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FIN13, a Novel Growth Factor-Inducible Serine-Threonine Phosphatase Which Can Inhibit Cell Cycle Progression

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We have identified a novel type 2C serine-threonine phosphatase, FIN13, whose expression is induced by fibroblast growth factor 4 and serum in late G_1 phase. The protein encoded by *FIN13* cDNA includes N- and C-terminal domains with significant homologies to type 2C phosphatases, a domain homologous to collagen, and an acidic domain. FIN13 expression predominates in proliferating tissues. Bacterially expressed FIN13 and FIN13 expressed in mammalian cells exhibit serine-threonine phosphatase activity, which requires Mn^{2+} and is insensitive to inhibition by okadaic acid. FIN13 is localized in the nuclei of transiently transfected cells. Cotransfection of FIN13-expressing plasmids with a plasmid that expresses the neomycin resistance gene inhibits the growth of drug-resistant colonies in NIH 3T3, HeLa and Rat-1 cells. In transiently transfected cells, FIN13 inhibits DNA synthesis and results in the accumulation of cells in G_1 and early S phases. Similarly, the induction of expression of FIN13 under the control of a tetracycline-regulated promoter in NIH 3T3 cells leads to growth inhibition, with accumulation of cells in G_1 and early S phases. Thus, overexpression and/or unregulated expression of FIN13 inhibits cell cycle progression, indicating that the physiological role of this phosphatase may be that of regulating the orderly progression of cells through the mitotic cycle by dephosphorylating specific substrates which are important for cell proliferation.

Central to the biological activity of a polypeptide growth factor is its ability to activate a signaling cascade which acts on the nucleus to induce the necessary genetic program for cell division (10, 31). Growth factors generate not only positive signals for stimulating cell division but also negative signals to protect against sustained or aberrant proliferation (24). Various positive and negative incoming signals are integrated at multiple checkpoints in the cell cycle, and depending on the quality and temporal relationship, the following possible cell fates are decided: (i) progression through the cell cycle, (ii) growth arrest, (iii) differentiation, and (iv) cell death (26). Pivotal to these outcomes is the signaling that arises from both kinases and phosphatases. Molecular switches that involve reversible phosphorylation of proteins constitute an important mechanism by which signals are transduced within cells and are an integral part of the positive and negative circuits that regulate cell proliferation (6, 19, 21, 23). A further consideration in the regulation of both positive and negative circuits is that genes which encode kinases and phosphatases that are transcriptionally induced by growth factor stimulation may amplify, attenuate, or modify additional signaling events as cells progress through the cycle. However, while it has been recognized that modification of protein activity or function by protein phosphatases may play a role as important as that of protein kinases in regulating cell growth or cycle progression, the elucidation of the specific functions of cellular phosphatases in growth regulation has lagged behind the studies of kinases (19).

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Phosphatases can be generally classified according to substrate specificity into serine-threonine and tyrosine phosphatases. In addition, dual-specificity phosphatases which dephosphorylate both serine-threonine and tyrosine residues and are structurally related to tyrosine phosphatases have previously been described. They can be cytosolic, nuclear, or membrane-spanning proteins (19). Recently, a number of phosphatases involved in cell cycle regulation have been described. For example, it has been shown in yeast that cdc25, a dual-specificity phosphatase, acts as an initiator of mitosis by activating $p34^{cdc2}$, and a similar role in controlling the cell cycle has been proposed for cdc25 mammalian homologs (12, 15). A feedback mechanism has been proposed for the highly conserved ras–mitogen-activated protein (MAP) kinase signaling pathway. The immediate-early growth factor-inducible gene MAP kinase phosphatase-1 (MKP-1) encodes a dual-specificity phosphatase that dephosphorylates MAP kinase in vivo and attenuates its activity (33). With few exceptions, however, the role attributed to specific phosphatases in growth control has been largely inferential.

In order to dissect the fibroblast growth factor (FGF)-regulated genetic program that mediates cell proliferation, we isolated 21 cDNAs containing FGF-inducible genes (FIN genes) (17). In this communication, we show that one of the novel FIN genes, *FIN13*, encodes a type 2C serine-threonine phosphatase which is localized to the nucleus and negatively regulates cell growth. Overexpression of FIN13 causes G_1/S arrest, indicating that it may have a role in regulating cell cycle progression at this boundary.

MATERIALS AND METHODS

Northern analysis. RNA extraction and Northern analysis were performed essentially as previously described (17). To stimulate folliculogenesis, immature (3-week-old) mice were injected subcutaneously with diethylstilbestrol (DES) or vehicle essentially as previously described (16) and RNAs were prepared from ovaries 4 days after stimulation.

/Construction of FIN13 expression plasmids. The pEThis-FIN13 and pEThis-FIN13-cat constructs were used for expressing full-length FIN13 and the catalytic domain of FIN13, respectively, as a fusion protein with a polyhistidine tag in bacteria. The *FIN13* open reading frame (from M-1 to D-392) was amplified by PCR from the original *FIN13* cDNA in Bluescript (17) with a 5′ primer containing a *Bam*HI restriction site (GAGAGGATCCCATGACTATTGAAGGC TGCTG) and a 3′ primer containing a *Not*I restriction site (CTCTCGCGGCC GCGTCCCTCTTGGCCTTCTTTTTG). The PCR product was digested with *Bam*HI and *Not*I and cloned into the *Bam*HI/*Not*I site of pET28a (Novagen) to give pEThis-FIN13. This construction resulted in the expression of a FIN13 fusion protein that contains both N- and C-terminal six-histidine tags, with the pET28a stop codon being utilized. pEThis-FIN13-cat was made by removing the sequences encoding the collagen homology (CH) domain and the acidic box by *Bam*HI/*Hin*dIII restriction enzyme digestion. The linearized plasmid was then religated with an adapter (sense primer, GATCCTGACTCTAGATGTGTGGT GTCCGAGGCTGCCAA) that preserved the *FIN13* open reading frame. This construction resulted in the expression of the catalytic domain of FIN13 from amino acids 195 to 392 (see Fig. 1) fused to both N- and C-terminal six-histidine tags. For expression of FIN13 in mammalian cells, the *FIN13* cDNA in Bluescript was subcloned into the pRK5 mammalian expression vector in several steps. An *Hga*I/blunt-ended *Spe*I fragment of the original *FIN13* cDNA was subcloned by using an *Xho*I/*Hga*I adapter made by hybridizing oligonucleotides with the sequences 5′-TCGAGGCCACCATGACTATTGAAGAGCTGCTGACGCGAT AT-3′ and 5′-TGCCCATATCGCGTCAGCAGCTCTTCAATAGTCATGGTG GCC-3′ into the *Xho*I/*Eco*RV site of pMJ30 to give pMJFIN13. A blunt-ended *Bgl*II/partial *Hin*dIII-digested fragment was released from pMJFIN13 and subcloned into the *Hin*dIII/*Sma*I site of Bluescript KS to give pBFIN13-15. pRKFIN13 was made by subcloning the *Cla*I/*Bam*HI fragment from pBFIN13-15 into the *Cla*I/*Bam*HI site of pRK5. pRKmyc-FIN13, which expresses FIN13 N terminally fused to the myc epitope, was made by subcloning hybridized oligonucleotides into the *Cla*I site of pRKFIN13. The sequence of the oligonucleotide for the sense strand was 5′-CGATGCCACCATGGAACAGAAACTG ATTTCCGAAGAAGATCTGAT-3′. Sequencing revealed that pRKmyc-FIN13 contained two copies of the myc epitope in frame with and N terminal to the *FIN13* open reading frame. The construct expressing FIN13 under the control of the *tet* operon, pUHDFIN13, was made by subloning the *Xba*I/blunt-ended *Cla*I *FIN13* cDNA from pRKFIN13 into the *Xba*I/blunt-ended *Eco*RI site of pUHD10-3 (14). The 5′ end of the long form of *FIN13* cDNA was cloned from NIH 3T3 cells by reverse transcription (RT)-PCR. One microgram of RNA was denaturated for 5 min at 65°C in the presence of 0.3 mM methylmercury and 0.05 M β-mercaptoethanol. RNA was reverse transcribed with random hexamer primers, and PCR was performed with a 3′ primer containing *FIN13* cDNA sequences flanking the *Xho*I restriction site (5′-TTAGCAACTCGAGGCAGC TTG-3′) and a 5′ primer corresponding to the 5′ portion of a murine expressed sequence tag (EST; GenBank accession no. W34891) (5′-CAGCCGCCGCCAT GGGTGCCTACCTCTCTC-3′). The PCR product was cloned into the TA vector (Stratagene) and digested with *Eco*RV and *Xho*I. The fragment of 766 nucleotides was cloned upstream to the *Xho*I site in the pRKFIN13 expression vector. The open reading frames of all FIN13 expression constructs were confirmed by sequencing.

Preparation of ^{32}P-labelled casein. Casein (10 mg; Sigma) was labelled with ^{32}P by using 10 U of the catalytic subunit of cyclic AMP-dependent protein kinase (Sigma) in 50 mM Tris-HCl (pH 7.0)–0.1 mM EGTA–10 mM magnesium acetate–0.1% β-mercaptoethanol–0.5 mCi of [γ-^{32}P]ATP for 12 h at 30°C. The reaction mixture was then precipitated at 4°C in 20% trichloroacetic acid (TCA). Labelled casein (specific activity, 43 nmol of ^{32}P/mg) was recovered by centrifugation, and the pellet was washed five times in 100% acetone and resuspended in 50 mM Tris-HCl–0.1 mM EGTA.

Phosphatase assay. The phosphatase activity of purified FIN13 expressed in bacteria as a fusion protein with N- and C-terminal His tags was assessed in vitro with ^{32}P-labelled casein as the substrate. For preparations of His-FIN13 and His-FIN13-cat from bacteria, BL21(DE3) strains transformed with pEThis-FIN13 and pEThis-FIN13-cat were induced with 0.2 mM IPTG (isopropyl-β-D-thiogalactopyranoside) for 2 h, after which cells were recovered by centrifugation, resuspended in column buffer (50 mM sodium phosphate [pH 8.0], 300 mM NaCl, 1 mM phenylmethylsulfonyl fluoride [PMSF]), and frozen overnight at −70°C. Bacteria were lysed by sonication at 4°C, and the extract was cleared by centrifugation. The supernatant was then adsorbed to Ni-nitrilotriacetic acid-agarose (Qiagen) at 4°C for 1 h. The resin was washed extensively in column buffer without PMSF, and His-FIN13 or His-FIN13-cat was eluted with 25 mM Tris-HCl (pH 7.2)–100 mM NaCl containing 0.2 mM imidazole (Sigma). In addition, the phosphatase activity of FIN13 expressed in HeLa cells was determined in an immune complex with ^{32}P-labelled casein as the substrate. For preparations of immune complexes of myc-FIN13, HeLa cells were transfected with 10 μg of pRKmyc-FIN13 or pRK5. Cells were lysed at 24 h after transfection in lysis buffer (10 mM sodium phosphate [pH 7.4], 100 mM NaCl, 1% Triton X-100, 10 mM EDTA) containing a protease-phosphatase inhibitor cocktail (1 mM PMSF, 1 μg of pepstatin per ml, 1 μg of leupeptin per ml, 1 μg of aprotinin, per ml, 10 mM sodium orthovanadate, 80 mM NaF, 80 mM β-glycerophosphate, 10 μM okadaic acid). Cleared lysates (100 μg of total protein) were immunoprecipitated with anti-myc monoclonal antibody (9E10) adsorbed to 30 μl of protein G-Sepharose (Zymed) or anti-FIN13 antiserum adsorbed to 30 μl of protein A-Sepharose (Zymed) for 1.5 h at 4°C. The anti-FIN13 polyclonal antiserum (630) was generated against the full-length His-FIN13 expressed and purified from bacteria. The immunoprecipitates (IPs) were washed five times with lysis buffer, once with 10 mM Tris-HCl (pH 7.0)–100 mM NaCl–5 mM $MnCl_2$, and twice with phosphatase reaction buffer (10 mM Tris-HCl [pH 7.0], 0.1 mM EGTA, 10 mM dithiothreitol, and 10 mM $MnCl_2$) containing 1 μM okadaic acid. Purified His-FIN13, His-FIN13-cat, and myc IPs were then examined for phosphatase activity by incubating the indicated FIN13 preparation in the presence of phosphatase reaction buffer containing ^{32}P-labelled casein (10^5 cpm; approximately 1 μl) at 30°C for up to 2 h. Okadaic acid (1 μM), poly-L-lysine (20 μg/ml), and protamine sulfate (20 μg/ml) were added where indicated and were preincubated with FIN13 preparations at 30°C for 5 min prior to addition of the ^{32}P-labelled substrate. After incubation, total protein was precipitated by adding TCA to a final concentration of 20% (wt/vol) and insoluble material was pelleted by centrifugation. Inorganic ^{32}P recovered in the supernatant was monitored by scintillation counting.

Transfection of cells. Cells were maintained in Dulbecco modified Eagle medium (DMEM)–10% calf serum as previously described (17). NIH 3T3, Rat-1, and HeLa cells were transfected for 12 to 19 h by $CaPO_4$ precipitation by standard procedures. For the isolation of stably transfected cell lines, cells were plated out 24 h after transfection in DMEM–10% calf serum containing 400 μg of G418 (Gibco-BRL) per ml or 250 μg of hygromycin B (Calbiochem) per ml. Single-cell-originated clones were selected after 7 to 10 days. To establish cell lines that express inducible FIN13, NIH 3T3 cells were cotransfected with 5 μg of pUHD15-1 (a construct expressing tTA) (14) and 0.2 μg of pRSVneo, which contains the G418 resistance gene. After 2 weeks of G418 selection, colonies were picked and screened for the expression of tTA by transient transfection of a construct that expresses luciferase under the control of the *tet* operon (pUHC13-3) by the method of Gossen and Bujard (14). Clones found to express inducible tTA in the luciferase assay were cotransfected with 5 μg of pUHDFIN13 and 0.2 μg of pCEP4, which contains the hygromycin B resistance gene, in DMEM–10% calf serum containing 1 μg of tetracycline (Sigma) per ml. Twenty-four hours after transfection, cells were plated in medium containing 1 μg of tetracycline per ml, 250 μg of hygromycin B per ml, and 400 μg of G418 per ml. The medium was changed every 4 or 5 days, and fresh tetracycline (1 μg/ml) was added every day. Drug-resistant clones were picked after 2 to 3 weeks and cultured by the same protocol.

Western blot analysis. Cells were lysed in lysis buffer containing a protease-phosphatase inhibitor cocktail (see above). Equal amounts of lysates (determined by Bradford protein staining reagent; Bio-Rad) were electrophoresed on a sodium dodecyl sulfate-polyacrylamide gel and transferred to a nitrocellulose membrane (MSI). Equal loading was confirmed by staining in 0.2% Ponceau–6% TCA. The membrane was blocked overnight at 4°C in 5% bovine serum albumin (BSA) in phosphate-buffered saline (PBS), incubated with primary and secondary antibodies for 1 h each at room temperature, and finally developed with an enhanced chemiluminescence kit (Amersham) by standard procedures. Anti-FIN13 polyclonal antibody (630) was used at a dilution of 1:500. Anti-myc monoclonal antibody (9E10 hybridoma supernatant) was used at 1:25.

Growth curves of tetracycline-inducible cells. Cell lines found to demonstrate FIN13-inducible expression after tetracycline removal were examined for their growth properties. Cells were plated in triplicate at 10,000 cells/ml on 24-well plates in DMEM–10% dialyzed calf serum in either the presence or the absence of 1 μg of tetracycline per ml. Cells were counted every day (Coulter counter), and the medium, with or without tetracycline, was changed every 2 days.

BrdU incorporation. The effect of FIN13 expression on bromodeoxyuridine (BrdU) incorporation was determined in HeLa cells growing asynchronously in DMEM–10% calf serum. Cells were plated at 10,000 cells/ml on coverslips and after 24 h were transfected with 10 μg of either pRKERK2-HA, CMVβ, or pRKFIN13. Twenty-four hours after transfection, the medium was changed and cells were incubated with BrdU labelling reagent (10 μl) for a further 12 h according to the supplier's (Boehringer Mannheim) instructions. After labelling, coverslips were rinsed in PBS and cells were fixed in 70% ethanol–50 mM glycine (pH 2.0) overnight at −20°C. Incorporated BrdU was detected with fluorescein isothiocyanate (FITC)-conjugated anti-BrdU (Boehringer Mannheim) by immunocytochemistry (see below).

Immunocytochemistry. Fixed cells on coverslips were washed twice with PBS and twice with PBS–0.02% BSA, followed by incubation with the primary antibody diluted in PBS–0.02% BSA for 1 h at 37°C (anti-FIN13 polyclonal serum at 1:200, anti-hemagglutinin (HA) monoclonal antibody 12CA5 at 1:200, or anti-β-galactosidase GAL40 monoclonal antibody at 1:500). After being washed, coverslips were incubated in secondary antibody as described above (anti-rabbit Texas red at 1:200 or anti-mouse Texas red at 1:200) (Molecular Probes). Coverslips were washed again and then incubated in 200 pg of (1:500) FITC-conjugated anti-BrdU (Boehringer Mannheim) per ml as described above. Where noted, cells were also stained with 1 μg of bisbenzimide (Hoechst 33342) per ml in PBS. Coverslips were mounted and examined with a fluorescence microscope.

FACS. HeLa cells growing asynchronously in DMEM–10% calf serum were cotransfected with 5 μg of a plasmid expressing a humanized version of green fluorescent protein (GFP) (pcDNA3hGFP) and 15 μg of either pRKERK2-HA, CMVβgal, or pRKFIN13. At 36 or 60 h after transfection, cells (5×10^6 to 10×10^6) were washed and harvested by trypsinization, and GFP-expressing cells were recovered by fluorescence-activated cell sorting (FACS). Cells were fixed in 80%

a

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MTIEELLTRY GQNCQKVPPH TKSGIGTGDE PGPQGLNGEA GPEDPSRETP 50
SQENGPTAKG HTGFSSNSEH GTEAGQISEP GTATGEAGPS CSSASDKLPR 100
VAKSKFFEDS EDESDEVEEE EDDSEECSED EDGYSSEEAE NEEDEDDTEE 150
AEEDDDEEMM VPGMEGKEEP GSDSGTTAVV ALIRGKQLIV ANAGDSRCVV 200
SEAGKALDMS YDHKPEDEVE LARIKNAGGK VTMDGRVNGG LNLSRAIGDH 250
FYKRNKNLPP QEQMISALPD IKVLTLTDDH EFMVIACDGI WNVMSSQEVV 300
DFIQSKISQR DENGELRLLS SIVEELLDQC LAPDTSGDGT GCDNMTCIII 350
CFKPRNTVEL QAESGKRKLE EALSTEGAED TGNSDKKKAK RD         392
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b

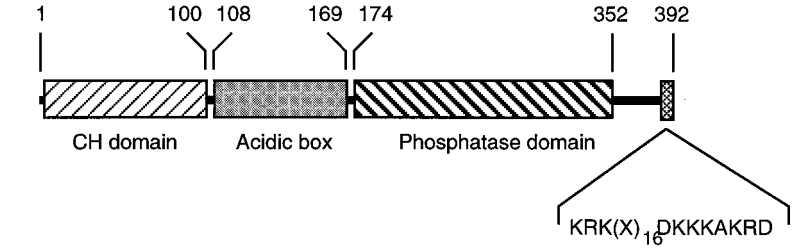

FIG. 1. (a) Deduced amino acid sequence of the FIN13 protein. (b) Schematic representation of the deduced FIN13 protein, showing the collagen homology (CH) domain, acidic box, phosphatase domain, and putative C-terminal dipartite nuclear translocation sequence.

ethanol at −20°C overnight and recovered by centrifugation. The fixed cell pellet was resuspended in 0.75 ml of PBS and DNA was stained with propidium iodide (200 μg of propidium iodide per ml, 40 mM sodium citrate [pH 7.0]) (Sigma) containing 100 μg of RNase A (Sigma) per ml at 37°C for 2 h. Cells were examined for DNA content with a FACScan flow cytometer, and the percentages of cells in G_0/G_1, S, and G_2/M phases were determined with ModFit software.

RESULTS

FIN13 structure and expression. *FIN13* was originally cloned as a murine FGF-inducible gene by a subtractive hybridization approach (GenBank accession no. U42383) (17). We have previously demonstrated that both serum and FGF4 induce FIN13 mRNA and that these changes are due to transcriptional activation (17). The 1,696-nucleotide sequence of the *FIN13* cDNA originally isolated contains a single long open reading frame encoding a 392-amino-acid protein with a predicted molecular mass of 42,292 Da (Fig. 1a). Since this cDNA contains no stop codons in frame with and 5′ of the putative translation initiation AUG, we could not be absolutely certain that the cDNA was full length. However, the putative initiator codon lies within a good context for translation initiation (GCTACCATGA [20]), a number of cDNA clones with similar 5′ ends were isolated (see Fig. 10), and RACE-PCR (11) also yielded clones with similar 5′ ends. We therefore decided to characterize the activity and biological effects of the protein encoded by this cDNA.

A comparison of the putative protein with entries in the GenBank database revealed the following four distinct homology domains: (i) an N-terminal CH domain with homologies to various collagens, including mouse alpha-2 (IV) collagen (29% identical) (29) and chicken alpha-3 (IX) collagen (29% identical) (18); (ii) a central acidic box rich in aspartic acid and glutamic acid residues (65% over 62 amino acids); (iii) a phosphatase domain with homologies to various type 2C phosphatases from *Saccharomyces cerevisiae* (46% identical) (30), *Caenorhabditis elegans* (45% identical) (37), rats (38% identical) (36), rabbits (36% identical) (21), and mice (35% identical) (34); and (iv) a C-terminal basic domain characteristic of dipartite nuclear translocation sequences (9) (Fig. 1b). The homology of FIN13 to type 2C phosphatases did not extend beyond the catalytic domains of these proteins, and no significant homologies were observed for other classes of phosphatases. Based on these data, the *FIN13* cDNA appears to encode a new member of this family of enzymes.

We have previously shown that while the *FIN13* gene is growth factor inducible in cultured fibroblasts and PC12 cells, its expression is largely restricted in adult mouse tissues to the testis (17). We now show that FIN13 RNA is also expressed in the embryo and is induced in a range of mouse tissues undergoing proliferation, such as the pregnant uterus, the placenta, and ovaries stimulated to undergo folliculogenesis with DES (Fig. 2). The induction of FIN13 by growth factors in cultured cells and its induction in diverse tissues undergoing proliferation could imply that FIN13 serves an important role in mediating the cellular response to growth factors.

FIN13 is a type 2C phosphatase localized in the nucleus. To determine whether FIN13 is a bona fide type 2C phosphatase, we examined the in vitro activity of purified FIN13 expressed in bacteria as a fusion protein with a histidine tag (His-FIN13). Type 2C phosphatases are characterized by their requirement for divalent cations and their insensitivity to the phosphatase inhibitor okadaic acid (1, 6, 19). His-FIN13 was able to dephosphorylate ^{32}P-labelled casein in the presence of Mn^{2+} ions, with maximal activity obtained at 10 mM $MnCl_2$ (Fig. 3). Only very low activity was observed in the presence of $MgCl_2$ or $CaCl_2$. The addition of 10 mM EDTA to the phosphatase assay greatly reduced activity. Although other type 2C phosphatases have been reported to be active in the presence of either Mn^{2+} or Mg^{2+}, His-FIN13 exhibited a preferential requirement for $MnCl_2$. The restricted requirement for $MnCl_2$ likely derives from the bacterial expression of His-FIN13, since it has also been observed for bacterially expressed protein phosphatase 2Cα (22) (see below). Furthermore, it is known that Mn is more active than Mg is in stimulating the activity of other 2C phosphatases (7). The catalytic domain of FIN13 (His-FIN13-cat) exhibited poor phosphatase activity at 10 mM

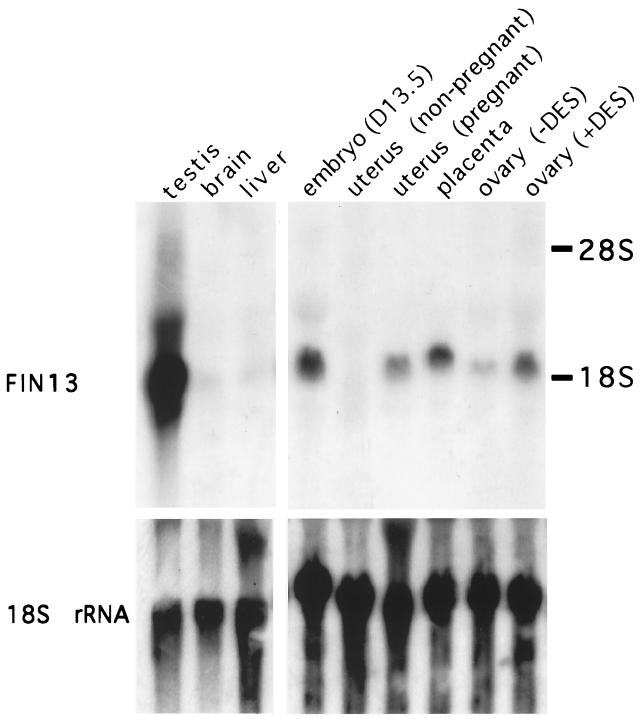

FIG. 2. Northern analysis of FIN13 expression in mouse tissues. Total RNA (15 μg) from each indicated tissue was examined for the expression of FIN13 mRNA by Northern blotting with a ^{32}P-labelled *FIN13* full-length cDNA probe. The positions of the 28S and 18S rRNAs are indicated on the right. Loading for each sample was confirmed by hybridization with a ^{32}P-labelled 18S rRNA probe. −DES, without DES; +DES, with DES; D13.5, day 13.5 of gestation.

$MnCl_2$, but activity was detectable at 400 mM. Thus, it appears that while the catalytic domain alone demonstrates phosphatase activity at high $MnCl_2$ concentrations, other domains are also important for activity or protein conformation, at least in bacterially expressed FIN13. As expected, the activity of His-FIN13 was not inhibited by the addition of okadaic acid. The presence of a highly charged acidic domain in FIN13 led us to examine the effects of highly charged basic molecules on phosphatase activity. Neither poly-L-lysine nor protamine sulfate significantly altered His-FIN13 activity.

The I phosphatase activity of IPs of FINI3 from transfected HeLa cells was also examined. HeLa cells were transfected with a construct that expresses FIN13 N terminally tagged with a myc epitope (pRKmyc-FIN13). myc-FIN13 was immunoprecipitated from HeLa cell extracts with 9E10 (anti-myc monoclonal antibody) or 630 (anti-FIN13 polyclonal antiserum), and the IP was washed extensively and then either used for Western analysis or examined in a phosphatase assay for its ability to dephosphorylate ^{32}P-labelled casein in vitro. Immunoprecipitations with either 9E10 or 630, followed by Western analysis with 630 antiserum, resulted in the detection of a single band of about 60 kDa in pRKmyc-FIN13-transfected cells (Fig. 4a). FIN13 expressed in bacteria or in NIH 3T3 cells also has an apparent molecular mass of 60 kDa (data not shown) (see Fig. 9). Although the predicted molecular mass of FIN13 is 42 kDa, the anomalous migration of FIN13 on a polyacrylamide gel could be due to the highly charged acidic box. Nonetheless, both 9E10 and 630 detect the same 60-kDa band in cells transfected with a FIN13 expression construct, confirming the specificity of these antibodies. IPs were then examined for phosphatase activity. Assays were performed in the presence or absence of 1 μM okadaic acid. myc-FIN13 IPs from HeLa cells with either 9E10 or 630 exhibited phosphatase activity in vitro (Fig. 4b). No significant phosphatase activity was ob-

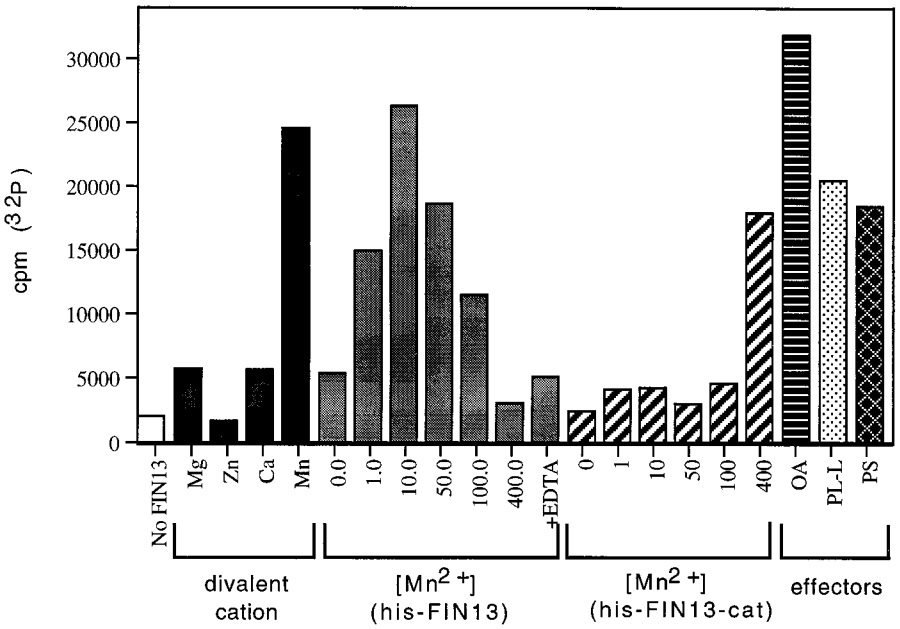

FIG. 3. Phosphatase activity of bacterially expressed FIN13. Purified, His-tagged full-length FIN13 (His-FIN13) and the His-tagged catalytic domain of FIN13 (His-FIN13-cat) were examined for the ability to dephosphorylate ^{32}P-labelled casein in vitro as described in Materials and Methods. Phosphatase assays were performed in the absence of His-FIN13 (No FIN13), with His-FIN13 incubated in the presence of 10 mM divalent cation ($MgCl_2$, $ZnCl_2$, $CaCl_2$, or $MnCl_2$), or in the presence of the indicated millimolar concentration of $MnCl_2$. +EDTA, His-FIN13 incubated in the presence of 10 mM EDTA and 10 mM $MnCl_2$. His-FIN13-cat was incubated in the presence of the indicated millimolar concentration of $MnCl_2$. His-FIN13 was also incubated in the presence of 1 μM okadaic acid (OA), 20 μg of poly-L-lysine (PL-L) per ml, or 20 μg of protamine sulfate (PS) per ml. All incubations were performed for 1 h at 30°C. The optimal phosphatase activity was 1.11 U/mg (1 U = 1 μmol of phosphate released/min).

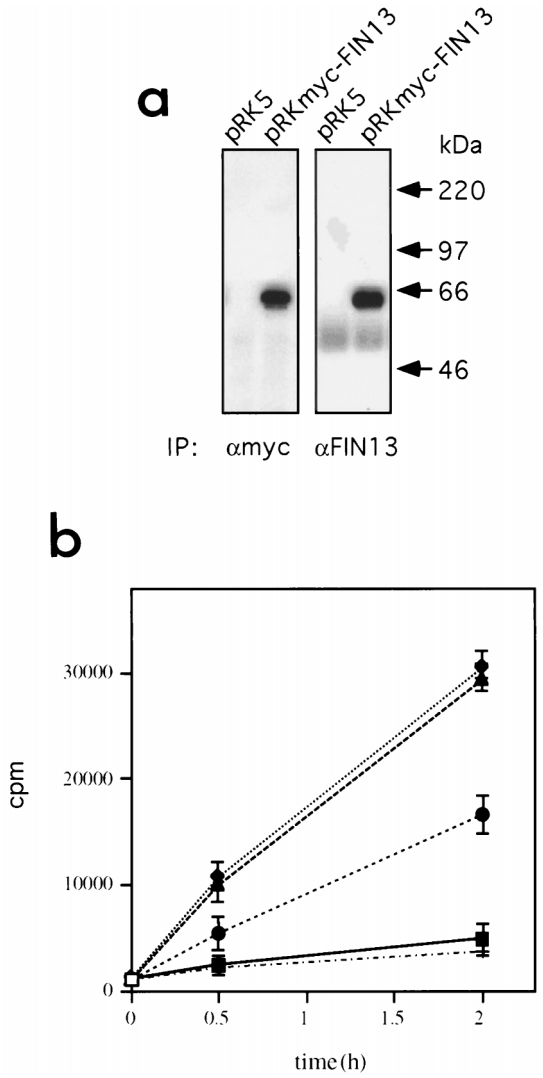

FIG. 4. Phosphatase activity and expression of FIN13 in mammalian cells. HeLa cells transfected with pRK5 vector or pRKmyc-FIN13 (expressing myc-FIN13) and harvested 24 h later were lysed in lysis buffer, and cleared extracts (100 μg) were immunoprecipitated with either anti-myc antibody (αmyc; 9E10) or anti-FIN13 antiserum (αFIN13) (630). IPs were divided and subjected either to Western analysis for the detection of FIN13 expression (a) or to a phosphatase assay (b). (a) For the detection of FIN13, one-fourth of the IP was boiled in sample buffer, loaded onto a 10% polyacrylamide gel, electrophoresed, and transferred to a nitrocellulose filter. Western blot analysis was performed with the 630 antiserum at 1:500 as described in Materials and Methods. (b) For an analysis of phosphatase activity, the ability of myc-FIN13 IPs (one-eighth of IP) to dephosphorylate ^{32}P-labelled casein was examined in vitro in the presence of okadaic acid and $MnCl_2$ as described in Materials and Methods. Transfections: pRK5, squares and circles; pRKmyc-FIN13, diamonds and triangles. IPs: 9E10, squares and diamonds; 630, circles and triangles. The IP from pRKmyc-FIN13-transfected HeLa cells with the 9E10 antibody was also subjected to a phosphatase assay in the presence of 10 mM EDTA (+).

served in 9E10 IPs from control pRK5-transfected cells. In addition, EDTA (10 mM) abolished the observed FIN13 activity in 9E10 IPs. Interestingly, FIN13 IPs from mammalian cells exhibited phosphatase activity in the presence of both Mn^{2+} and Mg^{2+} (data not shown), indicating that the specific requirement for Mn^{2+} (Fig. 3) is a feature of FIN13 that stems from bacterial expression. Reduced but significant phosphatase activity was detected in 630 IPs from pRK5-transfected cells. This activity could be due to immunoprecipitation of endogenous FIN13 by the 630 antiserum or immunoprecipitation of other cross-reacting 2C phosphatases. All these activities were insensitive to inhibition by okadaic acid (data not shown). Protein phosphatase 2A, which is a serine-threonine phosphatase of the type 2A family and is okadaic acid sensitive (21), was used as the control. As expected, the activity of protein phosphatase 2A was abolished in the presence of okadaic acid (data not shown).

Indirect immunofluorescence assay of pRKmyc-FIN13-transfected HeLa cells was performed to determine the subcellular localization of FIN13. In line with its C-terminal putative nuclear translocation signal, FIN13 was found by using the 630 antibody to be localized almost exclusively in the nucleus, as shown by colocalized fluorescence of the DNA-binding Hoechst 33342 stain (Fig. 5). Identical localization was observed with the 9E10 antibody, and omission of the primary antibody resulted in no significant fluorescence (data not shown). FIN13 was also localized in the nuclei of HeLa, NIH 3T3, and COS-7 cells transiently transfected with pRKFIN13 (see Fig. 7) (data not shown). The morphology of the nuclei of FIN13-expressing cells was somewhat abnormal, with nuclear enlargement. Hoechst staining was more diffuse compared to that of nontransfected cells (Fig. 5). Expression of FIN13 for more than 48 h was frequently associated with a number of more severe morphological abnormalities, such as nuclear vesiculation, nuclear membrane breakdown, and nuclear condensation.

The effect of FIN13 expression on cell growth. As *FIN13* was isolated as an FGF-inducible gene and is expressed in vivo in tissues undergoing proliferation, we decided to investigate the effects of FIN13 expression on cell growth. In order to generate stable cell lines that express FIN13, NIH 3T3 cells were cotransfected with a neomycin resistance plasmid (pRSVneo) and a construct that expresses FIN13 under the control of a constitutive cytomegalovirus promoter (pRKFIN13). After 2 weeks of drug selection, it was evident that the number of colonies in pRKFIN13 transfections was reduced relative to the number of colonies in control pRK5 transfections (Fig. 6a). The average numbers of colonies per plate in control and pRKFIN13 transfections were 86 ± 17 and 10 ± 2, respectively. A similar reduction in the number of drug-resistant colonies after transfection with pRKFIN13 was also observed for Rat-1 and HeLa cells (data not shown). Furthermore, although FIN13 expression was clearly detectable by Western analysis at 24 h posttransfection, no FIN13 protein was detected in pools of pRKFIN13-transfected colonies after 2 weeks of drug selection (Fig. 6b). Repeated attempts to isolate clones stably expressing FIN13 were not successful, indicating that constitutive expression of FIN13 caused either growth arrest or cell death.

It should be noted that a band of approximately 75 kDa was detected on some of the Western blots (Fig. 6b). This band was detectable with variable efficiency in mouse cells (data not shown) and could represent the endogenous FIN13 protein. However, its molecular weight was higher than that of the protein expressed from exogenously introduced FIN13 plasmids. This could be due to posttranslational modifications (such as phosphorylation or glycosylation) or translation from an alternatively spliced FIN13 mRNA. The nature of the 75-kDa band and expression of the endogenous FIN13 protein are discussed below.

Cells transiently transfected with FIN13 are blocked in the cell cycle. We utilized a transient-transfection approach to examine the effects of FIN13 expression on DNA synthesis and cell cycle progression. Asynchronously growing HeLa cells were plated on coverslips and transfected with a construct that

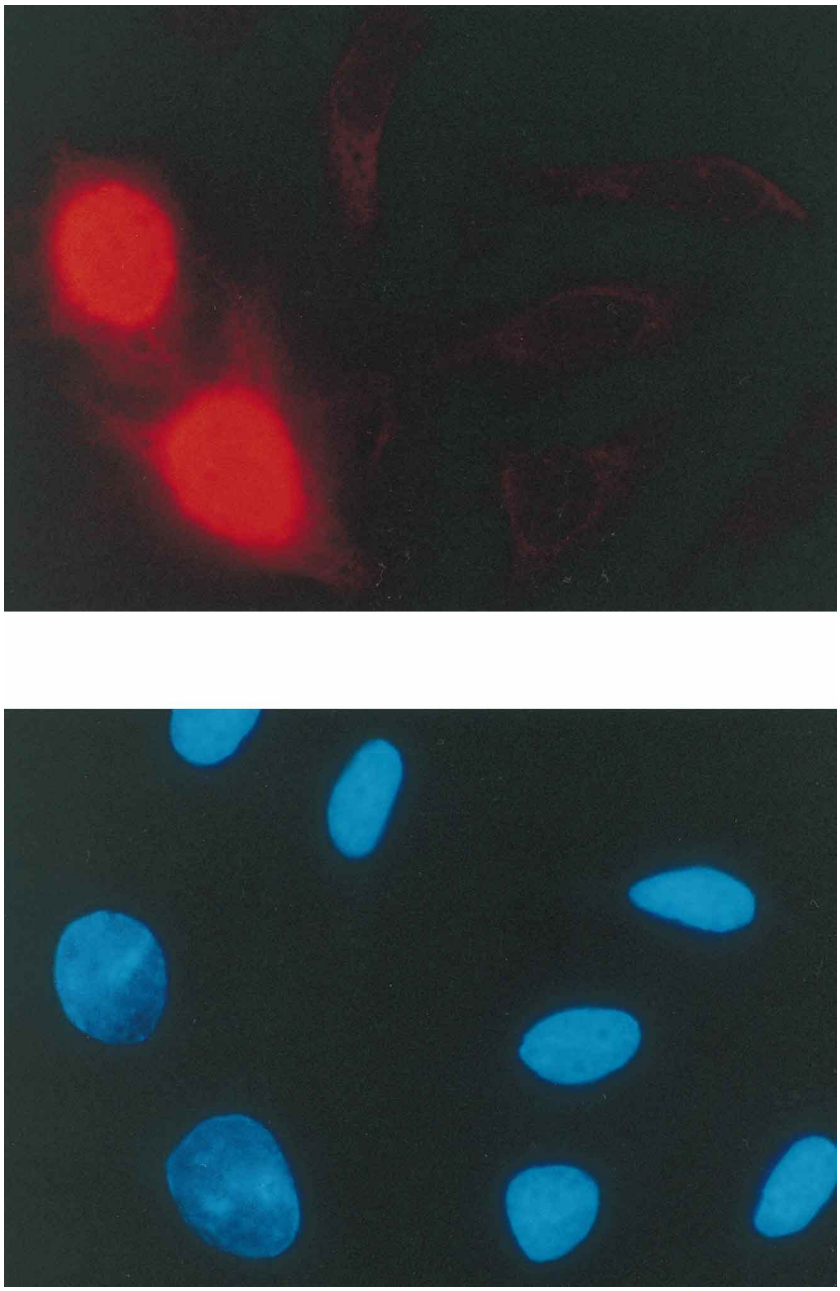

FIG. 5. Subcellular localization of FIN13. (Top) HeLa cells were transfected with pRKFIN13 as described in the legend to Fig. 4, fixed 36 h later, and subjected to an indirect immunofluorescence assay as described in Materials and Methods with the 630 antiserum (1:200). (Bottom) Cells were counterstained with bisbenzimide (Hoechst 33342) to visualize nuclei.

expresses either control protein ERK2 (pRKERK2-HA) or β-galactosidase (CMVβgal) or the FIN13-expressing construct pRKFIN13. ERK2 is expressed as a nuclear protein in HeLa cells in the presence of 10% serum (4), whereas β-galactosidase is predominantly cytoplasmic. At 24 h after transfection, the medium was changed, and cells were labelled for 12 h with BrdU. Cells were fixed and examined for (i) ERK2-HA expression with the anti-HA 12CA5 antibody, (ii) for β-galactosidase expression with the GAL40 antibody, and (iii) for FIN13 expression with the 630 anti-FIN13 antiserum. BrdU incorporation was detected with FITC-conjugated anti-BrdU antibodies.

Although nontransfected cells and ERK2-HA- and β-galactosidase-expressing cells were able to incorporate BrdU, strong inhibition of BrdU incorporation was observed for FIN13-overexpressing cells. The effects of ERK2-HA expression on BrdU incorporation are shown in Fig. 7a and b, and the effects of FIN13 expression on BrdU incorporation are shown in Fig. 7c and d. While >70% of cells expressing either ERK2-HA or β-galactosidase were observed to incorporate BrdU, only 10 to 17% of FIN13-expressing cells were observed to incorporate BrdU (Table 1). These data suggested that cells which overexpress FIN13 become blocked in the cell cycle and fail to synthesize DNA. This inhibition was presumably not due to a

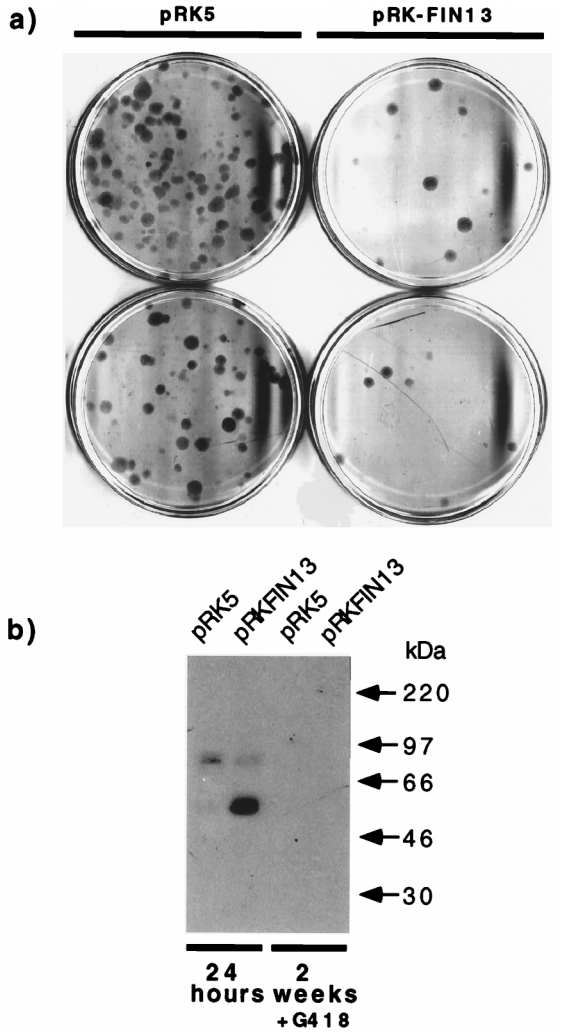

FIG. 6. Effects of FIN13 expression on the growth of neomycin-resistant cells. (a) NIH 3T3 cells were cotransfected with pRSVneo (0.2 μg) and either pRK5 or pRKFIN13 (5 μg), and neomycin-resistant transfectants were selected in G418 after being plated. Cells were stained after 2 weeks in crystal violet. The plates shown are from two independent experiments. (b) Whole-cell extracts prepared from NIH 3T3 cells at 24 h after transfection or from pools of drug-resistant colonies after 2 weeks of selection in G418 (+ G418) were examined for the expression of FIN13 by Western blot analysis with anti-FIN13 antiserum (630) as described in Materials and Methods.

nonspecific cytotoxic effect of protein overexpression, as the expression of another nuclear protein (ERK2-HA) or β-galactosidase did not significantly inhibit BrdU incorporation.

Although these studies indicated that DNA synthesis was blocked in FIN13-expressing cells, it was not clear at which point(s) in the cycle cells were arrested. To further investigate the nature of the cell cycle block, we examined the effects of FIN13 expression on cell cycle distribution by FACS. HeLa cells were transiently cotransfected with a reporter construct that expresses GFP (pcDNA3hGFP) together with constructs that express either ERK2 (pRKERK2-HA), β-galactosidase (CMVβgal), or FIN13 (pRKFIN13). Cells were harvested either 36 or 60 h after transfection, and GFP-positive cells were recovered by FACS, fixed, and stained with propidium iodide. Cells were examined for DNA content by flow cytometry. Cells harvested 60 h after transfection with ERK2-HA and β-galactosidase expression constructs exhibited similar cell cycle distributions (Fig. 8a and b; Table 2). Non-GFP-positive cells recovered by FACS (nontransfected cells) were found to have essentially the same cell cycle distribution as GFP-positive cells transfected with control vectors (Fig. 8a and b) (data not shown). However, pRKFIN13-transfected cells exhibited an accumulation of cells in G_1 phase and particularly in early S phase, accompanied by depletion of cells in G_2/M (Fig. 8c; Table 2). HeLa cells harvested 36 h after transfection exhibited qualitatively the same cell cycle distribution. These results indicate that FIN13 overexpression causes cell cycle arrest in G_1 and early S phases.

Inducible expression of FIN13 also inhibits cell cycle progression. To examine in more detail the cell cycle block induced by FIN13 expression, we utilized the tetracycline-regulated expression system for the isolation of NIH 3T3 cell lines that express FIN13 under the control of an inducible promoter. This inducible system employs a transactivator (tTA) that binds the *tet* operon upstream of a minimal promoter only in the absence of tetracycline to activate the expression of downstream sequences (14). In the presence of the antibiotic, ligand-induced conformational change to tTA prevents binding to the *tet* operon and the tTA-dependent expression unit is turned off. Thus, stable cell lines doubly transfected, initially with a construct that constitutively expresses tTA and subsequently with a construct that contains the *tet* operon-minimal promoter upstream of the *FIN13* cDNA, induce FIN13 expression after the removal of tetracycline.

Eight inducible clones derived from three independent tTA-expressing NIH 3T3 cell lines were studied. None of the inducible clones isolated showed detectable levels of leaky FIN13 expression in the off state (Fig. 9 and data not shown). Figure 9a shows a Western blot analysis of two clones that exhibit inducible FIN13 expression. While clone 8.3 demonstrated strong and sustained expression, clone 4.6 exhibited weaker induction with slower onset kinetics. Both clones exhibited reduced growth rates after FIN13 induction, and the growth inhibition correlated with the level of FIN13 expression (compare Fig. 9a and b). Although there was no observable effect on the cellular morphology of clone 8.3 after 2 days, widespread cell death was evident by day 5 (data not shown); clone 4.6 did not show significant levels of cell death.

Despite repeated efforts, we were not able to maintain FIN13-inducible cell lines in culture for more than 2 to 4 weeks. Of the eight clones isolated, all lost inducible expression. An examination of this phenomenon by immunocytochemistry with anti-FIN13 antibody revealed that the percentage of cells exhibiting inducibility became reduced over time. In all the tetracycline-regulated clones examined, detectable FIN13 induction was completely lost after 4 weeks in culture (data not shown). Although Western blot analysis indicated that the tetracycline system offered good inducibility of FIN13 with no detectable expression in the off state (Fig. 9a), a number of observations suggested that a low level of leaky expression in the presence of tetracycline resulted in the instability of inducible cell lines. Firstly, all inducible clones exhibited lower growth rates, even in the off state, than did parental cells and clones that exhibited inducible expression grew more slowly than did noninducible clones (data not shown). Secondly, it was consistently observed that after the loss of inducible expression (and presumably the loss of leaky expression in the off state), the growth rates of isolated clones increased, returning to rates comparable to that of parental cells. Together, these observations argue that even low levels of constitutive FIN13 expression may exert a strong negative effect on cell growth. Attempts to prevent the loss of inducible FIN13 expression by

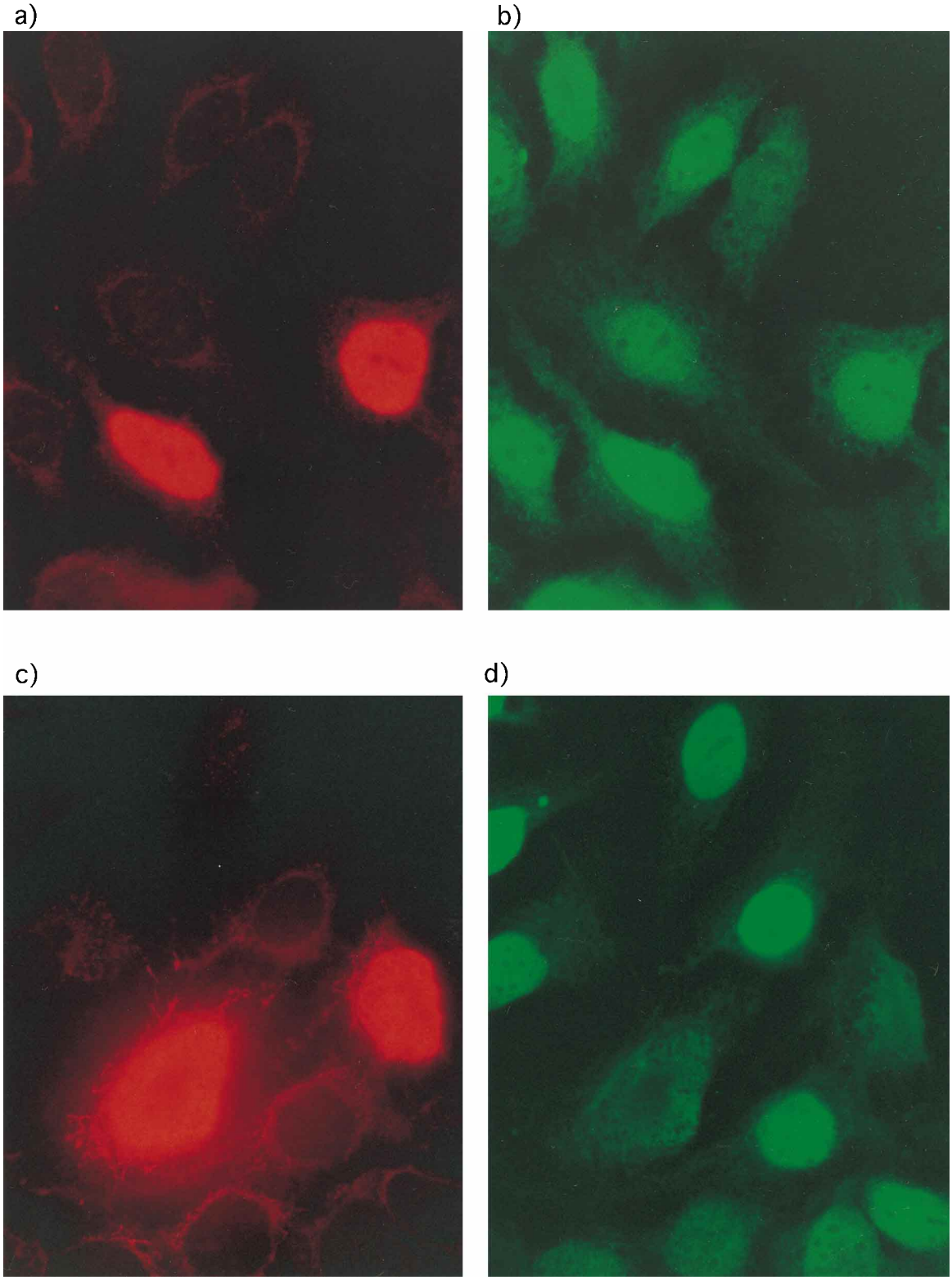

FIG. 7. Effects of FIN13 expression on BrdU incorporation. Asynchronously growing HeLa cells on coverslips were transfected with either pRKERK2-HA (a and b) or pRKFIN13 (c and d). At 24 h after transfection, the medium was changed, and cells were labelled for a further 12 h with BrdU. Cells were fixed and examined by immunofluorescence assay for either ERK2-HA (12CA5 antibody) (a) or FIN13 (630 antiserum [1:200]) (c) expression, followed by immunodetection of incorporated BrdU with an FITC-conjugated anti-BrdU antibody (1:500) (b and d). The same fields of view are shown in panels a and b and in panels c and d.

TABLE 1. Effects of FIN13 expression on BrdU incorporation

Expt and construct	Total no. of positive cells[a]	No. of BrdU-positive cells[b]	BrdU incorporation (%)
Expt 1			
pRKERK2-HA	366	260	71
CMVβgal	395	347	87
pRKFIN13	394	36	10
Expt 2			
pRKERK2-HA	379	291	77
CMVβgal	350	276	79
pRKFIN13	365	62	17

[a] Positive for expression of either ERK2-HA, β-galactosidase, or FIN13.
[b] Cells that expressed ERK2-HA, β-galactosidase, or FIN13 and incorporated BrdU.

increasing the amount of tetracycline in the culture medium or the frequency of tetracycline addition were not successful.

Although inducible expression was lost in clones 4.6 and 8.3 prior to cell cycle analysis, we were able to examine the cell cycle distribution of a clone which was induced to express FIN13 by withdrawing tetracycline shortly after the removal of cells from frozen stocks. The data obtained with the sp8.2 NIH 3T3 tetracycline-regulated clone, which had maintained FIN13 inducibility (determined by immunofluorescence assay) for a sufficient period to allow analysis of DNA content by flow cytometry, are shown in Fig. 8d and e. The data show that whereas the noninduced population had a cell cycle distribution of approximately 50% of cells in G_1 phase, 40% in S phase, and 10% in G_2 phase, the induced cell population showed virtually no cells in G_2, with an increase in cells with S-phase DNA content (Table 2). Most importantly, cells were not distributed evenly throughout the S phase but accumulated preferentially in early S phase. The similarity in cell cycle distribution due to induction of FIN13 expression to that observed in transiently transfected HeLa cells (Fig. 8a to c) is striking.

Identification of the endogenous FIN13 protein and cloning of a longer form of *FIN13* cDNA. As shown above (Fig. 6), on Western blots of protein extracts from NIH 3T3 cells with our anti-FIN13 antiserum, we observed a band of ~75 kDa which could represent the endogenous FIN13 protein. The same 75-kDa band was observed on Western blots of tissue extracts from the murine testis (Fig. 10), which shows very high levels of expression of FIN13 mRNA (Fig. 2). However, since the M_r of this immunoreactive protein was higher than that of the protein expressed from our *FIN13* cDNA plasmids, we first considered the possibility that it represented a posttranslational modification of FIN13. Pulse-chase experiments, however, failed to show conversion of the 60-kDa FIN13 protein to a 75-kDa form (data not shown). Therefore, it seemed possible that the *FIN13* cDNA, in spite of the isolation of multiple clones with similar 5′ ends, represented a truncated version of the full-length cDNA, perhaps because of the presence of RNA secondary structures which inhibited RT upstream of the common 5′ end of the cDNAs we isolated. Alternatively, the cDNAs we isolated could have represented an alternatively spliced mRNA whose relative abundance was increased by the aforementioned block to RT.

To resolve this dilemma, we cloned the murine *FIN13* genomic locus. Sequencing of the most 5′ of the genomic clones revealed an intron at about 50 nucleotides upstream of the initiator ATG of the *FIN13* cDNA. Sequences upstream of this intron appeared to show homologies to the 5′ ends of many type 2C phosphatases (data not shown). Furthermore, a recent entry in the EST database showed a murine sequence (accession no. W34891) which comprised the 5′ portion of our *FIN13* cDNA and continued upstream for about 400 nucleotides to a putative initiator ATG with a good consensus for translation initiation (GCCGCCATGG) (Fig. 10A). Therefore, by RT-PCR (as described in Materials and Methods), we cloned a 766-nucleotide cDNA fragment that partially overlaps our *FIN13* cDNA sequences. Insertion of this fragment in frame within the *FIN13* cDNA resulted in a plasmid (pRKFIN13L) with an open reading frame encoding 542 amino acids, including 150 amino acids upstream of the initiator methionine of *FIN13* cDNA (Fig. 10A). The first 120 amino acids of the predicted protein encoded by the *FIN13L* cDNA showed homology to a conserved domain of many type 2C phosphatases (7); thus, we believe that this cDNA encodes full-length FIN13. Accordingly, transfection of *FIN13L* cDNA in the pRK5 expression vector in HeLa cells produced a protein of 75 kDa, the same molecular mass as that of the endogenous protein detected in murine cells and tissues (Fig. 10c). We do not know at this point whether the short FIN13 protein represents a naturally occurring form of FIN13, resulting from an alternatively spliced mRNA or from internal translation initiation. Nevertheless, expression of the FIN13L protein had the same biological effects as that of the short form. FIN13L was localized in the nucleus, inhibited DNA synthesis in transiently transfected HeLa cells, and inhibited colony formation in cotransfection assays in Rat-1 and NIH 3T3 cells (Table 3 and data not shown).

Therefore, although the relationship between the short and long forms of the FIN13 protein remains to be resolved, it appears that the results obtained with the short form of FIN13 reflect the activities of both proteins. We have not been able to detect so far the short form of FIN13 on Western blots of cultured cells and tissues, but the limit of detection of our present antibodies probably would not allow identification of a protein which is expressed at a level corresponding to 10 to 20% of the 75-kDa FIN13 band.

DISCUSSION

In this study, we have identified a new gene, *FIN13*, which encodes a type 2C phosphatase that is able to negatively regulate cell proliferation by causing cell cycle arrest in G_1/S. *FIN13* was originally cloned in a screen for FGF4-inducible genes and was found to be induced in late G_1 phase by both FGF4 and serum in fibroblasts (17). While its expression in normal adult tissues appears to be largely restricted to the testis (17), the present study indicates that FIN13 expression is induced in a range of tissues undergoing proliferation, including the embryo, uterus at pregnancy, placenta, and ovaries of sexually immature mice after stimulation of folliculogenesis with DES.

An analysis of the deduced amino acid sequence of FIN13 revealed the following four distinct homology domains: (i) an N-terminal CH domain, which could perhaps play a role in protein interactions; (ii) a central acidic box; (iii) a phosphatase domain with homologies to numerous type 2C phosphatases; and (iv) a C-terminal putative bipartite nuclear translocation sequence. Additionally, the longer cDNA (*FIN13L*) that we eventually isolated encodes an N-terminal domain which is conserved in other 2C phosphatases (7) and precedes the CH region. As predicted from its homology to type 2C phosphatases, FIN13 exhibited phosphatase activity. Bacterially expressed and immune complexes of FIN13 precipitated from HeLa cells were able to dephosphorylate ^{32}P-labelled casein in vitro. In addition, the activity observed was type 2C like (1, 6)

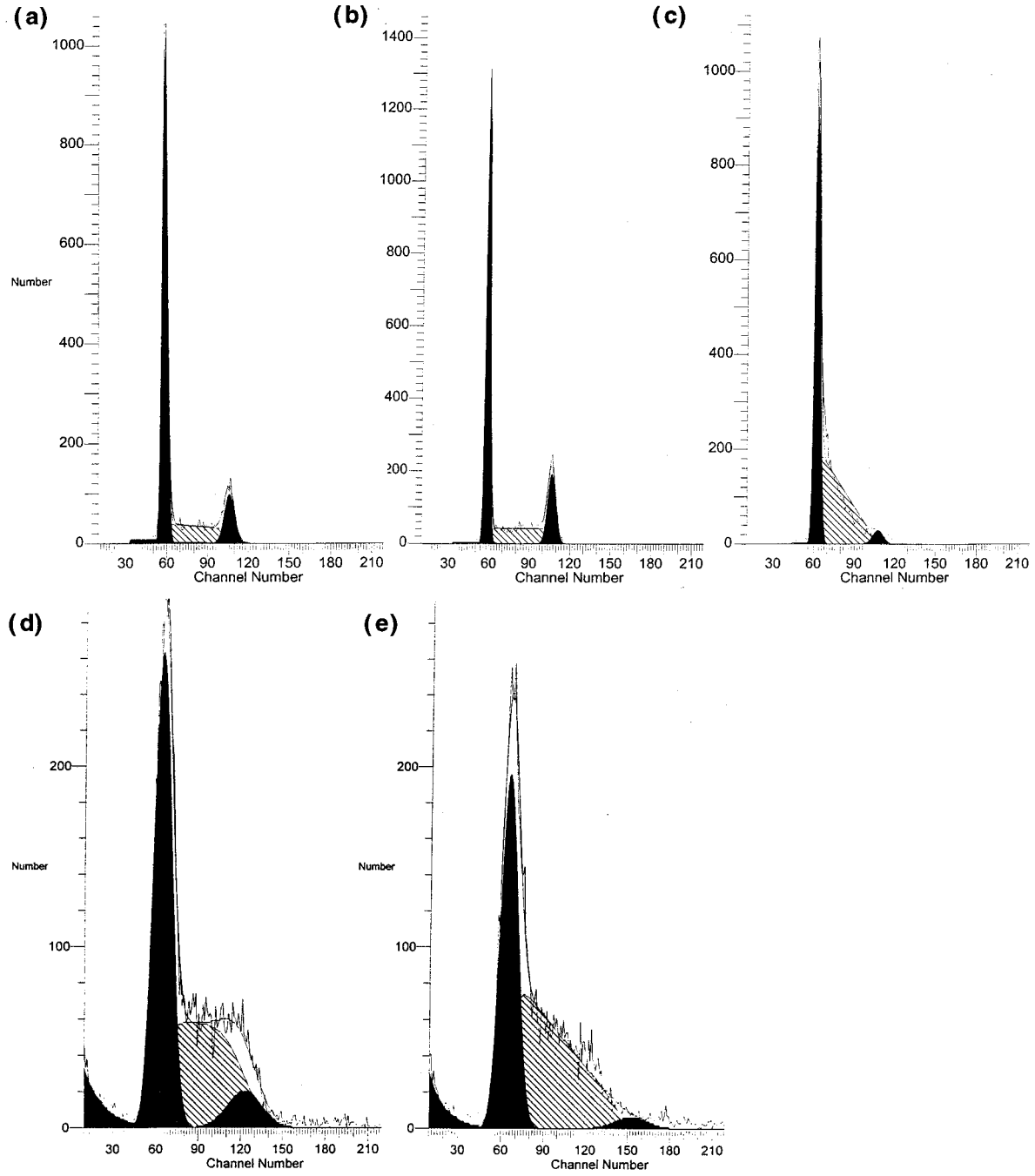

FIG. 8. Flow cytometric analysis of FIN13-expressing cells. HeLa cells were cotransfected with pcDNA3hGFP and either pRKERK2-HA (a), CMVβgal (b), or pRKFIN13 (c); at 60 h after transfection, cells were harvested and sorted for GFP-positive cells by FACS. Recovered cells were fixed, stained with propidium iodide, and analyzed for DNA content by flow cytometry. (d and e) Flow cytometric analysis of tetracycline-regulated clone sp8.2 after culture in the presence and absence of 1 μg of tetracycline per ml for 24 h, respectively.

in terms of its insensitivity to okadaic acid and its requirement of divalent cations. The prediction that FIN13 is a nuclear protein due to the presence of a C-terminal nuclear localization signal (9) was confirmed by indirect immunofluorescence. Other type 2C phosphatases are generally thought to be cytosolic, but it has been reported that protein phosphatase 2Cβ1 can exist in cytosolic and nuclear forms (35).

The induction of FIN13 mRNA in cultured cells in response to growth factors as well as in a number of tissues undergoing proliferation implies that FIN13 has a function related to cell growth. To investigate this possibility, we attempted to make stable cell lines that overexpressed FIN13. However, it was observed that transfection of either NIH 3T3, Rat-1, or HeLa cells with a FIN13 expression construct resulted in a decreased number of colonies compared to the number in control transfections. In addition, ectopic FIN13 expression was undetectable in all the clones isolated, further indicating that selection against FIN13 expression was a prerequisite for the prolifera-

TABLE 2. Effects of FIN13 expression on cell cycle distribution

Construct[a]	Cell cycle distribution (%)[b]			
	G_0/G_1 (1 N)	S		G_2/M (2 N)
		Early (1–1.5 N)	Late (1.5–2 N)	
pRKERK2-HA	65	12	10	13
CMVβgal	58	12	12	18
pRKFIN13	48	31	17	4
sp8.2 (+tet)	50	22	20	8
sp8.2 (−tet)	41	36	19	3

[a] HeLa cells were transfected with the indicated plasmids (profiles are shown in Fig. 8a to c). sp8.2 is a tetracycline-regulated NIH 3T3 clone (profiles are shown in Fig. 8d and e). +tet, with tetracycline; −tet, without tetracycline.
[b] All percentages were determined with ModFit software.

tion and expansion of drug-resistant clones. Together, these results indicate that FIN13 causes either cell cycle arrest or cell death. By using the tetracycline-inducible system (14) for regulated FIN13 expression in NIH 3T3 cells, it was found that the induction of FIN13 expression resulted in reduced cell growth. The reduced growth rates of clones 4.6 and 8.3 in the absence of significant cell death, even after 48 h of FIN13 induction, argue that the primary effect of FIN13 was not to induce programmed cell death. Furthermore, while cell death was observed with clones that express higher levels of FIN13 (such as clone 8.3), the delay in onset (>48 h) indicates that it was most likely a secondary consequence of nonviable arrest or delay in cell cycle progression.

Further attempts to analyze the effects of FIN13 expression on cell cycle progression with the tetracycline-inducible system were hampered due to the high rate at which tetracycline-regulated expression was lost. Clones that initially exhibited regulated FIN13 expression rapidly lost inducibility after 2 to 4 weeks in culture, presumably due to selective pressure against a low level of leaky expression in the off state. The rapid loss of inducible expression has also been observed for other gene products that are toxic or negatively regulate cell growth, including p16 and p53 (27a, 36a). Therefore, we employed an alternative transient-transfection approach to examine the effects of FIN13 expression on the cell cycle. In these experiments, FIN13 expression was found to inhibit DNA synthesis in HeLa cells. Furthermore, cotransfection of HeLa cells with

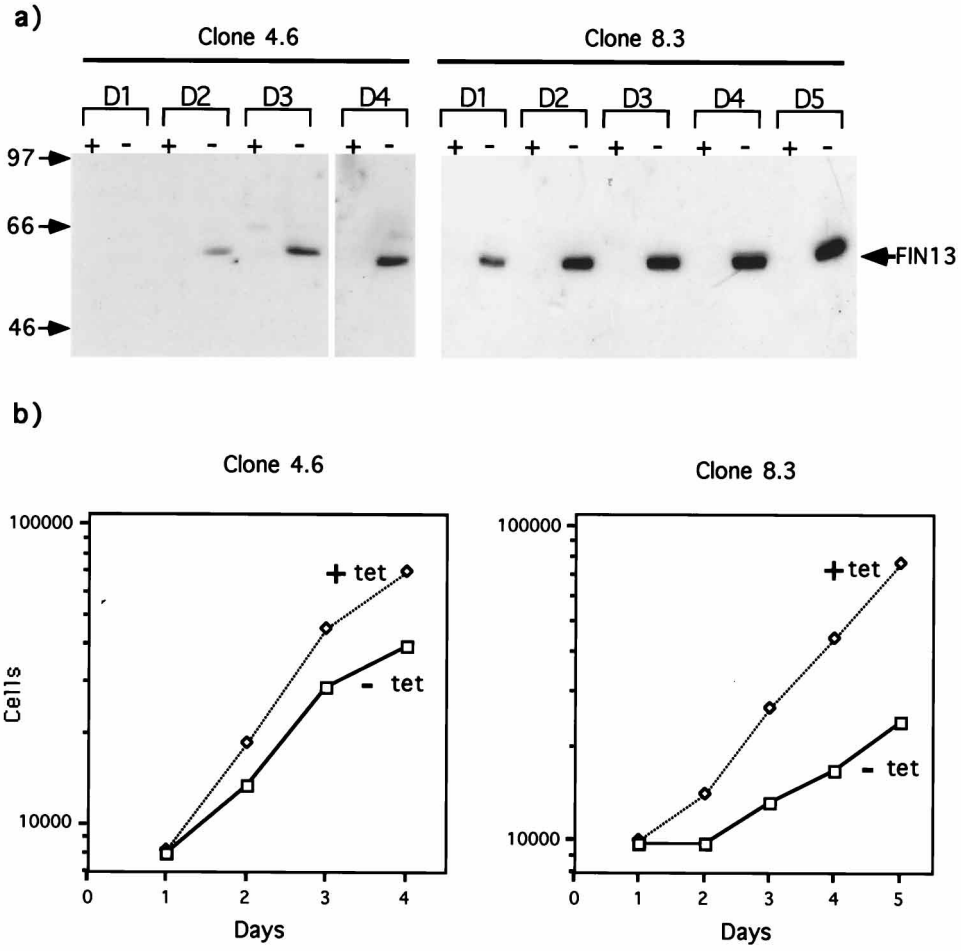

FIG. 9. Effects of induction of FIN13 expression on the growth rates of NIH 3T3 cells. Clones 4.6 and 8.3 were plated on day 0 at 10,000 cells/ml in the presence (+) and absence (−) of 1 μg of tetracycline per ml and harvested for Western blot analysis (a) and cell counting (b). (a) Cells were harvested daily for 5 days (D1 to D5), and extracts were examined by Western blotting with the 630 antiserum for FIN13 expression as described in Materials and Methods. Protein concentrations were determined by Bradford assay, and equal loading and transfer were confirmed by Ponceau staining. Molecular masses (in kilodaltons) are indicated on the left. (b) Growth rates of clones exhibiting FIN13-inducible expression. Triplicate cell counts were obtained daily (with a Coulter counter) until cells became confluent (4 days for clone 4.6 and 5 days for clone 8.3). The mean numbers of cells are plotted on a log scale over time. + tet, with tetracycline; −tet, without tetracycline.

a

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 1  gccgccgcc atg ggtgcctacctctctcagcccaacacggtgaagtgctccggggacggg
          M  G  A  Y  L  S  Q  P  N  T  V  K  C  S  G  D  G     17
61  gttggcgccccgcggctcccgctgccctacggcttctccgccatgcaaggctggcgcgtc
    V  G  A  P  R  L  P  L  P  Y  G  F  S  A  M  Q  G  W  R  V     37
121 tccatggaggatgctcacaactgtattcctgagctggacaatgagacagccatgttttct
    S  M  E  D  A  H  N  C  I  P  E  L  D  N  E  T  A  M  F  S     57
181 gtctacgatggacatggaggggaagaggttgccttgtactgtgccaaatatcttcctgat
    V  Y  D  G  H  G  G  E  E  V  A  L  Y  C  A  K  Y  L  P  D     77
241 attatcaaagatcagaaggcctacaaggaaggcaagcttcagaaggctttacaagatgcc
    I  I  K  D  Q  K  A  Y  K  E  G  K  L  Q  K  A  L  Q  D  A     97
301 ttcttggctattgatgccaagctgaccacagaggaagtcattaaggaactggcccagatt
    F  L  A  I  D  A  K  L  T  T  E  E  V  I  K  E  L  A  Q  I    117
361 gcagggagacccactgaagatgaggatgataaagacaaagtagcagatgaggatgatgtg
    A  G  R  P  T  E  D  E  D  D  K  D  K  V  A  D  E  D  D  V    137
421 gacaatgaggaggctgcattgttgcatgaagaggctacc atg actattgaagagctgctg
    D  N  E  E  A  A  L  L  H  E  E  A  T  M  T  I  E  E  L  L    157
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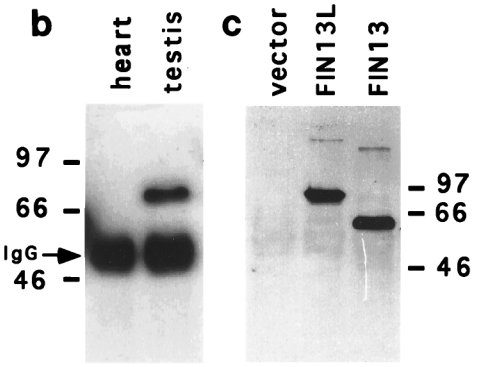

FIG. 10. Detection of the endogenous FIN13 protein and nucleotide and amino acid sequences of the 5′ portion of full-length *FIN13* cDNA. (a) Nucleotide and predicted amino acid sequences of the 5′ end of *FIN13L* cDNA, which continues into the sequence corresponding to the *FIN13* cDNA (Fig. 1). The sequence is interrupted at a position corresponding to amino acid 22 of the original FIN13 protein. The initiator ATGs of the *FIN13L* and *FIN13* cDNAs are in bold. The sequence within which the 5′ ends of most of the *FIN13* cDNAs isolated map is underlined. (b) Expression of the FIN13 protein in murine tissues. Whole-tissue extracts of heart or testis were immunoprecipitated with the 630 anti-FIN13 antibody, and the IPs were subjected to Western blotting with the same antibody. (c) Expression of the proteins encoded by plasmids pRKFIN13 (FIN13) and pRKFIN13L (FIN13L) in HeLa cells. HeLa cells were transfected with the indicated plasmids, and proteins were extracted 36 h later and subjected to immunoprecipitation and Western blotting with the 630 anti-FIN13 serum. The migrations of molecular mass (in kilodaltons) markers are indicated on the left (b) and right (c). The arrow in panel b indicates the position of immunoglobulin heavy chains, which are not visible in panel c because the exposure time of this blot was much shorter.

expression constructs for GFP and FIN13, followed by recovery of GFP-positive cells by FACS and analysis of sorted cells for DNA content by flow cytometry, revealed that FIN13 expression caused G_1/S arrest. A strikingly similar pattern of cell cycle arrest was observed for the sp8.2 tetracycline-regulated NIH 3T3 clone. In both HeLa and NIH 3T3 cells, it was observed that while some cells became arrested in G_1 phase and failed to enter S phase, entry was not completely blocked, with cells also arrested in early S phase. These results are consistent with a direct but partial block in the initiation of DNA synthesis. Such a block would cause the majority of cells to accumulate at the G_1/S boundary and in early S phase and concomitantly result in a very small proportion of cells in the late S phase, as these cells would have a higher probability of completing S phase (Table 2). On the other hand, the observed cell cycle distribution of FIN13-overexpressing cells could also be due to a partial block to S-phase progression, resulting from the reduced availability of substrate or enzymes necessary for DNA synthesis. FIN13 may also interfere with particular processes which are active in both late G_1 and early S phases; thus, cell cycle arrest occurs in a stochastic manner within this window. Regardless, overexpression of FIN13 can block S-phase entry and progression, but passage through G_2/M appears to be unaffected.

TABLE 3. Effects of FIN13 expression on DNA synthesis in transiently transfected HeLa cells[a]

Construct	Total no. of FIN13-positive cells counted[b]	No. of BrdU-positive cells[c]	BrdU incorporation (%)
pRK5 (vector)	273	262	96
pRKFIN13L	224	61	27
pRKFIN13	224	45	20

[a] HeLa cells were transfected with the indicated plasmids and labelled with BrdU from 24 to 38 h after transfection.

[b] The total number of cells positive for FIN13 expression was determined by immunofluorescence assay. In the case of pRK5, random cells were counted.

[c] Cells that expressed FIN13 and incorporated BrdU.

Numerous proteins have been identified as negative regulators of cell growth. The tumor suppressor genes constitute one such class of proteins and have been found either to cause cell cycle arrest after overexpression or to contribute through loss-of-function mutations to uncontrolled cell growth (24). For example, overexpression of the *p53* gene in a number of transformed cell lines causes apoptosis or cell cycle arrest in G_1 phase (5, 8). Loss-of-function mutations in both copies of the *p53* gene have been found to lead to uncontrolled cell growth and neoplasia (24). Genes that encode extracellular factors involved in growth inhibition signals, such as transforming growth factor β and leukemic inhibitory factor, have also previously been described (24). Transforming growth factor β causes cell cycle arrest in a number of cell types, and this system has proved invaluable for studying the mechanism by which growth inhibitory signals are transduced (28). Another group of proteins involved in the negative regulation of cell growth are the inhibitors of cyclin-dependent kinases (cdk's) (32). Progression through the cell cycle is tightly controlled by the kinase activity of a series of cdk's, which are subject to positive regulation by activating phosphorylation and/or dephosphorylation events as well as through association with cyclins. Negative regulation of cdk's is achieved through inhibitory phosphorylation and/or dephosphorylation events and through association with a number of cdk inhibitors. Altering the balance of cdk activity by overexpression of the cdk inhibitor $p21^{waf1/cip1}$ has been found to cause cell cycle arrest (13). Another point in the mitogen signalling pathway that can be negatively regulated is at the level of MAP kinase. Overexpression of MKP-1, which is proposed to inactivate MAP kinase by dephosphorylation, blocks entry into the S phase in fibroblasts (3, 33).

It appears to be somewhat counterintuitive for a mitogenic growth factor to induce the expression of a gene which in turn negatively regulates cell growth. Indeed, such a proposal is probably quite simplistic in regard to the true physiological function of FIN13. It should be noted that the ability of FIN13 to inhibit cell growth was observed under conditions of unregulated expression and/or overexpression; thus, it is possible that this activity does not entirely reflect the physiological activity of the protein. Nevertheless, there are a number of important precedents in which growth factor-inducible genes have been established as negative regulators of cell growth. For example, while $p21^{waf1/cip1}$ appears to negatively regulate growth factor signalling by inhibiting cdk activity, Michieli et al. (25) have reported that $p21^{waf1/cip1}$ is growth factor inducible in fibroblasts. Elevated levels of $p21^{waf1/cip1}$ may represent a threshold that growth factor signalling through cyclin-cdk complexes must overcome and a mechanism by which premature entry into S phase is prevented (25). MKP-1, another immediate-early growth factor-inducible gene product, as well as its rat homolog, erp, can block S-phase entry when it is overexpressed (3, 27, 33).

An important question that arises from the present studies concerns the possible physiological targets of FIN13 and the mechanism by which it causes cell cycle arrest. One possible explanation is that overexpression of FIN13 is toxic due to widespread nonspecific dephosphorylation of nuclear proteins. Although this possibility cannot be ruled out, one might expect cells to arrest at multiple points in the cell cycle and cell death to be more evident. This was clearly not the case with FIN13. Furthermore, the observed instability of tetracycline-inducible clones, even under conditions in which expression of exogenous FIN13 was biochemically undetectable, argues that low levels of unregulated expression of FIN13 are sufficient to inhibit cell growth. It is therefore possible that constitutive expression of FIN13 at an inappropriate time in the cell cycle causes arrest. Finally, the cell cycle arrest observed after overexpression of FIN13 could be due to constitutive dephosphorylation of physiological targets important in driving cells through G_1/S. The answer to these questions not only will necessitate identifying the physiological substrates for FIN13 but also will require a thorough understanding of the regulation of expression and function of the endogenous FIN13 protein. While this work is in progress, it is nevertheless tempting to propose that FIN13 is induced in response to growth factors and is important in regulating G_1/S events.

An additional consideration in regard to the inhibition of cell growth by FIN13 is that the mechanism by which the activity of FIN13 (or indeed any other type 2C phosphatase) is regulated in vivo is unknown (2, 6, 19). Because of the strong negative effect on cell growth, we predict that the biological activity of FIN13 is normally tightly regulated in vivo. Overexpression or unregulated expression is likely to uncouple FIN13 from its normal regulatory control, resulting in a constitutive negative signal that causes G_1/S arrest. Furthermore, FIN13 is itself a phosphorylated protein (data not shown); phosphorylation may contribute to the regulation of FIN13 activity. It should be emphasized that after this paper was submitted, we cloned a longer species of *FIN13* cDNA, which encodes a protein that is 150 amino acids longer than the one we originally characterized and that is likely the major form of the endogenous FIN13 protein. Although it seems unlikely that the long and short forms of FIN13 have exactly the same biological properties, the results of the experiments carried out so far indicate that overexpression of either protein causes cell cycle arrest. It is possible that the short form of FIN13 is an activated form of phosphatase and that the long and short forms of FIN13 are differently regulated or exhibit somewhat different substrate specificities. Overexpression and/or ectopic expression may minimize the differences in the biological activities of these two proteins. Work is in progress to answer these questions as well as to determine whether the short form of FIN13 is the product of an alternatively spliced FIN13 mRNA or internal translation initiation and whether it is produced only in specific phases of the cell cycle or in response to specific signals. Although the possibility that the short-form FIN13 protein is not a physiological gene product cannot be totally ruled out at the moment, the observation that this form retains phosphatase and potent growth inhibitory activities may be very useful in identifying the factors and protein regulatory domains which determine FIN13 activity during cell proliferation.

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