control cells or the cell line expressing high levels of exogenous FPPS were treated for the indicated times with FGF-2 (5 ng/ml). Raf-1 kinase activity was measured with an immune complex kinase assay using GST–MEK1 as a substrate. GST–MEK1 phosphorylation was determined by immunoblotting with a phospho-specific antibody. Similar results were obtained in three independent experiments. (**B**) Raf-1 hyperphosphorylation was analysed by SDS/PAGE mobility shift and immunoblotting of whole-cell lysates from stable vector and FPPS cell lines treated with FGF-2 (5 ng/ml) for the indicated times. Similar results were obtained in three independent experiments. (**C**) MEK1/2 activation in stable vector and FPPS cell lines treated with FGF-2 (5 ng/ml) for the indicated times was determined by immunoblotting of whole-cell lysates with a phospho-specific antibody. An antibody which detects total MEK2 was used to verify equal protein loading. Similar results were obtained in three independent experiments. (**D**) ERK1/2 activation in stable vector and FPPS cell lines treated with FGF-2 (5 ng/ml) for the indicated times was determined by immunoblotting of whole-cell lysates with a phospho-specific antibody. An antibody which detects total ERK1/2 was used to verify equal protein loading. Similar results were obtained in three independent experiments. (**E**) ERK1/2 activation in stable vector and FPPS cell lines treated with EGF (5 ng/ml) or FGF-2 (5 ng/ml) for the indicated times was determined by immunoblotting of whole-cell lysates with a phospho-specific antibody. Similar results were obtained in two independent experiments. Positions of molecular mass markers (kDa) are indicated on the left of all gels. P- denotes phosphorylated.

synthesis of isoprenoids, and it is therefore somewhat surprising that it binds to a growth factor receptor. Nevertheless, an increasing number of proteins have been identified that have multiple, disparate biological functions (e.g. [29]). For example, the enzyme aconitase, which participates in the citric acid cycle, also functions as a cellular iron sensor and a translational repressor of ferritin mRNA [30].

The interaction of many receptor tyrosine kinases with substrates and adapter proteins is regulated by ligand-induced receptor autophosphorylation. Although the binding of FPPS to FGFR1 is enhanced by treatment of cells with FGF-2, autophosphorylation of FGFR1 is not required for this interaction. Similarly, the docking protein FRS2 interacts with FGFR1 independently of receptor activation [31]. However, the progressive increase in the binding of FPPS to FGFR1 induced both by starvation and by FGF-2 treatment suggests that the interaction is regulated. Although it is somewhat surprising that both starvation and FGF-2 treatment increase the binding of FPPS to FGFR1, preliminary experiments indicate that both treatments decrease the phosphorylation of FPPS on serine and threonine residues (results not shown). However, whether this decrease in FPPS phosphorylation is responsible for the increase in FGFR1 binding remains to be determined.

The subcellular localization of FPPS may also play a role in regulating its interaction with FGFR. FPPS has been localized to peroxisomes, along with several other enzymes involved in isoprenoid biosynthesis [32]. However, unlike the peroxisomal marker catalase, FPPS can readily move from peroxisomes into the cytosol, and this translocation has been proposed as a regulatory mechanism for cellular FPP synthesis [12]. Although the mechanism and regulatory factors for FPPS transport out of peroxisomes remain to be elucidated, starvation and FGF-2 treatment may play roles in this phenomenon.

Overexpression of FPPS prolongs the FGF-2-induced activation of the Ras/ERK cascade. Despite an increase in FPPS activity in FPPS-transfected fibroblasts, no increase in the basal level of Ras farnesylation or activation was detected. Thus our data support a model in which FGF-2 treatment results in enhanced farnesylation of Ras, prolonging FGF-2-induced Ras activation. This effect is then transmitted through the kinase

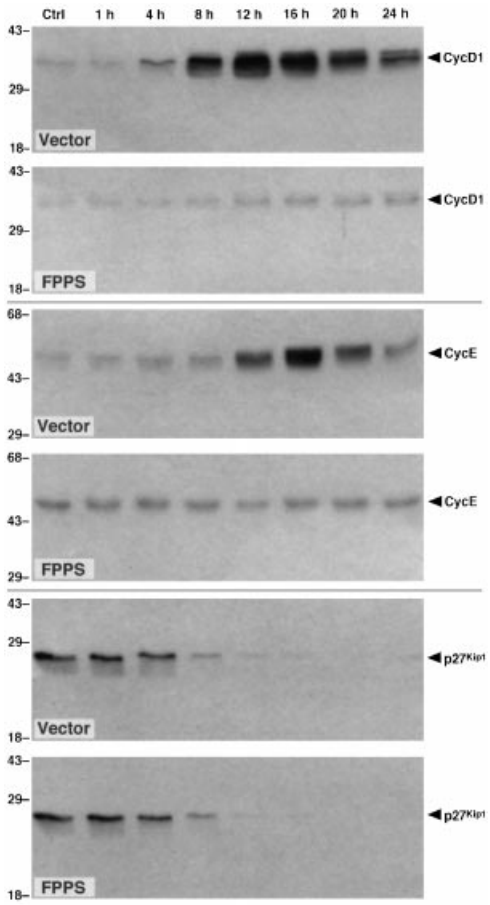

Figure 6 Effects of FPPS on cell cycle regulatory proteins

Quiescent cultures of stable NIH 3T3 vector control cells or the cell line expressing high levels of exogenous FPPS were treated for the indicated times with FGF-2 (5 ng/ml). Equal amounts of protein from whole-cell lysates were separated by SDS/PAGE, transferred to nitrocellulose and immunoblotted for cyclin D1 (top), cyclin E (middle) or $p27^{Kip1}$ (bottom). Similar results were obtained in three independent experiments. Positions of molecular mass markers (kDa) are indicated on the left.

cascade, as shown by the prolonged activation of Raf, MEK and ERK. Stimulation of Ras farnesylation has been proposed as an additional mechanism for the regulation of cellular Ras activity, enhancing Ras activation and modifying gene expression [33]. The observation that a 33% increase in FPPS activity dramatically affected signalling through FGFR1, but had no effect on signalling through other growth factor receptors that also use Ras (e.g. Figure 5), suggests that there may be different pools of Ras that are relatively stably associated with specific receptors and are regulated by distinct mechanisms. This idea is supported by the observation that, in quiescent fibroblasts, Ras is organized into ordered but inactive signalling modules [34].

Our data do not rule out the possibility that overexpression of FPPS affects signalling through other farnesylation-dependent pathways in addition to Ras. One such possibility is the small GTPase RhoB. Although most small GTPases require geranylgeranylation for their activity, RhoB can be either farnesylated or geranylgeranylated [35,36]. Both forms are capable of inhibiting cell proliferation [37], and therefore enhanced RhoB farnesylation may contribute to the effects of FPPS on FGF-mediated signalling.

The enhanced farnesylation of Ras detected in FGF-2-treated FPPS-transfected cells is likely to require the activity of farnesyl transferase (FTase), a ubiquitous enzyme that catalyses the attachment of the farnesyl moiety to Ras [9]. Basal FTase activity results in farnesylation of 25–50% of total cellular Ras, and treatment with insulin induces phosphorylation of FTase and enhancement of its activity [15]. In contrast with the effects of insulin, FTase activity is not enhanced by EGF, PDGF or insulin-like growth factor-1 [38]. The effects of FGF-2 on FTase have not been investigated, but an FGF-2-stimulated increase in FTase activity may underlie the enhanced Ras farnesylation observed in the present study. An increase in the cellular FPP concentration could also play a role in the enhanced Ras farnesylation. Although earlier data suggested that the total cellular FPP concentration is saturating for FTase [39,40], more recent work suggests that FPP is compartmentalized within the cell [41], and thus may be limiting for FTase activity at the site of Ras farnesylation.

Alternatively, the effect of FPPS overexpression on Ras activation may not require the activity of the enzyme. For example, since Ras activation is dependent upon the balance between RasGEF and RasGAP activity (where GEF is guanine-nucleotide-exchange factor and GAP is GTPase-activating protein), if FPPS competed with a RasGAP for binding to FGFR1 [42], then prolonged activation of Ras would result. On the other hand, FPPS could be acting simply as an adapter which links FGFR1 to the Ras/ERK cascade. Unfortunately, determining whether the effects of FPPS overexpression on Ras activation and FGF-2-dependent cell proliferation require FPPS activity is not as straightforward as it appears at first glance. Catalytically inactive FPPS can form heterodimers with catalytically active FPPS, and heterodimers between active and inactive FPPS are enzymically active [43]. Thus it would require an impossibly high level of overexpression of a catalytically inactive form of FPPS in order to give a preponderance of inactive homodimers.

In addition to prolonging the FGF-2-induced activation of the Ras/Erk cascade, overexpression of FPPS resulted in the specific inhibition of FGF-2-induced cyclin D1 and E expression, and inhibition of FGF-2-stimulated cell proliferation. Given the strong association of the Ras/Erk cascade with cell proliferation [44], it is likely that these effects are related. Activation of the Ras/Erk cascade can induce cell proliferation or cell cycle arrest and differentiation, depending upon the cell type and the time course of activation. For example, treatment of PC12 cells with EGF activates the ERK cascade for less than 1 h and induces proliferation, whereas treatment with nerve growth factor activates the cascade for more than 90 min and induces differentiation [45]. These observations led to the hypothesis that transient ERK activation signals proliferation, whereas sustained ERK activation signals differentiation [46]. In other cell types, transient ERK activation also leads to proliferation, whereas sustained or chronic ERK activation results in cell cycle arrest [47–50]. For example, in FGF-2-treated MCF-7 cells, ERK activation returns to baseline within 1 h and the cells proliferate [50]. Overexpression of Ha-Ras in these cells prolongs the activation of ERK beyond 1 h and inhibits FGF-2-induced proliferation. Similarly, treatment of hepatocytes with nerve growth factor induces ERK activation for less than 30 min and stimulates proliferation, whereas co-treatment with ethanol extends the activation of ERK beyond 2 h and inhibits proliferation [49]. In fibroblasts, the normal time course of ERK activation is much longer than in the previously discussed cell lines. Although growth factor-induced activation of ERK in NIH and Swiss 3T3 cells persists for 4–12 h (Figure 5D; [11,47]), FGF-2 strongly stimulates proliferation of these cells. In the

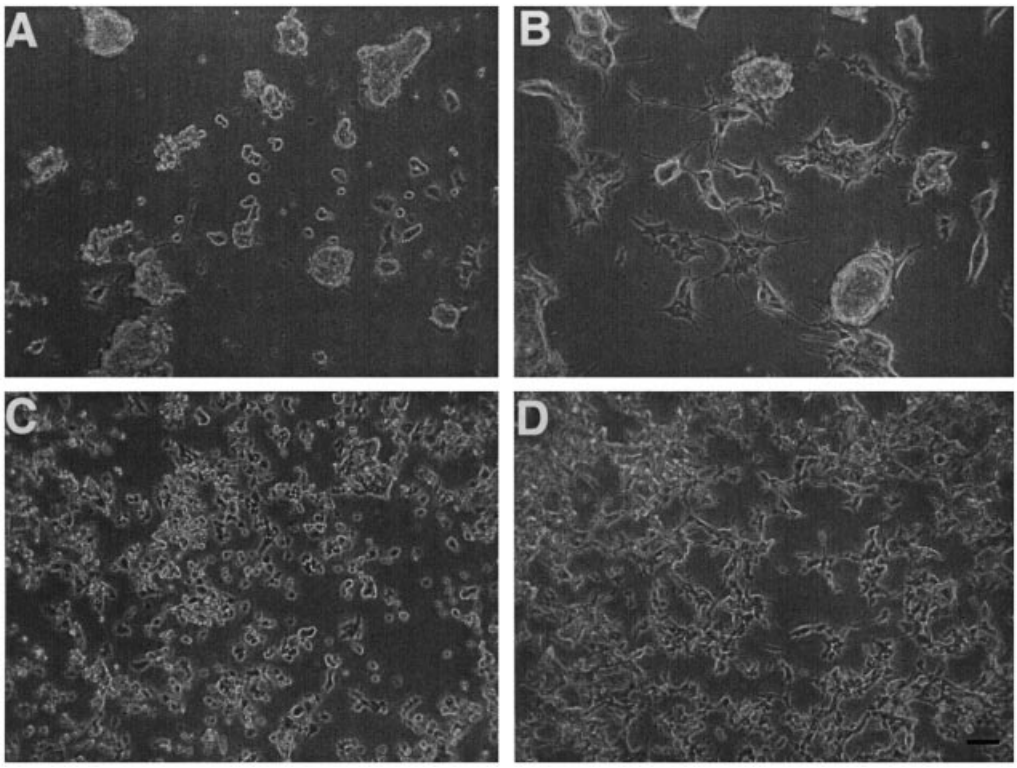

Figure 7 FPPS enhances the FGF-2-induced differentiation of PC12 cells

Cultures of stable PC12 vector control cells or a cell line expressing exogenous FPPS were treated for 24 h with FGF-2 (25 ng/ml), after which time they were examined and photographed by phase-contrast microscopy. (**A**) Untreated FPPS-transfected cells; (**B**) FGF-2-treated FPPS-transfected cells; (**C**) untreated vector-transfected cells; (**D**) FGF-2-treated vector-transfected cells. All cells were photographed at the same magnification. Similar results were obtained in two independent experiments with several different FPPS-transfected PC12 cell lines. Bar = 40 μm.

present study, overexpression of FPPS in fibroblasts prolonged the activation of ERK beyond the normal 4–12 h period, and inhibited proliferation. In contrast, overexpression of FPPS in PC12 cells enhanced FGF-2-induced differentiation, consistent with the association between differentiation and sustained ERK activation. Thus our data are consistent with the opposing effects of transient compared with sustained activation of the Ras/Erk cascade over a time course appropriate for the cell lines used in this study.

Prolonged or high-intensity activation of Raf has been shown to induce cell cycle arrest via the induction of $p21^{Cip1}$ [48,51]. Increased expression of this CKI inhibits the activity of the cyclin-dependent kinase partners of cyclins D1 and E. In the present study, no differences in $p21^{Cip1}$ expression were seen between the control and FPPS-transfected cell lines. However, since cyclins D1 and E are not induced by FGF-2 treatment of FPPS-overexpressing cells, CKIs are unlikely to be involved in the inhibition of FGF-2-stimulated proliferation. High-intensity activation of Ras was recently shown to induce cell cycle arrest by enhancing the proteasome-dependent degradation of cyclin D1 [52]. However, proteasome inhibitors did not increase the levels of cyclin D1 in the FGF-2-treated FPPS-overexpressing cells (results not shown). Thus the exact mechanism whereby prolonged FGF-2-induced ERK activation inhibits cell proliferation remains to be elucidated.

In conclusion, in the present study we demonstrate that FPPS interacts with FGFR and modulates FGF-2-mediated signal transduction. Our data suggest that this interaction promotes the farnesylation of Ras, which prolongs downstream signalling through the ERK cascade. The ubiquitous expression of FPPS highlights the intriguing possibility that this interaction is involved in regulating the duration of FGF-2-induced ERK activation in a variety of cell types. Thus the binding of FPPS to FGFR1 may play a role in determining the cellular responses to FGF-2 treatment.

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