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Complementation of Growth Factor Receptor-Dependent Mitogenic Signaling by a Truncated Type I Phosphatidylinositol 4-Phosphate 5-Kinase

J. NATHAN DAVIS,[1] CHARLES O. ROCK,[2] MANGENG CHENG,[1] JOSEPH B. WATSON,[1] RICHARD A. ASHMUN,[3] HEATHER KIRK,[4] ROBERT J. KAY,[4] AND MARTINE F. ROUSSEL[1]*

Departments of Tumor Cell Biology,[1] Biochemistry,[2] and Experimental Oncology,[3] St. Jude Children's Research Hospital, Memphis, Tennessee 38105, and Department of Medical Genetics, University of British Columbia, and The Terry Fox Laboratory, British Columbia Cancer Agency, Vancouver, British Columbia, Canada V5Z 4E6[4]

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Substitution of phenylalanine for tyrosine at codon 809 (Y809F) of the human colony-stimulating factor 1 (CSF-1) receptor (CSF-1R) impairs ligand-stimulated tyrosine kinase activity, prevents induction of c-*MYC* and cyclin D1 genes, and blocks CSF-1-dependent progression through the G_1 phase of the cell cycle. We devised an unbiased genetic screen to isolate genes that restore the ability of CSF-1 to stimulate growth in cells that express mutant CSF-1R (Y809F). This screen led us to identify a truncated form of the murine type Iβ phosphatidylinositol 4-phosphate 5-kinase (mPIP5K-Iβ). This truncated protein lacks residues 1 to 238 of mPIP5K-Iβ and is catalytically inactive. When we transfected cells expressing CSF-1R (Y809F) with mPIP5K-Iβ (Δ1-238), CSF-1-dependent induction of c-MYC and cyclin D1 was restored and ligand-dependent cell proliferation was sustained. CSF-1 normally triggers the rapid disappearance of CSF-1R (Y809F) from the cell surface; however, transfection of cells with mPIP5K-Iβ (Δ1-238) stabilized CSF-1R (Y809F) expression on the cell surface, resulting in elevated levels of ligand-activated CSF-1R (Y809F). These results suggest a role for PIP5K-Iβ in receptor endocytosis and that the truncated enzyme compensated for a mitogenically defective CSF-1R by interfering with this process.

Growth factor receptor mutants have provided a means of defining signaling pathways that control the cell's decision to enter the cell cycle, commit to DNA synthesis, and ultimately divide. Colony-stimulating factor 1 (CSF-1) is required for the proliferation, differentiation, and survival of macrophages and their immediate progenitors (39), acting by signaling through high-affinity receptors (CSF-1 receptor [CSF-1R]) with intrinsic tyrosine kinase activity (38). CSF-1R is a member of the type III receptor family that includes the platelet-derived growth factor receptor (PDGF-R), the KIT ligand receptor, and FLT3/FLK2. Type III receptors are glycoproteins with a single hydrophobic transmembrane segment, an extracellular moiety composed of five immunoglobulin-like domains that mediate ligand binding, and a cytoplasmic portion composed of a tyrosine kinase split by a kinase insert (KI), which is a hallmark of this receptor family (37). Ligand binding triggers receptor dimerization, activation of the intrinsic tyrosine kinase, and phosphorylation in *trans* of specific tyrosine residues (P-Tyr) within the intracellular kinase domain. These phosphorylated residues, in turn, function as docking sites for proteins containing *src* homology 2 (SH2) (36) and phosphotyrosine binding domains (47). The effector proteins that bind to P-Tyr-containing docking sites within the human CSF-1R are SRC kinases at P-Tyr-561, GRB-2 at P-Tyr-692, and phosphatidylinositol 3-kinase (PI3-K) at P-Tyr-723 (27). Candidate molecules that bind to P-Tyr-571, P-Tyr-708, and P-Tyr-809 have not been identified. The phosphorylation of Tyr-809 is considered particularly important. Phosphorylation of this residue is required for SRC kinase activation and for MYC induction (28) and likely controls the overall level of receptor kinase activity.

Ligand-mediated receptor dimerization, activation, and effector protein binding are followed by receptor aggregation into clathrin-coated pits, internalization into endosomes, and transport to lysosomes where receptor degradation takes place, thereby terminating receptor signaling. Unlike receptors for nutrients such as low-density lipoprotein and transferrin, which recycle to the cell surface, CSF-1R is one of the most rapidly downregulated receptors of its kind. Proper sorting and targeting of CSF-1R and PDGF-R to the lysosomal compartment may be mediated in part by PI3-K, because mutants of these receptors that are unable to bind PI3-K recycle to the cell surface and are not degraded (7, 17, 18). Other molecular events regulated by phosphatidylinositol kinases are also likely to involve receptor trafficking (4). For example, dynamin, a component of the endocytic apparatus, has recently been implicated in the endocytosis of the epidermal growth factor receptor through its interaction with GRB2 and PLC-γ and is necessary for extinguishing epidermal growth factor receptor signaling (41, 42).

Previously, we created a mutant human CSF-1R (Y809F), in which we substituted phenylalanine (F) for tyrosine (Y) at codon 809. Ectopic expression of this mutant receptor in NIH 3T3 cells supports cell survival but not proliferation or anchorage-independent growth in response to human CSF-1. The CSF-1-stimulated cells are unable to progress through the first gap (G_1) phase of the cell cycle, and this G_1 block is associated

* Corresponding author. Mailing address: Department of Tumor Cell Biology, St. Jude Children's Hospital, 322 North Lauderdale, Memphis, TN 38105. Phone: (901) 495-3481. Fax: (901) 495-2381. E-mail: martine.roussel@stjude.org.

† Present address: Department of Biochemistry and Molecular Biology and Center for Excellence in Cancer Research, Treatment and Prevention, Louisiana State University Medical Center, Shreveport, LA 71130-3932.

with the inability of these cells to express c-MYC (34). Enforced c-MYC expression restores CSF-1-dependent proliferation in these cells, which suggests that c-*MYC* transcription is required for the CSF-1-mediated proliferative response (30). Genetic complementation of the mitogenically defective mutant CSF-1R (Y809F) has enabled the identification of downstream effector molecules that participate in c-MYC activation. By using a combination of enforced expression of either wild-type or dominant-negative forms of genes known to be involved in G_1 progression, we have shown that *ETS-1*, *ETS-2*, and any of the D-type cyclins can restore CSF-1-induced mitogenesis (19, 31, 35). In each case, complementation reinitiated c-MYC expression, which was required for S-phase entry and mitogenesis, although the kinetics of c-MYC induction differed for each complementing gene.

To identify additional effector molecules involved in receptor tyrosine kinase signaling pathways, we developed a genetic screen based on complementation of the mitogenically defective CSF-1R (Y809F) mutant with retrovirally transduced mammalian cDNA libraries. The efficiency of the screen was enhanced by use of a clonal NIH 3T3 cell line bearing CSF-1R (Y809F) and engineered to express human CSF-1, establishing a potential autocrine loop. In this way, any cDNAs that complement the defective receptor will be identified because the cells they infect will form transformed foci on cell monolayers and colonies in soft agar.

By using this system, we recovered a cDNA encoding a truncated form of murine phosphatidylinositol 4-phosphate 5-kinase type Iβ (mPIP5K-Iβ). This truncated form of the enzyme is catalytically inactive and appears to act as a dominant-negative mutant in inhibiting receptor internalization. We discuss the implications of this finding for CSF-1R-mediated signal transduction.

MATERIALS AND METHODS

Cell lines, infections, agar cloning, and focus formation. NIH 3T3 fibroblast cell lines were maintained in Dulbecco's modified Eagle's medium supplemented with 10% fetal bovine serum (FBS), 2 mM glutamine, 100 U of penicillin per ml, and 100 μg of streptomycin per ml. Cell lines expressing the wild-type CSF-1R and CSF-1R (Y809F) were previously described (34). Purified recombinant human CSF-1 was generously provided by Steven C. Clark (Genetics Institute, Cambridge, Mass.). To generate a cell line expressing both CSF-1R (Y809F) and human CSF-1, a clonal cell line bearing the mutant receptor was cotransfected with a feline sarcoma-based retroviral vector expressing the full-length, secreted form of human CSF-1 (26), together with a puromycin-expressing plasmid, pJ6ΩPuro, a generous gift from Hartmut Land (Imperial Cancer Research Fund, London, United Kingdom) (24). Stable transfectants were established by a modified calcium phosphate precipitation technique (9) and selected and maintained in medium containing 7.5 μg of puromycin (Sigma Chemical Co., St. Louis, Mo.) per ml. Growth in semisolid media was tested by suspension of 5×10^3 cells in 1 ml of 0.3% Noble agar in the presence or absence of 10 ng of recombinant human CSF-1 per ml as previously described (35). Virus was produced by cotransfecting 293T cells with ecotropic psi2$^-$ retroviral plasmid DNA and with pSRαMSV at a ratio of 1:1. Viruses were harvested as previously described (1), and cells were infected with viral supernatants in the presence of 10 μg of Polybrene per ml. Six hours after virus addition, fresh medium was added and the cells were incubated at 37°C overnight in an 8% CO_2 humidified chamber. Fresh medium was added the next day.

The retroviral library screen and focus formation assays were performed by infecting 2×10^5 cells at a multiplicity of infection of <1 in Dulbecco's modified Eagle's medium containing 10% FBS and 10 μg of Polybrene per ml. Forty-eight hours after infection, cells were cultivated in complete medium containing 5% FBS, which was replaced every 3 days thereafter. Foci of transformed cells were scored after 2 weeks and cloned by using microcylinders after 3 to 4 weeks, and the resulting cultures were expanded into 75-cm^2 flasks. High-molecular-weight DNA from these clones was isolated as previously described (33).

Recovery of retroviral cDNA inserts and plasmid construction. Construction and use of the pCTV vectors and recovery of the cDNA inserts have been described elsewhere (43). The sequences of the oligonucleotide primers used were 5′-CTCACTCCTTCTCTAGCTC-3′ and 5′-AACAAATTGGACTAATC GATACG-3′ for the pCTV1 libraries and 5′-TCGAATCAAGCTTATCATAC G-3′ for the pCTV3 libraries. The amplification reactions contained 20 mM Tris (pH 8.8), 85 mM potassium acetate, 1 mM $MgCl_2$, 8% glycerol, 200 μM dideoxynucleotides, 100 ng of each primer, 100 ng of cellular DNA, 2.5 U of *Taq* polymerase, and 0.05 U of *Pfu* polymerase in a total volume of 25 μl. Specific amplification products were identified by Southern blotting followed by hybridization with a probe specific for the bacterial *supF* gene placed downstream of the pCTV cloning site. The amplified cDNAs were digested with *Bsi*WI and *Mlu*I, gel purified with a Geneclean Kit (Bio 101, La Jolla, Calif.), and cloned into a modified pCTV vector that lacks the *supF* gene. cDNAs of interest were subsequently excised by restriction endonuclease digestion with *Sal*I and cloned into pSKBluescript for DNA sequencing. The cDNAs encoding potentially interesting genes were recloned (*Sal*I fragment) into the pcDNA3 vector at the *Xho*I site and transfected either into 293T cells to produce virus or into NIH 3T3 cells expressing the mutant CSF-1R (Y809F) alone.

Immunoprecipitation, immunoblotting, and metabolic labeling. Metabolic labeling with [^{35}S]methionine, cell lysis, immunoprecipitation, and polyacrylamide gel electrophoresis (PAGE) in the presence of sodium dodecyl sulfate (SDS) were performed as described in detail elsewhere (32). For immunoblot analysis, proteins were separated by SDS-PAGE, transferred to nitrocellulose (Millipore, Bedford, Mass.), and immunoblotted with the indicated primary antibodies as described previously (29). The antibodies were against human CSF-1R (3), c-MYC (06340 antibody; Upstate Biotechnology Institute, Lake Placid, N.Y.), murine cyclin D1 (35, 40), and phosphotyrosine (antibody 4G10 from the Upstate Biotechnology Institute). Reactive products were visualized by using protein A-conjugated horseradish peroxidase (Ex Laboratories, San Mateo, Calif.), followed by chemiluminescence detection (ECL detection kit; Amersham, Arlington Heights, Ill.). In some experiments, filters were stripped in accordance with the manufacturer's instructions and reprobed with different antibodies.

Isolation and construction of full-length mPIP5K-Iβ. The mouse spleen cDNA library (1.0 kb) average insert constructed in Lambda Zap II, an excisable cloning vector (Stratagene), was plated and screened in accordance with the manufacturer's instructions. Nitrocellulose membranes were hybridized to ^{32}P-labeled probes (10^6 cpm/ml) derived from the truncated mPIPK-Iβ cDNA for 16 h at 42°C in 10 mM Tris–3× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate)–1× Denhardt's solution–100 μg of boiled salmon sperm DNA per ml–0.1% SDS–35% deionized formamide. Positive phage plaques were isolated, and cDNA fragments were excised with helper phage to generate subclones in pBluescript SK(−). Based on the DNA sequence of the two overlapping cDNA clones, the full-length cDNA was constructed, excised from pSK by digestion with *Xho*I and *Xba*I, blunt ended with Klenow, and cloned into the pCTV vector, which had been digested with *Sal*I and end filled.

mPIP5K-Iβ antibodies. A peptide corresponding to a unique sequence in the carboxy-terminal domain of mPIP5K-Iβ (CSSTLEKLDVAESEFTH, prepared with an additional cysteine residue on the amino terminus) was synthesized and coupled to keyhole limpet hemocyanin. Rabbit polyclonal serum against this peptide was generated by Rockland Laboratories and tested in our laboratory against extracts of COS-7 cells that had been transfected with the full-length mPIP5K-Iβ plasmid expression vector. Primary antiserum was used at a 1:2,000 dilution to visualize the protein.

Assay for mPIP5K-Iβ. The full-length and truncated mPIP5K-Iβ cDNAs were cloned into the pcDNA3 vector (Invitrogen, Carlsberg, Calif.) and sequenced to verify that they were cloned in the correct orientation. The cDNAs were transfected into COS-7 cells by using the DEAE dextran method (23). Cells were then incubated for 48 h to allow protein expression. PIP5K assays were performed with cell extracts essentially as previously described (5). Briefly, transfected COS-7 cells were washed with phosphate-buffered saline (PBS), scraped in 1 ml of cold PBS, and pelleted. The cell pellet was resuspended in 50 mM Tris-HCl (pH 7.5)–0.25 M sucrose–0.1 M NaCl–5 mM EDTA–0.5 mM EGTA–3 μg of leupeptin per ml–3 μg of pepstatin A per ml–1 mM phenylmethylsulfonyl fluoride. Cells were lysed by sonication in a cup horn (three 30-s bursts), and the protein concentration was determined. Reaction mixtures contained 50 mM Tris-HCl (pH 7.5), 0.5 mM EGTA, 12 mM $MgCl_2$, 80 μM phosphatidylinositol (PtdIns)-4-phosphate [PtdIns(4)P], 25 μM [γ-^{32}P]ATP (specific activity, 20 μCi/nmol; Dupont, New England Nuclear, Boston, Mass.), and 1 to 20 μg of protein in a final volume of 50 μl. The reactions proceeded for 10 min at 37°C and were stopped by addition of 50 μl of 12 N HCl. The reaction product was extracted by addition of 500 μl of chloroform-methanol-water (15:15:5), followed by 500 μl of methanol–1 N HCl (1:1). After thorough mixing and centrifugation, the upper phase was discarded and the lower phase was dried under a stream of nitrogen. After resuspension in chloroform-methanol (1:1), the entire sample was spotted on a Silica Gel 60 thin-layer plate that had been sprayed with 60 mM EDTA–2% sodium tartrate–50% ethanol and developed with chloroform-methanol-ammonium hydroxide-water (90:90:7:22). The plate was dried, and the amount of phosphatidylinositol-4,5-biphosphate [PtdIns(4,5)P2] formed was quantitated by using a PhosphorImager.

The cellular levels of polyphosphoinositides were measured by labeling COS-7 cells with [^{3}H]inositol. Twenty-four hours after transfection, the medium was changed to medium containing 3 μCi of [^{3}H]inositol per ml and the cells were incubated for an additional 24 h. The cells were then washed with PBS, harvested, extracted, and analyzed by thin-layer chromatography as described above.

Flow cytometric analysis of CSF-1R expression. Detection of cell surface CSF-1R by flow cytometry was performed as previously described (2). Briefly, cells were stained with an excess of human CSF-1R-specific monoclonal antibody, washed, and then stained with an excess of fluorescein-conjugated goat

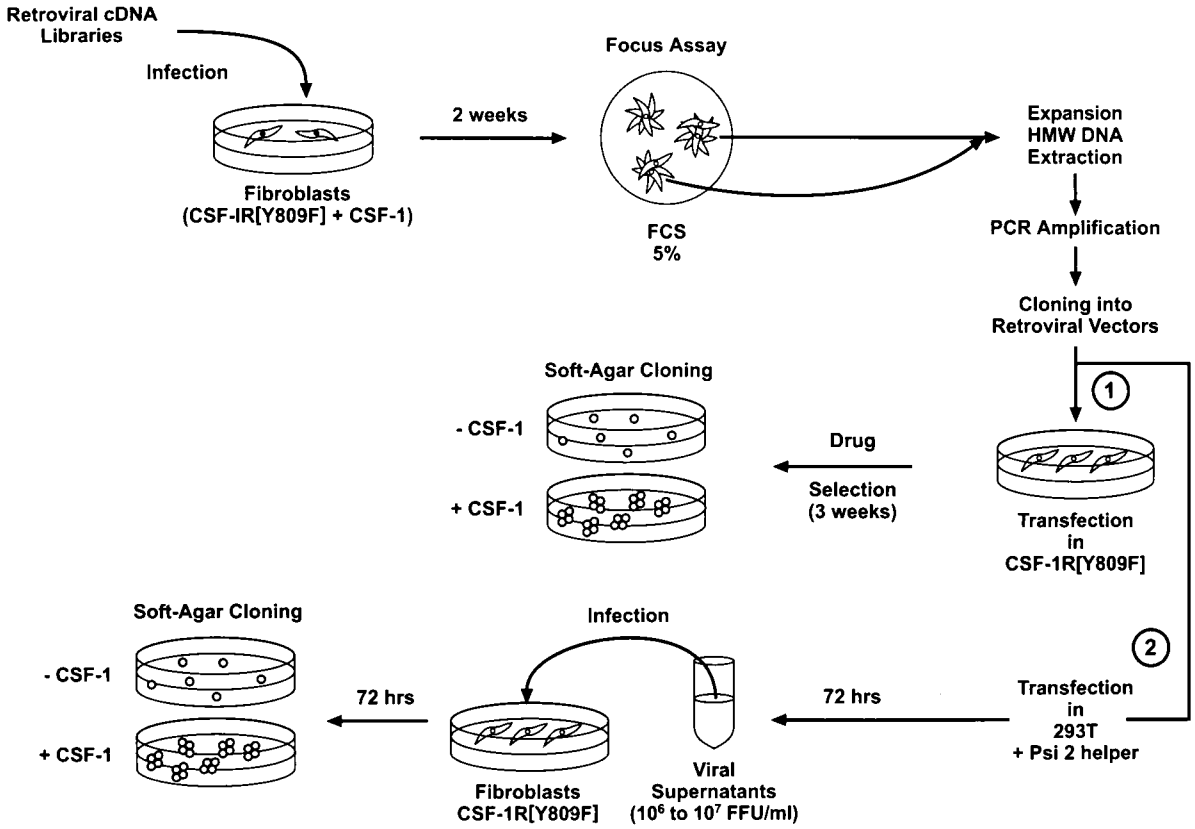

FIG. 1. Schematic representation of the genetic screen with mammalian retroviral cDNA libraries. HMW, high molecular weight; FCS, fetal calf serum; FFU, focus-forming units.

anti-rat immunoglobulin. Fluorescence from approximately 10,000 cells was detected by using a Becton Dickinson FACS Vantage and compared to that of cells stained with an isotype-matched control primary antibody.

RNA isolation and Northern blotting analysis. Total RNA was isolated from NIH 3T3 cells as previously described (34). Total RNA samples (20 μg) were fractionated on a 2.2 M formaldehyde–1.5% agarose gel and immobilized on nitrocellulose by capillary transfer. The nitrocellulose was then hybridized with a ^{32}P-labeled probe with high specific activity (10^6 cpm/ml) in 10 mM Tris–3× SSC–1× Denhardt's solution–100 μg of sheared, boiled salmon sperm DNA per ml–0.1% SDS–50% deionized formamide, for 16 h at 42°C. The blots were washed in 2× SSC–0.5% SDS at room temperature and then exposed to Kodak XAR-5 film. The ^{32}P-labeled probe was prepared by using a random priming kit in accordance with the manufacturer's instruction (Boehringer Mannheim). The probe comprised the C-terminal 181 bp of coding sequences and 127 bp of an untranslated sequence present in the mPIP5K-Iβ (Δ1-238) cDNA. A cDNA encoding glyceraldehyde-3-phosphate dehydrogenase was used as a probe to normalize for RNA loading.

RESULTS

Library screening. cDNAs from the murine erythroleukemia cell line GM979 were cloned into retroviral vector pCTV1 (43). Pooled plasmid DNA from the library was transfected into the BOSC23 packaging cell line, and viruses collected from the culture supernatants were used to infect NIH 3T3 cells that had been previously engineered to express CSF-1R (Y809F) and CSF-1 (Fig. 1). A parallel infection was performed with an equivalent number of viruses derived from pCTV1 lacking cDNA inserts. Two weeks after the infection, nine foci of transformed cells were detected in the library-infected cell monolayers, while no transformed foci were found in the culture infected with the pCTV1 empty vector. Therefore, the transformation events were not the result of retroviral insertional mutagenesis or a consequence of other variables of the infection protocol. Rather, they were presumably induced by expression of specific cDNAs from the library.

Foci of transformed cells were individually isolated and expanded. Virally transmitted cDNAs were recovered by PCR amplification using pCTV1-specific primers. The amplified cDNAs were then recloned into the pCTV3K retroviral vector. The plasmid DNAs were either (i) transfected with a puromycin-containing vector into cells expressing CSF-1R (Y809F) or (ii) converted to viruses by transfection into 293T with ecotropic psi2$^-$ plasmid DNA. These viral supernatants were then used to infect NIH 3T3 cells that express CSF-1R (Y809F) but do not express the human CSF-1 protein. Drug-selected transfected or infected cells were then tested for transformation in the presence or absence of exogenous CSF-1 to determine whether individually recovered cDNAs conferred transforming activity and whether that activity was dependent on ligand stimulation of the mutant receptor. This secondary screen used soft agar growth as an independent assay for transformation. In all cases in which recovered cDNAs conferred transformation, they did so in a CSF-1-dependent manner, indicating that the screen was successful in identifying cDNAs that specifically complemented the receptor mutation.

Molecular characterization of a cDNA encoding a truncated form of mPIP5K-Iβ. One of the positive cDNAs conferred CSF-1-dependent colony formation in agar when transfected into NIH 3T3 cells expressing CSF-1R (Y809F) (Table 1). The cloning efficiency of the mass population of transfected cells was three- to fourfold greater than the background but about fivefold less than that of wild-type CSF-1R. When transformed colonies from CSF-1-containing plates of the mass population

TABLE 1. Colony formation in soft agar[a]

Receptor and plasmid	Colony formation	
	Without human CSF-1	With human CSF-1
CSF-1R	0	17.5
CSF-1R (Y809F) plus:	0	<0.1
pCTV3	0	0
pCTV3-mPIP5K-Iβ (Δ1-238)		
Mass population	0	3.6
Subclone 1	0	12.2
Subclone 2	0	7.7
Subclone 4	0	6.7
Subclone 6	0	11.5
Subclone 7	0	13.1
Subclone 8	0	15.4

[a] NIH 3T3 cells expressing the wild-type CSF-1R or the mutant CSF-1R (Y809F) alone or transfected with either an empty vector (pCTV3) or a vector containing mPIP5K-Iβ (Δ1-238) were cloned in soft agar in the presence or absence of exogenous CSF-1. The mass population of cells coexpressing the CSF-1R (Y809F) and truncated mPIP5K-Iβ [pCTV3-mPIP5K-Iβ (Δ1-238)] formed colonies only in the presence of recombinant CSF-1. Individual colonies, subclones 1 to 8, isolated from the CSF-1-containing plates from the mass population were reseeded in soft agar. Colonies, which appeared only in dishes containing CSF-1, were counted 2 and 3 weeks later. The efficiency of colony formation was calculated as the number of colonies per total number of cells plated (5×10^3) × 100. Subclones 7 and 8 were analyzed further.

of cells expressing the cDNA clone were replated in soft agar (subclones 1 to 8), their cloning efficiency was equivalent to that of cells expressing the wild-type CSF-1R, indicating that expression of the cDNA could fully complement the receptor mutation. The entire cDNA was sequenced on both strands and contained an open reading frame predicted to encode a 280-amino-acid protein followed by an extended 3′ untranslated sequence and a nucleotide polyadenylation sequence. Because the clone did not contain any stop codon 5′ to the first encoded methionine, we reasoned that it probably encoded a partial protein. In vitro transcription and translation of the cDNA produced a 30-kDa protein consistent with the initiation of translation at the first encoded ATG (data not shown). In the course of this work, the sequences of mPIP5K-Iα and mPIP5K-Iβ were published (15). Comparison of our cDNA to the published mPIP5K-I sequences revealed that our clone matched the type Iβ sequence but contained 515 bp of 3′ untranslated sequences and lacked 44% of the full-length cDNA at its 5′ end. We concluded that the cDNA we pulled from our screen encoded a truncated version of mPIP5K-Iβ, starting at amino acid 239 and lacking the first 238 amino acids (Fig. 2). This cDNA will be referred to throughout this report as mPIP5K-Iβ (Δ1-238). The mPIP5K-Iβ (Δ1-238) cDNA also contained an in-frame deletion between amino acids 473 and 498 in the C-terminal coding region. This deletion could represent a spliced variant expressed in the murine erythroleukemia cell line GM979.

The PtdIns(4)P5-kinase (PIP5K) family appears to share a conserved putative kinase domain (referred to as the kinase homology domain (KHD), between amino acids 97 and 320 and amino acids 383 and 430, that is interrupted by a KI region (22) (Fig. 2). mPIP5K-Iβ (Δ1-238) lacks most of the first KHD, including the predicted nucleotide binding motif represented by the SGS sequence (amino acids 71 to 74) and the lysine (K) residue at codon 179. However, it retains the sequences that correspond to the putative catalytic domain, which is represented by the sequence KYDLKGST (amino acids 242 to 249), followed by an aspartic acid (D) residue at position 307.

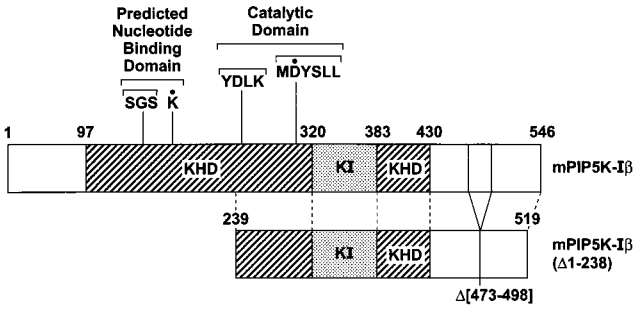

FIG. 2. Schematic protein structure of full-length mPIP5K-Iβ and its truncated (Δ1-238) form. The hatched bar represents the KHD, and the dotted bar represents the KI. The empty boxes are regions of the gene that are unique to each PIP5K family member.

mPIP5K-Iβ (Δ1-238) also contains a PtdIns binding domain homologous to that found in α-actinin (11).

We confirmed the expression of endogenous mPIP5K-Iβ mRNA in CSF-1R-expressing NIH 3T3 cells by Northern blot analysis. To avoid cross-hybridization with the endogenous mPIP5K-Iα mRNA, we used a 308-bp fragment of the mPIP5K-Iβ (Δ1-238) cDNA as a probe. This fragment corresponds to the C-terminal 181 bp of type Iβ, which are unique to this isozyme, and 127 bp of the 3′ untranslated sequence. Expression of mPIP5K-Iβ (Δ1-238) in the two CSF-1R (Y809F)-transfected clonal cell lines (subclones 7 and 8) was confirmed by detection of its 5-kb mRNA (Fig. 3, lanes C and D, respectively). Expression of the endogenous 4-kb mPIP5K-Iβ mRNA was detected, as expected, in the NIH 3T3 cell lines expressing the wild-type CSF-1R (Fig. 3, lane A), the mutant (Y809F) (Fig. 3, lane B), and the two subclones coexpressing CSF-1R

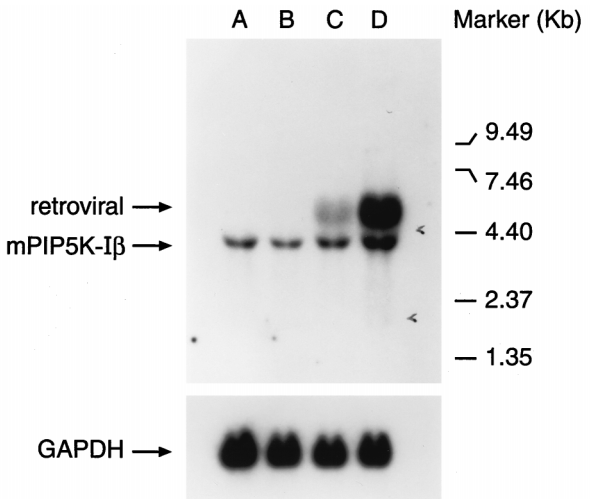

FIG. 3. Expression of mPIP5K-Iβ RNA in NIH 3T3 cells. Total RNA (20 μg/lane) was size selected by agarose gel electrophoresis, transferred to nitrocellulose, and hybridized with a ^{32}P-labeled probe. The probe corresponded to the 3′ end of mPIP5K-Iβ, comprising 181 bp of the unique 3′-end coding sequence and 127 bp of the untranslated sequence. Shown is mRNA from NIH 3T3 cells expressing the wild-type CSF-1R (lane A) or the mutant CSF-1R (Y809F) alone (lane B) or coexpressing the CSF-1R (Y809F) and mPIP5K-Iβ (Δ1-238) subclone 7 (lane C) or 8 (lane D). The positions of endogenous and retroviral mRNAs are shown by arrows. RNA loading was controlled by rehybridizing the same blot with a cDNA encoding glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

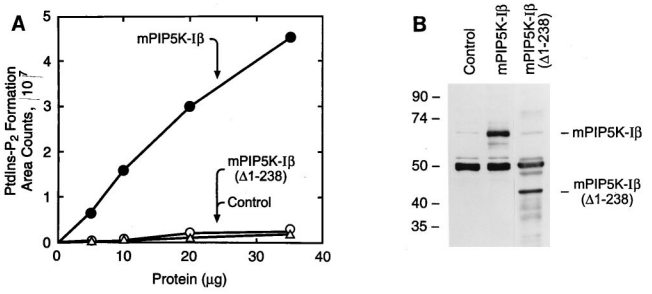

FIG. 4. Biochemical activities of full-length mPIP5K-Iβ and its truncated form. COS-7 cells were transfected with plasmids expressing either full-length mPIP5K-Iβ (●), mPIP5K-Iβ (Δ1-238) (○), or an empty vector control (△). Forty-eight hours later, the cells were harvested and cell extracts were prepared and assayed for PIP5K activity as described in Materials and Methods. (A) The specific activity of PIP5K in extracts from each of the transfected cell cultures. (B) Cell extracts fractionated by SDS-PAGE and immunoblotted with antipeptide serum directed against the carboxy-terminal 15 amino acids of mPIP5K-Iβ. The locations of the full-length and truncated versions of mPIP5K-Iβ are noted. The values to the left of the gel are molecular sizes in kilodaltons.

(Y809F) and mPIP5K-Iβ (Δ1-238) (subclones 7 [Fig. 3, lane C] and 8 [Fig. 3, lane D]).

Biochemical characterization of mPIP5K-Iβ. Because mPIP5K-Iβ (Δ1-238) lacked sequences at the predicted active site, it was unlikely to be catalytically active. To verify this point and to determine whether the deletion between amino acid residues 473 and 498 influenced kinase activity, we cloned the full-length and truncated cDNAs into the pcDNA3 mammalian expression vector and transiently transfected COS-7 cells. Protein from the reconstructed full-length version of mPIP5K-Iβ exhibited robust kinase activity with PtdIns(4)P but not with PtdIns as the substrate (Fig. 4). As predicted from its amino acid sequence, the expressed mPIP5K-Iβ (Δ1-238) protein lacked kinase activity (Fig. 4A). Cells transfected with wild-type mPIP5K-Iβ had 10-fold higher kinase specific activities than those transfected with mPIP5K-Iβ (Δ1-238), although both cell populations expressed their constructs at approximately the same level (Fig. 4). We also performed a mixing experiment to determine if extracts from cells expressing the mPIP5K-Iβ (Δ1-238) protein inhibited the kinase activity in extracts from cells expressing the mPIP5K-Iβ construct. Extracts from both control and truncated mPIP5K-Iβ cells had an inhibitory effect on kinase activity when added to extracts from cells expressing mPIP5K-Iβ. However, there was no difference in inhibitory effect between the control and truncated mPIP5K-Iβ cell extracts. Expression of the recombinant proteins in COS-7 cells was confirmed by immunoblotting with polyclonal rabbit antisera raised against the C-terminal 15 amino acids of mPIP5K-Iβ. The antisera detected proteins with apparent molecular masses of 68 kDa for full-length recombinant mPIP5K-Iβ and 44 kDa for the truncated form (Fig. 4B), demonstrating significant levels of recombinant protein expression in each cell lysate. These data confirm that the lack of kinase activity was not due to the absence of mPIP5K-Iβ (Δ1-238) expression but rather to an intrinsic defect in the truncated protein.

Transient overexpression of wild-type and truncated mPIP5K-Iβ does not alter cellular PtdIns(4,5)P2 levels. The expression of active mPIP5K-Iβ may increase the levels of PtdIns(4,5)P2; and the expression of mPIP5K-Iβ (Δ1-238) may decrease the levels of PtdIns(4,5)P2. On the other hand, there may be no effect of these proteins on cellular PtdIns(4,5)P2 levels if the activity of the lipid kinase is limited by the supply of substrate or by a mechanism that controls its translocation to the membrane. These possibilities were examined by determining the effect of full-length or truncated mPIP5K-Iβ overexpression on the levels of PtdIns(4,5)P2. COS-7 cells were transfected with the plasmid expression vectors used in Fig. 4 and labeled 24 h later with [^{3}H]inositol (3 μCi/ml) for an additional 24 h. Cells were harvested and extracted as described for the PIP5K assay, and the phosphoinositides were separated and quantitated by thin-layer chromatography. There was no difference in the amounts of [^{3}H]inositol incorporated in PtdIns, PtdIns(4)P, PtdIns(4,5)P2, and lyso-PtdIns between the control COS-7 cells and those transfected with either full-length or truncated mPIP5K-Iβ. Specifically, the PtdIns(4,5)P2 content of COS-7 cells transfected with the empty control vector was 4.72% ± 0.64% inositolphospholipids, whereas the cellular PtdIns(4,5)P2 content was 4.64% ± 0.39% in cells expressing full-length mPIP5K-Iβ and was 4.90% ± 1.06% in cells expressing mPIP5K-Iβ (Δ1-238). These data show that enforced expression of full-length mPIP5K-Iβ did not affect the overall cellular levels of PtdIns(4,5)P2. Similarly, overexpression of mPIP5K-Iβ (Δ1-238) did not decrease total PtdIns(4,5)P2 levels.

mPIP5K-Iβ causes reinduction of c-MYC and cyclin D1 in the CSF-1R (Y809F) mutant. Because expression of both c-MYC and cyclin D1 is required for CSF-1-dependent S-phase entry and proliferation, we expected that cells coexpressing CSF-1R (Y809F) and mPIP5K-Iβ (Δ1-238) would reinduce MYC and cyclin D1 in response to CSF-1 stimulation. Cells were starved of growth factors for 18 h and restimulated to enter the cell cycle with CSF-1 in chemically defined medium. Under these conditions, cells synchronously reinitiate DNA synthesis 12 h after CSF-1 addition, as confirmed by flow cytometric analysis of DNA content (data not shown). Cells expressing the wild-type CSF-1R showed induction of c-MYC protein, which reached a maximum after 4 h of stimulation, followed by elevated cyclin D1 expression, which reached a peak at the G_1-to-S transition 12 h following ligand stimulation (Fig. 5A). As reported previously, cells expressing CSF-1R (Y809F) did not express sustained levels of c-MYC or cyclin D1 when cultured in CSF-1-containing serum-free medium (Fig. 5B) (35). In contrast, two selected clonal cell lines coexpressing CSF-1R (Y809F) plus mPIP5K-Iβ (Δ1-238) showed CSF-1-dependent reinduction of c-MYC and cyclin D1 with kinetics indistinguishable from those obtained with cells expressing the wild-type receptor (Fig. 5C and D). Therefore, expression of truncated mPIP5K-Iβ reactivated the CSF-1R signaling pathways that lead to c-*MYC* and cyclin D1 transcription.

Coexpression of CSF-1R (Y809F) and mPIP5K-Iβ (Δ1-238) does not affect receptor processing in the absence of CSF-1. Yeast PIP5Ks are involved in vesicular and protein trafficking (10), suggesting that the truncated form of mPIP5K-Iβ might affect CSF-1R (Y809F) transport. Truncated mPIP5K-Iβ could alter receptor levels at the cell surface either by modulating its rate of transport to the plasma membrane or by decreasing its rate of endocytosis and/or degradation after ligand stimulation. Tyr-809 is a major autophosphorylation site of the CSF-1R and is predicted to reside in the regulatory T loop (16). Therefore, substitution of phenylalanine for Tyr-809 would be predicted to reduce the overall ligand-induced tyrosine kinase activity (12). We previously reported no significant differences in the overall activity of the Y809F receptor mutant compared with that of the wild-type receptor in transfected cell populations (34). However, isolation of a clonal cell line suitable for the biological screen (i.e., cells that are unable to grow in soft agar, form foci on monolayers, or grow in serum-free medium containing CSF-1 but are rescuable by c-MYC) showed a marked decrease (about eightfold less) in ligand-induced tyrosine ki-

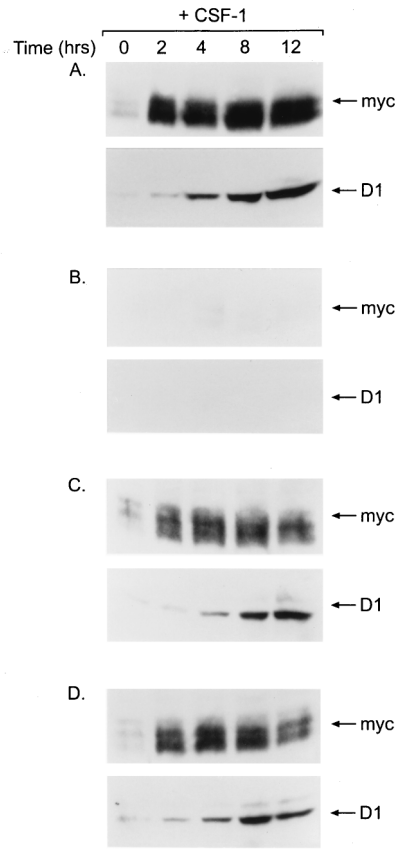

FIG. 5. Kinetics of CSF-1-dependent c-MYC and cyclin D1 protein expression. NIH 3T3 cells expressing wild-type CSF-1R (A) or CSF-1R (Y809F) (B) or coexpressing the mutant CSF-1R (Y809F) with mPIP5K-Iβ (Δ1-238) subclone 7 (C) or 8 (D) were serum-starved for 24 h and then stimulated with recombinant human CSF-1 in chemically defined medium for the various times as indicated. Cells were trypsinized and lyzed in radioimmunoprecipitation assay lysis buffer. Proteins from cell lysates were separated by SDS-PAGE, transferred to nitrocellulose, and blotted successively with antibodies against MYC and cyclin D1. Proteins were revealed by chemiluminescence with the ECL detection kit (Amersham).

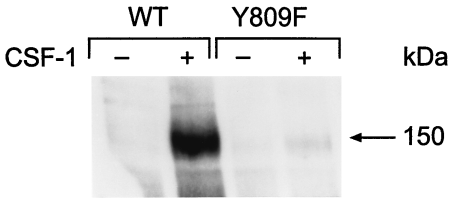

FIG. 6. Antiphosphotyrosine immunoblot of CSF-1R. NIH 3T3 cells expressing the wild-type (WT) or mutant (Y809F) human CSF-1 receptor were stimulated (+) or not stimulated (−) with human recombinant CSF-1. Detergent lysates were prepared, and CSF-1R was immunoprecipitated from equal amounts of protein from each lysate with an antiserum to the human CSF-1R gene product. After electrophoretic separation in polyacrylamide gels, the CSF-1R was transferred to nitrocellulose and immunoblotted with an antibody to phosphotyrosine (4G10) and detected by the ECL detection kit.

nase activity compared with that of the wild-type receptor, as determined by immunoblotting with an antibody to phosphotyrosine (Fig. 6). These results confirm previously published data (16) and the predictions based on the crystal structures of SRC tyrosine kinases (45). Hence, one mechanism to complement the receptor (Y809F) growth phenotype might be to maintain the mutant receptor on the cell surface, thus increasing the total signal output in response to ligand stimulation.

The effect of mPIP5K-Iβ (Δ1-238) expression on the rate of CSF-1R processing to the cell surface was determined in NIH 3T3 cells expressing the wild-type or mutant CSF-1R alone or CSF-1R (Y809F) together with truncated mPIP5K-Iβ. Cells were metabolically labeled with [^{35}S]methionine for 15 min, after which the medium was replaced with medium containing a 100-fold excess of nonradioactive methionine. Detergent lysates were prepared at various time intervals during the chase, and CSF-1R was immunoprecipitated and analyzed on denaturing polyacrylamide gels. The human CSF-1R immature glycoprotein (130 kDa) underwent modification of its N-linked oligosaccharide chains during transport to the cell surface to yield mature 150-kDa receptor molecules (Fig. 7) (25). As reported previously, in cells expressing the wild-type (Fig. 7A) or mutant (Fig. 7B) CSF-1R, the 130-kDa immature form of the receptor is converted almost completely to the mature 150-kDa form after 60 min, and this mature form has a half-life of 2 to 3 h (25). These results suggest that ectopic coexpression of mPIP5K-Iβ (Δ1-238) with CSF-1R (Y809F) did not affect receptor processing to the cell surface or receptor turnover in the absence of CSF-1 (Fig. 7C).

mPIP5K-Iβ (Δ1-238) alters CSF-1R endocytosis. To assay for receptors at the cell surface, viable cells were examined by fluorescence-activated flow cytometry. CSF-1R was readily detected on the cell surface with an antibody specifically recognizing epitopes in the extracellular domain of the receptor, whereas a control isotype-matched antibody gave a negative fluorescence profile (Fig. 8). When cells were incubated with recombinant human CSF-1 at 37°C, the cells expressing the wild-type (Fig. 8A) or mutant (Fig. 8B) CSF-1R displayed a significant decrease in cell surface expression of the receptor. In contrast, the clonal cell lines coexpressing CSF-1R (Y809F) and mPIP5K-Iβ (Δ1-238) failed to internalize CSF-1R, even after 60 min in the presence of CSF-1 (Fig. 8C and D). Several single cell clones were evaluated and showed the same inhibition of internalization in the presence of the growth factor (data not shown). To eliminate the possibility that expression of truncated mPIP5K-Iβ masked the epitope recognized by the

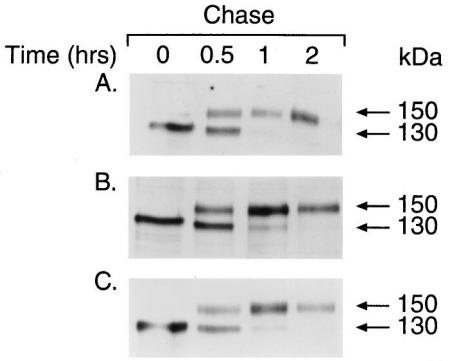

FIG. 7. Human CSF-1 receptor processing and turnover. NIH 3T3 cells expressing the human wild-type (A) or mutant (Y809F) (B) CSF-1R or coexpressing the mutant CSF-1R (Y809F) and truncated mPIP5K-Iβ (C) were metabolically pulse-labeled for 15 min with [^{35}S]methionine and then incubated in excess unlabeled-methionine-containing medium for various times. Detergent lysates were prepared, and the CSF-1R was immunoprecipitated with an antiserum to the human CSF-1R gene product. The radiolabeled CSF-1R was denatured in SDS, separated electrophoretically in polyacrylamide gels, and detected by fluorography. Chase intervals are shown above the lanes. The mobilities of the immature human CSF-1R protein (130 kDa) and the mature cell surface form (150 kDa) are on the right.

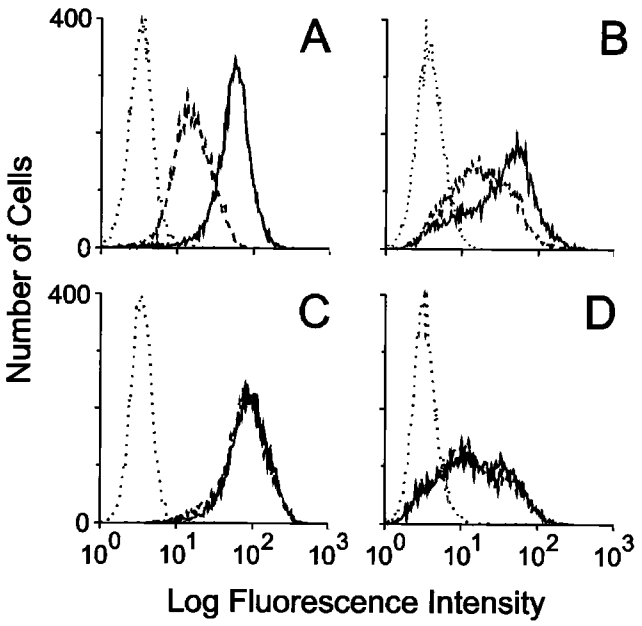

FIG. 8. CSF-1R internalization in response to ligand stimulation. Receptor expression, measured by flow cytometry, was determined for NIH 3T3 cells expressing the wild-type CSF-1R (A) or the mutant CSF-1R (Y809F) (B) or coexpressing the mutant CSF-1R (Y809F) and truncated mPIP5K-Iβ (C and D, representing two independent single-cell clones, subclones 7 and 8, respectively). CSF-1R fluorescence from unstimulated cells (——) or cells stimulated with recombinant human CSF-1 for 60 min (– – –) was compared to the background fluorescence from cells stained with a control antibody (· · · · ·).

specific monoclonal antibody, we used several monoclonal antibodies, as well as polyclonal antisera recognizing different epitopes of the human CSF-1R extracellular domain. In all of the cases, the data (not shown) were identical.

These results predicted that receptor kinase activity would also be increased in cells coexpressing CSF-1R (Y809F) and truncated mPIP5K-Iβ. Cells were stimulated with recombinant CSF-1 and harvested at different times thereafter, as indicated in the legend to Fig. 9. The CSF-1R was immunoprecipitated with an antibody to the CSF-1R, run on a polyacrylamide gel, and immunoblotted with either the same antibody or an antibody to phosphotyrosine. Figure 9 shows that although both the immature 130-kDa and mature 150-kDa forms of the receptor were detected with antibodies to the receptor itself (top), only the mature cell surface form underwent time-dependent autophosphorylation (bottom). As predicted, expression of mPIP5K-Iβ (Δ1-238) restored CSF-1R (Y809F) tyrosine phosphorylation (Fig. 9C) to levels approaching those detected for the wild-type CSF-1R (Fig. 9A). Taken together, these results show that enforced expression of truncated mPIP5K-Iβ altered ligand-mediated CSF-1R endocytosis, resulting in sustained expression of the activated receptor on the plasma membrane in the presence of ligand.

DISCUSSION

In this report, we describe the isolation and characterization of a cDNA clone isolated through a genetic screen that was designed to identify genes that complement a mitogenically defective CSF-1R. The complementing cDNA encoded a truncated form of mPIP5K-Iβ. Ectopic expression of the truncated lipid kinase restored CSF-1R (Y809F) mitogenic activity and allowed sustained CSF-1-dependent proliferation and ligand-stimulated induction of MYC and cyclin D1 protein expression with essentially the same kinetics as those elicited by the wild-type receptor. Sequencing of the cloned cDNA revealed that it corresponds to the carboxy-terminal half of mPIP5K-Iβ but lacks residues 1 to 238. Biochemical characterization confirmed that the full-length mPIP5K-Iβ protein possesses lipid kinase activity and demonstrated that the truncated protein is catalytically inactive. The isolation of mPIP5K-Iβ was surprising, not only because polyphosphoinositide turnover does not contribute to signaling by the CSF-1R (38), but also because mPIP5K-Iβ (Δ1-238) lacked kinase activity. Indeed, expression of full-length mPIP5K-Iβ did not complement CSF-1-dependent proliferation of cells expressing CSF-1R (Y809F) (data not shown). However, coexpression of truncated mPIP5K-Iβ and CSF-1R (Y809F) led to impaired CSF-1-dependent receptor internalization, resulting in elevated and sustained levels of tyrosine-phosphorylated CSF-1R (Y809F) on the cell surface. These data suggest that mPIP5K-Iβ (Δ1-238) complemented CSF-1R (Y809F) by interfering with receptor endocytosis, thereby compensating for the reduced overall activity of the mutant receptor and restoring the CSF-1-dependent mitogenic response.

There is ample evidence that polyphosphoinositides are involved in the regulation of vesicular trafficking, and the identification of the precise roles of the lipids and kinases in yeast and mammalian cells is an area of intense investigation (6, 22). In yeast, mutant forms of a nonessential PIP5K homolog, Fab1p, exhibit defective vacuole function and morphology (46). In mammals, the list of polyphosphoinositide-binding proteins implicated in almost every aspect of vesicular movement within cells is constantly growing (10). Many of the pro-

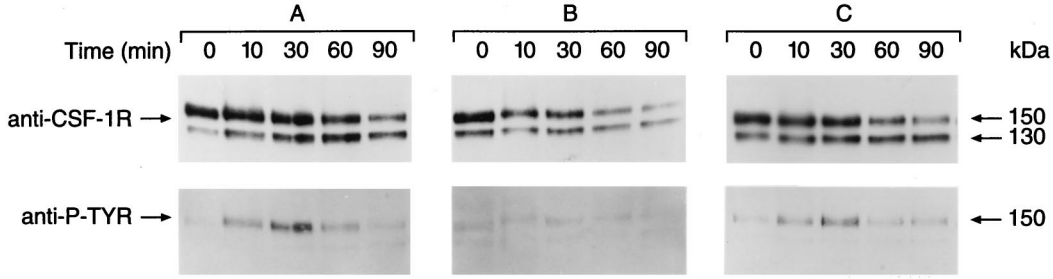

FIG. 9. Ligand-induced CSF-1R tyrosine kinase activity. NIH 3T3 cells expressing the wild-type human CSF-1R (A), the mutant CSF-1R (Y809F) (B), or the mutant CSF-1R (Y809F) with mPIP5K-Iβ (Δ1-238) (C) were stimulated for different times in medium containing purified recombinant human CSF-1. Detergent cell lysates were prepared, and the CSF-1R was immunoprecipitated with an antiserum to the CSF-1R. Equal amounts of protein were used per immunoprecipitation. The CSF-1R was separated by SDS-PAGE, transferred to nitrocellulose, and immunoblotted successively with antibodies to the human CSF-1R and to phosphotyrosine. The mobilities of the immature form of the human CSF-1R protein (130 kDa) and the mature cell surface form (150 kDa) are noted to the right.

teins that interact with polyphosphoinositides do so through pleckstrin homology domains, which selectively bind to specific inositol phospholipids (14, 20). Current thinking is that PtdIns 4-kinase, which is associated with vesicle membranes, works in concert with cytosolic PIP5K to generate PtdIns(4,5)P2. PtdIns (4,5)P2, in turn, can function as a molecular tag to recruit additional cytosolic components that are required for the proper sorting and targeting of vesicles (10). Moreover, PtdIns (4,5)P2 is a substrate for PtdIns 3-kinase (PI3-K), which, through the formation of PtdIns(3,4,5)P3, is thought to play a key role in growth factor-stimulated targeting of receptors to lysosomes. Deletion of the PI3-K binding site in either the murine CSF-1R or PDGF-R blocks the sorting of these receptors to lysosomes, causing them to be recycled to the cell surface after ligand-induced internalization (7, 17, 18). Therefore, it is possible that inhibition of PtdIns(4,5)P2 synthesis by truncated mPIP5K-Iβ (Δ1-238) might affect PI3-K function by limiting the supply of one of its key substrates. Since recycling to the cell surface and the inhibition of internalization will both maintain receptors on the cell surface, we cannot distinguish whether the effect of mPIP5K-Iβ (Δ1-238) was due to interference with receptor internalization or the inhibition of subsequent sorting. Our finding that expression of a truncated form of mPIP5K-Iβ obstructs ligand-mediated trafficking of CSF-1R (Y809F) suggests that type Iβ PIP5K and phosphoinositide metabolism are intimately involved in receptor endocytosis.

The fact that mPIP5K-Iβ (Δ1-238) is catalytically inactive suggests that it exerts its effects on receptor metabolism by impeding the normal function of mPIP5K-Iβ. Whereas mammalian PtdIns 4-kinases are predominantly membrane-bound enzymes (8, 21, 44), PIP5Ks are found distributed between the cytosolic and membrane fractions (22). Cells can be depleted of type I PIP5K isozymes by using permeabilization techniques designed to remove cytosolic components (13). These isozymes are easily extracted from membrane fractions with salt and purified as soluble proteins in the absence of detergents or phospholipid vesicles (5, 22). The type I PIP5K enzymes are also potently stimulated by phosphatidic acid (15, 22), illustrating that catalytic activity is enhanced by the presentation of substrate in association with anionic phospholipid vesicles. Our finding that mPIP5K-Iβ overexpression in COS-7 cells does not increase the cellular levels of PtdIns(4,5)P2 is consistent with the idea that the access of mPIP5K-Iβ to its substrate is tightly controlled. Likewise, expression of truncated mPIP5K-Iβ in COS-7 cells did not reduce the steady-state level of PtdIns(4,5)P2, showing that the truncated protein does not affect basal PtdIns(4,5)P2 synthesis. These properties suggest that an important component of type I PIP5K regulation may be its reversible association with membranes. The truncated mPIP5K-Iβ protein may act as a dominant-negative inhibitor by binding to the membrane docking sites for mPIP5K-Iβ and blocking the recruitment of catalytically competent mPIP5K-Iβ to the vesicle. This mechanism would disrupt the highly localized synthesis of PtdIns(4,5)P2 and, subsequently, PtdIns (3,4,5)P3 and would impede endocytosis or sorting by retarding the recruitment of additional cytosolic components of the vesicle. If this hypothesis is correct, it identifies the carboxy terminus of mPIP5K-Iβ as the critical region involved in the regulation of mPIP5K-Iβ translocation and targeting. It is important to note that the primary structure of the carboxy terminus of the Iα isoform is quite different from that of the Iβ isoform (15, 22).

The precise biochemical mechanism by which truncated mPIP5K-Iβ inhibits receptor internalization remains to be determined; however, our studies provide genetic evidence that mPIP5K-Iβ is intimately involved in receptor endocytosis.

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