Structure of pp32, an Acidic Nuclear Protein Which Inhibits Oncogene-induced Formation of Transformed Foci

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pp32 is a nuclear protein found highly expressed in normal tissues in those cells capable of self-renewal and in neoplastic cells. We report the cloning of cDNAs encoding human and murine pp32. The clones encode a 28.6-kDa protein; approximately two-thirds of the N-terminal predicts an amphipathic α helix containing two possible nuclear localization signals and a potential leucine zipper motif. The C-terminal third is exceptionally acidic, comprised of approximately 70% aspartic and glutamic acid residues; the predicted pI of human pp32 is 3.81. Human and murine pp32 cDNAs are 88% identical; the predicted proteins are 89% identical and 95% similar. Although the structure of pp32 is suggestive of a transcription factor, pp32 did not significantly modulate transcription of a reporter construct when fused to the Gal4 DNA-binding domain. In contrast, in cotransfection experiments, pp32 inhibited the ability of a broad assortment of oncogene pairs to transform rat embryo fibroblasts, including ras + myc, ras + jun, ras + E1a, ras + mutant p53, and E6 + E7. In related experiments, pp32 inhibited the ability of Rat 1a-myc cells to grow in soft agar, whereas it failed to affect ras-induced focus formation in NIH3T3 cells. These results suggest that pp32 may play a key role in self-renewing cell populations where it may act in the nucleus to limit their sensitivity to transformation.

INTRODUCTION

In 1990, Malek *et al.* described two related nuclear phosphoproteins, pp32 and pp35, in a neoplastic B-lymphoblastoid cell line, A₂₀ (Kim *et al.*, 1979). The pattern of protein expression in vivo and in cell lines suggested a dual association with self-renewing stem-like cell populations and with neoplastic cells. Whereas the majority of A₂₀ cells stained positively for pp35 and pp32, the distribution in normal tissues was highly restricted. Both intestinal crypt cells and the

basal cells of squamous epithelium stained positively with great specificity; these cell populations are well known to contain the cells which continually renew their respective tissues (Cairnie *et al.*, 1965; Bickenbach and MacKenzie, 1984). Since the initial antibodies to murine pp32 and pp35 reacted well with their human counterparts, additional disease-related studies were undertaken which concluded that increased frequency and intensity of pp32 staining in human cancers corresponded to increased malignant potential.

Recently, Walensky *et al.* (1993) explored the relationship between pp32 expression and self-renewing cell populations using androgen to manipulate hormonally dependent rat tissues in a highly physiological model in vivo. In the intact prostate, pp32 protein and mRNA were found primarily in the peripheral regions thought to be roughly equivalent to intestinal

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crypts in that they contain the self-renewing cell populations (Hayashi *et al.*, 1991); overall, approximately 15% of the epithelial cells stained positively for pp32. In contrast, virtually all epithelial cells in the involuted prostate of castrated rats were positive for pp32 mRNA by *in situ* hybridization and approximately 55% were antibody positive. Calculations of epithelial cell mass suggested that the residual pp32-positive population in castrates was roughly equivalent, numerically, to the pp32-positive population in the intact gland. Simply stated, pp32 appeared to mark the stem-like cells capable of renewing the gland upon subsequent androgen stimulation. When androgen was administered to castrated rats, the pp32-positive cells appeared to dilute out to their original proportion and reach their original anatomical distribution. Consistent with the dual finding of pp32 in stem-like self-renewing cell populations and cancer, pp32 levels and the number of pp32-positive cells increased with increasing Gleason grade in human prostate cancer.

The expression patterns of pp32 and pp35 raise interesting questions about their function. The initial biochemical studies of pp32 provided few clues to its function. Characterization of pp35 and pp32 phosphorylation showed that they are casein kinase II substrates (Malek *et al.*, 1990), but casein kinase II phosphorylations do not specifically point toward any single function or class of functions (Pinna, 1990; Allende and Allende, 1995).

In this study, we explored pp32 function through cloning, analysis of its sequence, and transfection studies. We report the cloning of murine and human pp32 as part of an ongoing effort to characterize the related family of pp42, pp35, and pp32 (Malek *et al.*, 1990). Because of the close relationship between pp35 and pp32, a probe based on pp35 sequence facilitated detection of related sequences in pp32 cDNA. We further demonstrate that pp32 inhibits transformation by a series of mechanistically distinct oncogene pairs.

MATERIALS AND METHODS

Materials

A poly(T)-primed cDNA library was constructed by Clontech (Palo Alto, CA) from A₂₀ cell mRNA purified with the guanidinium isothiocyanate method (Sambrook *et al.*, 1989) in λ gt11. A randomly primed cDNA library from HL-60 cells in Lambda-Zap and a poly(T)-primed A₂₀ cell cDNA library in Uni-Zap were purchased from Stratagene. All cDNAs were subcloned into Stratagene pBluescript II KS⁺, except for clones *in vivo* excised from the HL-60 Lambda-Zap library which were in pBluescript I SK⁻. A glyceraldehyde-3-phosphate dehydrogenase probe was purchased from Clontech. Oligonucleotides were synthesized by Genosys. Enzymes and kits for molecular biology were purchased from Amersham (Arlington Heights, IL), Collaborative Research (Bedford, MA), Boehringer Mannheim (Indianapolis, IN), Clontech, Life Technologies (Gaithersburg, MD), Stratagene (La Jolla, CA), Promega (Madison, WI), Schleicher & Schuell (Keene, NH), and United States

Biochemicals (Cleveland, OH). Other chemicals were purchased from Sigma (St. Louis, MO) and J.T. Baker (Phillipsburg, NJ). Radiochemicals were obtained from Amersham and ICN (Costa Mesa, CA). Cell culture media and reagents were purchased from Life Technologies and serum supplements were obtained from Hyclone (Logan, UT).

Plasmids and Constructs

pHyg (Sugden *et al.*, 1985), pEJ-ras, pMLV-myc, pSVcJun (Wisdom and Verma, 1990), and pCT-E1a (Ruppert *et al.*, 1991) were kindly donated by Dr. Chi Dang and John Barrett. pSV40, a pUC19-derived vector containing the SV40 early promoter, was donated by Dr. Sangram Sisodia. pCMVE6 (Kessis *et al.*, 1993) and pCMVE7 (Slebos *et al.*, 1994) were gifts from Drs. Lora Hedrick and Kathleen Cho. pC53-175 (Eliayhu *et al.*, 1984) was a kind gift from Dr. Ken Kinzler. pCMVneo (Baker *et al.*, 1990) was donated by Dr. Ruth Craig. Bacterial expression vectors PRX-1/2/3 (Rimm and Pollard, 1989) were donated by Drs. Steven Dobberstein and Thomas Pollard.

Cell Culture

A₂₀ cells (American Type Culture Collection, Rockville, MD; Kim *et al.*, 1979) were maintained in RPMI 1640 supplemented with 10% fetal bovine serum, 50 U/ml penicillin, and 50 μ g/ml streptomycin and passed twice weekly by diluting 1:10 in fresh medium. HL-60 cells (Collins *et al.*, 1978) were maintained in the same medium and passed twice weekly by diluting 1:10. Primary rat embryo fibroblasts were either purchased through BioWhittaker (Walkersville, MD) or were generously donated by Dr. Chi Dang. Cells were grown in low-glucose DMEM containing 10% fetal bovine serum, 50 U/ml penicillin, and 50 μ g/ml streptomycin. All cells were grown in humidified incubators at 37°C in 5% CO₂.

Purification of Total RNA and mRNA

For the A₂₀ library, total RNA was isolated from cultured A₂₀ cells using the guanidinium-cesium chloride method (Sambrook *et al.*, 1989). Poly(A)⁺ mRNA was purified using an oligo(dT)-cellulose column (Sambrook *et al.*, 1989). Total RNA and mRNA from HL-60 cells were isolated using a modified protocol of the single-step acid-phenol extraction (Chomczynski and Sacchi, 1987) and the MiniRiboSep kit from Collaborative Biomedical, respectively.

Library Screening

Initial clones were obtained by screening the A₂₀ library with a long, nondegenerate oligonucleotide probe designed by back-translating, using the codon usage frequencies of Lathe (1985), the phosphopeptide from murine pp35 sequenced earlier in the study of Malek *et al.* (1990; see RESULTS AND DISCUSSION for sequence). The oligonucleotide was synthesized by Genosys and purified by high-performance liquid chromatography. Six hundred thousand plaque-forming units (pfus) of the previously described A20 λ gt11 library were initially plated on *Escherichia coli* strain Y1090, transferred onto Nytran filters (Schleicher & Schuell), and fixed by baking for 2 h in an 80°C vacuum oven. The screening oligonucleotide guesser was end labeled using T4 polynucleotide kinase (New England Biolabs, Beverly, MA) and used to probe the library at moderate stringency. Filters were hybridized overnight in 6 $\times$ SSC, 1 $\times$ Denhardt's solution, 0.05% sodium pyrophosphate, and 100 μ g/ml salmon sperm DNA at 42°C and then washed twice at room temperature in 6 $\times$ SSC and twice at 50°C in 6 $\times$ SSC. Thirteen clones positive on duplicate filters were plaque purified and the DNA was isolated from liquid lysates on cesium chloride gradients (Sambrook *et al.*, 1989). cDNA inserts were excised using *Eco*RI, gel purified, and ligated into pBluescript II KS⁺ (Stratagene). Cesium-banded plasmid was sequenced using T7 and T3 primers (Stratagene) and Sequenase II (United States Biochemicals) and nested deletion constructs were made using the Exo/Mung deletion kit (Stratagene).

The initial partial murine pp32 clone isolated by this strategy was used as a probe to identify the complete human homolog from the randomly primed HL-60 library (Stratagene). Five hundred thousand pfus were plated using XL1-Blue cells, transferred onto Nytran filters, and UV cross-linked with a Stratalinker machine (Stratagene). The partial murine insert was used as a probe and labeled by random priming using Prime-It (Stratagene) and hybridized with the filters in 50% formamide, 5× SSPE, 0.1% SDS, and 100 µg/ml salmon sperm DNA at 42°C. Filters were washed twice at room temperature in 2× SSC and twice at 55°C in 0.2× SSC. Plaque-purified clones were excised in vivo using XL1-Blue cells and R408 helper phage (Stratagene). Sequencing was performed as described above. Finally, the complete murine clone was isolated by using a 5' *Hind*III fragment from the human clone as a probe to screen the Stratagene A₂₀ cDNA library.

Northern Blots

Ten micrograms of total RNA and 0.5 µg of poly(A)⁺ mRNA from A₂₀ or HL-60 cells were run in formaldehyde-agarose gels using MOPS running buffer (Sambrook *et al.*, 1989). RNA was transferred to Hybond nylon filters (Amersham) by capillary transfer in 20× SSC. After overnight transfer, the blot was UV cross-linked and hybridized using the same conditions for probing the HL-60 library with randomly primed probes. Filters were washed twice at room temperature in 2× SSC and twice at 55°C in 0.2× SSC. Autoradiography was performed at -80°C.

In Vitro Transcription and Translation

Plasmids containing cDNA were linearized and RNA transcripts were synthesized using RiboMAX T7 polymerase (Promega). Three micrograms of pp32 RNA was in vitro translated using rabbit reticulocyte lysate (Promega) with [³⁵S]cysteine (ICN) at 30°C for 1 h. Twenty-five microliters of lysate were loaded onto a 10% SDS-polyacrylamide gel which was run, transferred to nitrocellulose, and exposed overnight at -80°C. A₂₀ lysate was run alongside to compare the size of the in vitro-translated products with proteins recognized by anti-pp35 antibody.

Protein Purification and Analysis

Murine pp32 was purified from A₂₀ cells as described previously (Malek *et al.*, 1990). A recombinant partial murine pp32 fragment was expressed and purified from CAG456 *E. coli* cells using the pRX expression vector (Rimm and Pollard, 1989) containing cDNA-encoding amino acids 67 through 247 of murine pp32 (pRX32). A single colony of CAG456 containing the pRX32 was grown in 1 ml LB media with 50 µg/ml ampicillin at 30°C with vigorous shaking. The saturated culture was then transferred into 50 ml LB with ampicillin and allowed to reach saturation overnight. The saturated culture was then added to 500 ml M9CA media (Sambrook *et al.*, 1989) with ampicillin and allowed to grow with vigorous shaking at 30°C for 3.5 h. Indol acrylic acid was added to the cells to achieve 10 µg/ml final concentration, and growth was continued for another 4.5 h. The cells were then pelleted at 4000 × g at 4°C, washed with 100 ml of 10 mM Tris-HCl (pH 8.0) at 4°C, pelleted again, and lysed in ice-cold 140 ml STET lysis buffer consisting of 8% sucrose, 10 mM Tris-HCl (pH 8.0) at 4°C, 50 mM EDTA, 0.5% Triton X-100, 0.5 mM diisopropylfluorophosphate, and 10 µg/ml each of chymostatin, leupeptin, antipain, and pepstatin. The cell lysate was kept on ice and sonicated 10–11 times with 30-s bursts at intensity level 6 at 15-s intervals using a Brinkmann Sonicator. Cell debris was removed by centrifugation at 13,000 × g for 10 min at 4°C, the supernatant was brought to 25% ammonium sulfate, and the lysate was allowed to precipitate overnight on ice. The precipitate was pelleted at 13,000 × g for 10 min at 4°C; the supernatant was then brought to 65% ammonium sulfate and allowed to further precipitate. The protein precipitate was pelleted as before, resuspended in 20 ml of

200 mM NaCl, 20 mM Tris-HCl (pH 7.5) at 4°C, 1 mM EDTA, 1 mM 2-mercaptoethanol, and 0.1 mM phenylmethylsulfonyl fluoride and dialyzed overnight in the same buffer to remove ammonium sulfate. The dialyzed lysate was applied to a HR-5/5 MonoQ FPLC column (Pharmacia) at a flow rate of 1 ml/min. The column was washed and developed with a 50-ml linear gradient of 200 mM to 1.0 M NaCl in the same buffer and 1-ml fractions were collected. Samples were analyzed by SDS-PAGE (Laemmli, 1970) and Western blots probed with anti-pp32 polyclonal antibodies. Pure partial recombinant murine pp32 was usually obtained after only one column run. Material was rechromatographed under the same conditions as needed to obtain >95% purity by SDS-PAGE. Protein concentration was assayed using the BCA assay (Pierce, Rockford, IL).

Polyclonal Antibody Production and Purification

Purified partial murine recombinant pp32 protein as above was used to prime and boost two *Pasteurella*-free New Zealand White rabbits using biweekly 300-µg injections in Freund's adjuvant to produce polyclonal antibodies. For affinity purification of antirecombinant pp32 from the rabbit serum, an affinity column was prepared and run as previously described for native pp32 (Malek *et al.*, 1990). Affinity-purified antibodies to native and denatured pp35 and pp32 have been described previously (Malek *et al.*, 1990).

Western Blots

All protein samples were run on a 10% SDS-PAGE system (Laemmli, 1970). They were transferred to nitrocellulose filters (Schleicher & Schuell) in standard Tris/glycine/20% methanol transfer buffer and blocked overnight in 3% bovine serum albumin/phosphate-buffered saline. Primary antibodies were hybridized onto blots at room temperature in 1× Tris-buffered saline containing 0.1% Tween 20 (TBS-T) for 1 h with continuous shaking. After washing the blot at room temperature in TBS-T, a donkey anti-rabbit horseradish peroxidase secondary antibody was applied. The ECL Western blot detection kit (Amersham) was used to visualize binding of the antibodies.

Transcriptional Activity of pp32

Human pp32 coding sequence was amplified from cloned cDNA in Bluescript by the polymerase chain reaction (Perkin Elmer-Cetus, Norwalk, CT) maintained at 95°C for 1 min, cycled 34 times through 95°C for 1 min, 54°C for 1 min, and 72°C for 1 min, and then incubated for a final 7 min at 72°C. The upstream and downstream primer sequences (cgacatattggagatgggcagac and agtttctagaggcaggatagagg) were chosen to introduce *Nde*I and *Xba*I sites into the amplified sequence. Following cleavage and low-melt agarose gel purification, amplified pp32 was ligated into the GalO vector (Kato *et al.*, 1990) to create a fusion protein of pp32 downstream of the Gal4 DNA-binding domain under control of the thymidine kinase promoter. The effects of pp32 on transcription were determined by DEAE/dextran cotransfecting the Gal-pp32 construct into CHO cells along with the luciferase reporter plasmid pG5L2, a modification of the pGL2-promoter vector (Promega) containing five Gal4-binding sites in the multiple cloning site (Kato *et al.*, 1990). Luciferase activity was measured on the fourth day following transfection and normalized to total cellular protein.

Transfection and Analysis of Rat Embryo Fibroblasts

For each experiment, approximately 1 × 10⁶ cells were plated per T75 flask and incubated for 2 to 3 d before transfection to achieve approximately 40% confluency. For each flask of primary rat embryo fibroblasts, the plasmids indicated in each experiment were added in the following amounts: pEJ-ras, pCMVE6, or pCMVE7, 5 µg and pMLV-c-myc, pCMV32, pCMVneo, pSV32, pSV40, pc53-175,

pCT-E1a, or pSVcjun, 10 μ g. Plasmids were prepared in two volumes of Lipofectin (2 * total μ g DNA = μ l Lipofectin) and then gently mixed by inversion in 1.5 ml OPTIMEM in sterile 15-ml polystyrene tubes and allowed to incubate at room temperature for >15 min. For experiments with more than one flask, mixtures of all reagents were increased in proportion to the numbers of flasks required for each transfection. Cells were washed once with OPTIMEM (Life Technologies) and then fed with 6 ml of OPTIMEM and 1.5 ml of the DNA/Lipofectin mix. After overnight incubation, the cells were grown in standard media and refed with fresh media twice weekly. Foci were counted 14 d posttransfection, except for the E6/E7 experiments which were counted after 10 d. To determine the dose dependence on pCMV32, pCMV vector and pCMV32 DNA were transfected such that the sum of the two was a constant 12 μ g. Ratios of 0:12, 3:9, 6:6, 9:3, and 12:0 μ g pCMV: μ g pCMV32 were cotransfected into triplicate flasks and foci were counted 2 wk later.

To determine whether any of the plasmids used were toxic, rat embryo fibroblasts were cotransfected with pCMV, pCMV32, pSV40, or pSV32 along with pHyg (Sugden *et al.*, 1985). If any of the pp32-containing constructs were lethal, the number of hygromycin-resistant colonies from cells transfected with it would be significantly less than control blank-plasmid-transfected cells. Ten micrograms of test vector and 1 μ g of pHyg were transfected per flask of cells using the protocol described above. Each transfection was done in duplicate. Forty-eight hours after transfection, 12.5 μ g/ml of hygromycin was added to the medium and the cells were allowed to grow for 1 mo, with a weekly medium change. The dose of hygromycin is the optimal dose for selection of rat embryo fibroblasts transfected with pHyg (Resar *et al.*, 1993). Resistant colonies were stained with crystal violet and counted.

Sequence Analysis

Sequences were analyzed using GCG version 7 software.

Production of Human pp32 in Baculovirus

pp32BAC is a recombinant baculovirus into which we subcloned full-length pp32 under the polyhedrin promoter using the pVL1393 transfer plasmid (PharMingen, San Diego, CA). SF9 cells were maintained in Grace's insect medium supplemented with 10% heat-inactivated fetal bovine serum and 0.05 mg/ml gentamicin at 27°C. Initially, SF9 cells were cotransfected with the transfer plasmid and Baculogold virus by calcium phosphate precipitation. The resultant pp32BAC stocks were amplified from a single pfu obtained by end point dilution. For production of recombinant pp32, SF9 cells infected with pp32BAC were grown in two 1-l spinner cultures stirred at 80–90 rpm at 27°C; cells were infected at the beginning of log phase growth and collected on d 4 postinfection, after having reached a density of 1.5×10^6 cells/ml. Cells were harvested and pelleted at $1000 \times g$ at 4°C, washed with 100 ml medium without serum, pelleted, and lysed in ice-cold lysis buffer consisting of 20 mM Tris-HCl (pH 7.5) at 4°C containing 1% Triton X-100, 1 mM EDTA, 10 mM sodium pyrophosphate, 2 mM sodium vanadate, 3 mM ATP, 50 mM NaF, 0.5 mM diisopropylfluorophosphate, and 0.1 mM phenylmethylsulfonyl fluoride at 2×10^8 cells/ml with vortexing every 3 min over the course of a 15-min incubation. Cell debris was removed by centrifugation at $17,800 \times g$ for 20 min at 4°C and recombinant pp32 was filtered at 0.45 μ m and 0.22 μ m and purified by anion exchange high-performance liquid chromatography (Malek *et al.*, 1990).

Analysis of pp32 Effects on ras-Transformed NIH3T3 Cells

NIH3T3 cells (American Type Culture Collection) were maintained in DMEM containing 4.5 g/l D-glucose, 10% fetal bovine serum, 50 U/ml penicillin, and 50 μ g/ml streptomycin. For analysis of pp32 effects, cells were grown to approximately 35–40% confluency and

then transfected with 5 μ g of pEJ-ras and 10 μ g pCMV or pCMV32 by lipofection as described above, except that the incubation was for 5 h. Following transfection, the cells were washed once with Hanks balanced salt solution without phenol red and then incubated in supplemented DMEM for 10 d. The medium was changed after overnight incubation and twice thereafter during incubation. Foci were enumerated at 10–12 d posttransfection as described for rat embryo fibroblasts.

Analysis of pp32 Effects on Rat 1a-myc Cells

Rat 1a-myc cells were a generous gift from Dr. Chi Van Dang. Cells at 40–50% confluence in 100-mm Petri dishes in the same DMEM used for rat embryo fibroblasts were transfected with 10 μ g of either pCMV or pCMV32 DNA simultaneously with 1 μ g pBABE containing a selectable puromycin resistance marker. The transfection protocol was essentially as described for NIH3T3 cells. Following a 5-h lipofection incubation, the cells were washed twice with $1 \times$ phosphate-buffered saline and then incubated in supplemented DMEM. Once the cells achieved 100% confluence, they were divided into two daughter plates and grown in medium containing 0.8 μ g/ml puromycin. Individual clones were isolated using glass cloning rings, and pp32 overexpression was documented with Northern blot analysis. Soft agar assays were carried out on individual clones as described (Barrett *et al.*, 1995). After incubation for 14 d, colonies were enumerated by photographing each plate, digitizing each photograph, laser printing color-reversed, contrast-enhanced 243% enlargements of the digitized images, and then manually counting all colonies regardless of size visible in a standardized sector representing one-eighth of the plate area.

RESULTS AND DISCUSSION

cDNA Sequences of Murine and Human pp32

Figure 1A shows the resultant cDNA sequences of human and murine pp32. The clones used to derive those sequences are diagrammed in Figure 1B. The cloning strategy employed a 42-bp nondegenerate oligonucleotide probe back-translated from an independently sequenced murine pp35 peptide to screen a murine cDNA library from A₂₀ cells; the initial murine clones were used to obtain human cDNA and to complete the murine sequence (see MATERIALS AND METHODS for details). The evidence that the cDNAs thus obtained encode pp32 is severalfold: 1) the polypeptide expressed from a partial murine cDNA clone reacts preferentially with antibodies to native murine pp32; 2) antibodies to the expressed murine pp32 fragment react specifically with pp32 in Western blot analysis of total cellular lysates of A₂₀ cells (Walensky *et al.*, 1993); 3) recombinant human pp32 expressed in baculovirus reacts with antibodies to murine pp32 and pp35; and 4) the *in vitro* translation product of human pp32 cDNA comigrates with pp32 and not pp35 in Laemmli gels. As additional supporting evidence, the murine pp32 sequence encodes three peptides independently sequenced from purified murine pp35 which are apparently common to both proteins.

The immunological identification of the murine and human cDNA clones bears further discussion. Figure 2 shows immunological similarity between native

pp32 and the full-length protein product of the human cDNA expressed in baculovirus and purified from infected SF9 cells. The purified recombinant human polypeptide (Figure 2, lanes 1) comigrates with native human pp32 purified from HL-60 cells (Figure 2, lanes 2) and with murine pp32 detected in total lysates of A₂₀ cells (Figure 2, lanes 3). Since the human pp32 sequence predicts a polypeptide of 28,585 Da, migration at $M_r = 32,000$ on SDS-polyacrylamide gels likely reflects a retardative influence of the acidic domain of pp32, which would be unlikely to bind SDS well. All three polypeptide species react with antibodies specific for pp32 (Figure 2A) and with the original antibodies designated anti-pp35d (Malek *et al.*, 1990), which first detected the existence of pp32. Analogous studies likewise confirm the identity of the murine pp32 cDNA (our unpublished observations). As further evidence, the in vitro translation product of the full-length murine clone also comigrates with pp32 (Figure 3). Finally, previously published studies show that polyclonal antibodies raised to and affinity puri-

fied with the expressed murine recombinant fragment (v.s.) preferentially react with pp32 in Western blots of A₂₀ cell lysates (Walensky *et al.*, 1993). The confluence of size and immunologic data thus support the identification of the murine and human cDNA clones as pp32.

Sequence data also indirectly support the identification of the cDNA clones as pp32. The underlined sequences in Figure 1 are identical to peptides independently sequenced from purified native pp35 (Malek *et al.*, 1990 and our unpublished observations) and thus are likely to represent peptides common to both pp32 and pp35. This supposition is reasonable, given the close correspondence between the peptide maps of the two proteins and the strong cross-reactivity between antibodies raised against either pp32 or pp35 (Malek *et al.*, 1990). Furthermore, the peptide sequence between residues 138 and 151 closely corresponds to the previously reported sequence from the major pp35 phosphopeptide (Malek *et al.*, 1990); this same pp35 phosphopeptide sequence gave rise to the

Figure 2. Native human pp32, recombinant human pp32, and murine pp32 comigrate and react with anti-pp32 antibodies. This figure shows a Western blot of a 10% SDS-polyacrylamide gel that was split and developed alternatively with affinity-purified polyclonal antibody to a recombinant murine pp32 fragment (A) or affinity-purified polyclonal antibodies to denatured pp35 (B), which originally detected pp32 (Malek *et al.*, 1990). Lanes 1, 4.5 μ g recombinant human pp32; lanes 2, 0.45 μ g purified native pp32 from HL-60 cells; and lanes 3, 120 μ g from murine A₂₀ total cell lysate protein. A was exposed for 35 s and B for 5 min. Lane 3B shows the detection of both pp35 and pp32 in A₂₀ lysate.

nondegenerate 42-bp oligonucleotide probe used to obtain the initial pp32 clones. The murine peptide and nucleotide sequences, shown below, are close but non-identical (differences are shown in **bold**) in pertinent ways. There is sufficient correspondence between the two nucleotide sequences to explain how the pp32 sequence was obtained with a pp35 probe. The pp35 sequence contains a serine at the position equivalent to the M¹⁴³ in the pp32 sequence; it is this serine which appears to be phosphorylated in murine pp35 by casein kinase II (Malek *et al.*, 1990). The absence of this casein kinase II phosphorylation site in the pp32 peptide is consistent with the observation that pp32 and pp35 phosphopeptides run distinctly from one another on reversed phase chromatography (Malek and Pasternack, unpublished observations) and precludes the possibility that the cDNA could encode pp35. It remains uncertain whether pp32 and pp35 represent

splice variants or are products of discrete genes: pp35 sequence, L L P Q L S Y L D G Y D D E; pp32 sequence, L L P Q V M Y L D G Y D R D; screening probe, ctgctgccccagctgtctacctggatggctatgatgatgag; and pp32 sequence, ctctgccccaggtcatgtacctgatggctatgacagggac.

The sequence of pp32 predicts several interesting features illustrated in Figure 4. Overall, pp32 is divided into two domains. The N-terminal two-thirds (residues 1–167) is generally amphipathic and has a high probability of α helical conformation according to Chou-Fasman predictions; approximately at its midpoint, the α helical domain has a potential leucine zipper composed of Leu-69, Val-76, Leu-83, and Leu-90 (Landschulz *et al.*, 1988). The putative leucine zipper sequence is conserved in murine pp32 with one difference: isoleucine is conservatively substituted for the second valine. These features suggest that pp32 might act through self-association or through association with other molecules. Preliminary evidence obtained using size exclusion chromatography indicates that both purified native human pp32 and purified full-length human pp32 produced in baculovirus exist in trimeric form in solution (Romantsev and Pasternack, unpublished observations). The N-terminal region also contains two potential nuclear localization sequences commencing at amino acids 61 and 108, respectively; each sequence is comprised of a proline followed by a cluster of basic residues (Kalderon *et al.*, 1984). The C-terminal third of pp32 is highly acidic, composed of approximately 70% aspartic acid and glutamic acid residues; the predicted pI of human pp32 is 3.81. Although the function of this domain is presently unknown, similar acidic regions have been found on proteins as diverse as neurofilament triple helical L protein (Lewis and Cowan, 1985) and the major centromere-binding protein CENP-B (Earnshaw *et al.*, 1987). Molecules with acidic domains are regularly detected in homology searches due to the compositional monotony of the acidic regions. Similarly, neurofilament triple helical L protein is identified since it shares the general organizational feature of an N-terminal amphipathic α helix coupled with a C-terminal acidic tail.

Human and murine pp32 are quite homologous to one another: human and murine cDNA are 88% identical and the predicted proteins are 89% identical with conservative substitutions accounting for most of the differences, yielding 95% similarity. The human pp32 sequence is essentially identical to a sequence termed PHAPI (Vaesen *et al.*, 1994) which appeared while preparing this manuscript for publication. PHAPI was one of two distinct peptides detected through its ability to bind to a synthetic peptide derived from HLA class II sequence. The authors report no functional data, but do comment on subcellular localization. Antibodies to a synthetic peptide representing amino

Figure 3. In vitro translation product of human pp32 cDNA comigrates with murine pp32. ³⁵S-labeled in vitro translation products and A₂₀ lysate were electrophoresed on a 10% Laemmli gel and transferred to nitrocellulose. This figure compares a ³⁵S autoradiograph of the filter with the corresponding chemiluminescent Western blot image. Lane A, in vitro translation products of sense RNA from the HL2 human pp32 clone; lane B, A₂₀ lysate probed with the same antibody recognizing both pp35 and pp32 illustrated in Figure 3; and lane C, in vitro translation product of antisense RNA from the HL2 human pp35 clone.

acids 31–46 of PHAPI labeled the nucleus, cytoplasm, and membranes of H2LCL cells; only a small fraction of label appeared to codistribute with HLA-DR α . We have not observed plasma membrane or cytosolic localization of pp32. In the course of preparing the revised manuscript, we became aware of two additional studies. Li *et al.* (1996) found that an inhibitor of protein phosphatase 2A termed I_1^{PP2A} is identical in sequence to PHAPI and, by our analysis, to pp32. Since I_1^{PP2A} shares an acidic tail with a second specific inhibitor of protein phosphatase 2A termed I_2^{PP2A} , the authors speculated that the acidic tail is responsible for the observed phosphatase inhibition. Meanwhile, Matsuoka *et al.* (1994) reported cloning of essentially the same sequence from rat cerebellar brain. Identifying it through its transient postnatal expression in developing cerebellum, these workers termed the protein leucine-rich nuclear acidic protein (LANP) and showed that its expression occurred principally in Purkinje cells. No function was identified in this study, although a role in differentiation was suggested. Other than PHAPI, LANP, and I_1^{PP2A} , no close homologies can be found between pp32 and other molecular species listed in the GenBank, European Molecular Biology Laboratory, and SwissProt databases.

Expression of pp32 Message

Human pp32 hybridizes with three RNA species in Northern blots of poly(A)⁺ RNA from HL-60 cells and hybridizes reciprocally with two murine species from A₂₀ cells under highly stringent conditions. Figure 5 shows that when the entire coding sequence is used as a probe, a 1.3-kb species apparently corresponding to the cloned species is identified as well as additional species at 2.2 and 2.3 kb (Figure 5, lane A). The explanation for this phenomenon likely lies in alternative 3' polyadenylation signals, since one additional human pp32 mRNA species has been identified which is 881 nucleotides longer at its 3' end than the HL.2 clone shown in Figure 1. Clone HL.13 is a 1766-bp cDNA fragment from HL-60 cells which begins on nucleotide 176 of the HL.2 clone of pp32 (Figure 1) and extends for an additional 881 nucleotides beyond its termination; no poly(A) tail is present in HL.13 cDNA, although the 3' extension hybridizes with poly(A)⁺ RNA. Except for the 3' extension, it is completely identical to the pp32 sequence. The 1.3-, 2.2-, and 2.3-kb messages all hybridize in blots of human poly(A)⁺ RNA probed with a 5' *Hind*III fragment of HL.2 (Figure 5, lane E), whereas only the 2.2- and 2.3-kb messages hybridize with an *Eco*RI fragment representing the 3' terminal 273 bp of HL.13 (Figure 5, lane F). Based on its hybridization pattern, it is probable that the 2.3-kb species also represents a polyadenylation variant. In human tissues and cell lines, the

Figure 4. Structure of human and murine pp32. The schematic of pp32 illustrates the following features: P, potential casein kinase II phosphorylation sites; N, potential nuclear localization signals; LVLL (human) and LILL (murine), potential leucine zippers. Murine pp32 is apparently phosphorylated on a single phosphoserine (Malek *et al.*, 1990); the phosphorylation state of human pp32 has not yet been characterized.

three pp32 mRNA species appear by Northern blot analysis to be roughly coequal in their expression. In contrast, only 1.3- and 2.2-kb species are seen in murine cell lines such as A₂₀, with the 1.3-kb species predominating (Figure 5, lane D). The predominance of the 1.3-kb form may account for why no murine cDNA clones with extended 3' untranslated regions have been encountered so far.

pp32 Fails to Modulate Transcriptional Activity

The combination of an amphipathic α helix with an acidic domain suggests that pp32 might act through regulation of transcriptional activity. Although both native and recombinant pp32 fail to bind to DNA cellulose (our unpublished results), pp32 could interact with DNA through a second protein to which it binds, permitting it to modulate transcription. To test this possibility, we transfected CHO cells with an

Figure 5. Human and murine pp32 RNA analyzed by Northern blot. This figure shows that similar RNA species are reciprocally identified in human and murine RNA in blots probed with human and murine pp32 probes and washed at moderately high stringency. Lanes A and C, 10 μ g of total human HL-60 RNA; lanes B and D, 10 μ g of total murine A₂₀ RNA. Lanes A and B were probed with human cDNA (clone HL2), and lanes C and D were probed with murine cDNA (clone 35.7). Lanes E and F, 1 μ g poly(A)⁺ human HL-60 RNA probed either with the 5' *Hind*III fragment from clone HL2 (lane E) or with an *Eco*RI fragment from HL13 representing the extended 3' untranslated sequences. All filters were washed at 55°C in 0.2 $\times$ SSC.

expression vector encoding a fusion protein of pp32 downstream of the Gal4 DNA-binding domain. Transcriptional modulatory activity was evaluated by cotransfection of the Gal-pp32 construct into CHO cells along with a reporter plasmid containing the luciferase gene under control of the thymidine kinase promoter along with five tandem Gal4 DNA-binding sequences. The thymidine kinase promoter was chosen because its detectable basal activity permits analysis of both positive and negative transcriptional regulatory activity. Controls consisted of a Gal-VP16 fusion as a positive regulator and Gal-wt1 as a negative regulator. All constructs were compared with the activity of the unfused Gal-O plasmid or buffer. Figure 6 shows that pp32 caused only fourfold transcriptional activation in comparison to the nearly 420-fold activation of the Gal-VP16 construct. Since the Gal4-pp32 construct produced only marginal activity, the data indicate that pp32 is unlikely to act through transcriptional activation. It does remain possible that pp32 may modulate transcription of selected genes through specific interactions not replicated by the Gal4 system.

pp32 Inhibits Transformation

The high levels of pp32 expression seen in normal self-renewing cell populations and in cancer cells lead to two disparate potential explanations. In normal

Figure 6. Modulation of transcription by pp32. CHO cells were cotransfected with the indicated constructs and pG5L2 containing a luciferase reporter under the thymidine kinase promoter. Luciferase activity was measured 4 d after transfection and normalized to total cellular protein to obtain the relative transcriptional activity. This figure shows the average $\pm$ range of duplicate determinations.

tissues *in vivo*, pp32 is selectively expressed in those cells capable of self-renewal, which, from a teleological standpoint, should be resistant to transformation. By this reasoning, pp32 might potentially act to suppress one or more events leading to transformation. In contrast, pp32 is highly expressed in at least several forms of human neoplasia including prostate cancer (Walensky *et al.*, 1993) at cellular levels and in proportions of cells which seem to increase with increasing severity of clinical disease. These data lead to the alternative suggestion that increased pp32 expression either favors tumorigenesis or confers a selective advantage to neoplastic cells. To begin to resolve these questions, we tested the effects of increased pp32 expression on the well-characterized system of transformation of rat embryo fibroblasts by *ras* and *myc* (Land *et al.*, 1993). pp32 and control plasmid were cotransfected in varying ratios with *ras* and *myc* into rat embryo fibroblasts. Figure 7 shows that pp32 inhibits *ras-myc* transformation of rat embryo fibroblasts in a dose-dependent manner. The inhibition of *ras-myc*

Figure 7. Dose-dependent inhibitory effect of pp32 on formation of transformed foci in rat embryo fibroblasts transfected with *ras* and *myc*. Rat embryo fibroblasts were cotransfected with pEJ-*ras*, pMLV-*c-myc*, and the indicated mixtures of pCMV32 and pCMV DNA such that the total amount of transfected DNA was constant in all samples. Foci were counted at 2 wk posttransfection. This figure shows the mean of triplicate measurements $\pm$ SE.

Table 1. pp32-mediated inhibition of transformed focus formation is independent of vector and promoter

Experiment	Plasmid	FLASK				% Inhibition
		I	II	III	IV	
I	pCMV	49	44	48	ND ^a	70.9
	pCMV32	8	15	18	ND	
II	pCMV	42	52	47	ND	63.1
	pCMV32	14	20	18	ND	
III	pSV40	25	24	20	28	73.2
	pSV32	6	6	5	9	
Control	None	43	47	ND	ND	5.6
	pCMV	48	37	ND	ND	
	pSV40	43	40	ND	ND	

Rat embryo fibroblasts were transfected with pEJras and pMLV-c-myc with either blank plasmids (pCMV or pSV40) or human pp32 cDNA (HL2)-carrying plasmids (pCMV32 or pSV32). Transformed foci were counted 2 wk posttransfection. Control experiments were performed to show that blank plasmids had no effect on focus formation.

^a ND, not determined.

transformation by pp32 is highly reproducible and is independent of both vector and promoter, since similar results were obtained in constructs using an SV40 promoter (Table 1). Rat embryo fibroblasts transfected with pp32 alone were indistinguishable from cells transfected with blank vector in terms of focus formation, cell density, and morphology.

We considered the possibility that pp32 expression may be directly toxic to cells; however, additional evidence suggests that this is not the case. Cotransfection of pCMV32 or pSV32 plasmids with the hygromycin-selectable plasmid pHyg (Sugden *et al.*, 1985) did not reduce the average number of dually hygromycin- and G418-resistant stably transfected colonies over those obtained with pCMV or pSV40 vectors alone: pCMV + pHyg, 58.5; pCMV32 + pHyg, 72.5; pSV40 and pHyg, 84.0; and pSV32 and pHyg, 73.5. In point of fact, stably transfected cells are resistant to drug-induced programmed cell death (Chen *et al.*, manuscript in preparation). Taken together, these observations suggest that pp32 exerts its effects in a specific manner and not through toxic effects or induction of apoptosis.

Additional experiments evaluated whether pp32 inhibits transformation only by ras and myc, by additional subsets of oncogenes possessing specific structural motifs such as a leucine zipper, or by oncogene subsets which act through specific pathways, such as E6 and E7, which involve Rb and p53. When tested against mutant p53 (Eliayhu *et al.*, 1984), E1a (Ruley, 1983; Ruppert *et al.*, 1991), or human c-jun (Schutte *et al.*, 1989; Wisdom and Verma, 1990) in combination with ras, pp32 inhibited focus formation to an extent similar to the inhibition of ras and myc (Figure 8). To exclude the possibility that pp32 simply acts through the ras signaling pathway, which is common to all of

the foregoing oncogene combinations, pp32 was also tested against E6 and E7. Figure 9 shows that transformation by this combination is also suppressed by pp32.

Figure 8. pp32 inhibits formation of transformed foci in rat embryo fibroblasts transfected with E1a, mutant p53, and c-jun in combination with ras. Rat embryo fibroblasts were cotransfected with pEJ-ras (ras), with pMLV-myc (myc), pC53-175 (mutant p53), pCT-E1a (E1a), or pSVcjun (jun) as described in the text. Cultures were also simultaneously cotransfected with either vector (pCMV) or a pp32 construct (pCMV32). Foci were scored following 2 wk of incubation. Error bars, SE of the mean of triplicate determinations. This figure demonstrates that pp32 suppresses focus formation not only by myc, but by c-jun, E1a, and mutant p53 also.

Figure 9. pp32 inhibits formation of transformed foci in rat embryo fibroblasts transfected with E6 and E7. Rat embryo fibroblasts were cotransfected with pEJ-ras, pMLV-myc, and either vector (pCMV) or a pp32 construct (pCMV32). Alternatively, cells were cotransfected with pCMV16E6 and pCMVE7 along with either pCMV or pCMV32. Foci were counted after 10 d of incubation. Error bars, SD of triplicates for the ras/myc combination and the range of duplicates for the E6/E7 experiment. These data indicate that inhibition of transformed focus formation by pp32 is independent of the ras signaling pathway, since ras is not required as one of the cotransfection partners.

Two additional independent and complementary lines of evidence support our hypothesis that pp32 acts through a nuclear pathway rather than the ras signaling pathway. Transient transfection of pp32 into NIH3T3 cells along with ras does not affect focus formation (Table 2). In contrast, stable transfection of pp32 into Rat 1a-myc cells greatly diminishes their ability to grow in soft agar and diminishes colony size (Table 3). Rat 1a-myc cells (Barrett *et al.*, 1995) are a subline of Rat 1a stably transfected with myc. Phenotypically, these cells appear stably transformed as judged by their constitutive ability to grown in an

Table 2. Effect of pp32 on ras-induced formation of transformed foci in NIH3T3 cells

Experiment	ras + pCMV	ras + pp32	pCMV	pp32
1	32.3 ± 3.0	28.6 ± 1.1	4.0 ± 0.8	n.d. ^a
2	23.0 ± 1.2	17.6 ± 0.8	5.3 ± 0.8	4.6 ± 0.4
3	31.6 ± 0.4	43.3 ± 0.6	7.3 ± 0.3	n.d.

NIH3T3 cells were transiently transfected with ras, pp32, or control vectors as indicated and then plated. Transformed foci were enumerated at 10–12 d posttransfection. The data represent the mean of triplicate determinations ± SE.

^a n.d., not determined.

Table 3. Effect of pp32 on growth of rat 1a-myc cells in soft agar

Cell line	Mean colonies/dish ± SE
Rat 1a-myc-pCMV	1674 ± 26.4
Rat 1a-myc-pCMV32	792 ± 71.0

Rat 1a-myc cells were stably transfected with either pp32 (Rat 1a-myc-pCMV32) or blank vector (Rat 1a-myc-pCMV). Following selection, individual clones were plated and analyzed for their ability to form colonies in soft agar. For each vector, two cell lines were stably transfected as indicated, then both cell lines for each vector were tested in duplicate in a single experiment. The data represent the mean of the resultant four determinations for each construct ± SE.

adhesion-independent manner and form colonies in soft agar. The data indicate that overexpression of pp32 diminishes both colony number and size. In Table 3, Rat 1a-myc cells stably transfected with pp32 produced markedly fewer colonies than did cells transfected with vector alone. The colonies that do form are dramatically smaller (Figure 10). Because Rat 1a-myc cells were transfected with myc substantially prior to their stable transfection with pp32, the data not only show an inhibitory effect on myc-mediated adhesion-independent growth, but show that under some circumstances pp32 can act to reverse previously established phenotypic changes.

The ability of pp32 to inhibit the formation of transformed foci induced by a series of mechanistically disparate oncogenes is intriguing. Relatively few molecules have been reported to inhibit focus formation caused by ras and myc (Eliyahu *et al.*, 1989; Finlay *et*

Figure 10. Effect of pp32 on morphology of rat 1a-myc cell colonies in soft agar. Rat 1a-myc cells were stably transfected with either pp32 (Rat 1a-myc-pCMV32) or control vector (Rat 1a-myc-pCMV) and assayed for growth in soft agar. This figure shows the pronounced difference in colony morphology between pp32-transfected and control cells at 14 d after plating (250×).

al., 1989; Ginsberg *et al.*, 1991; Makela *et al.*, 1992; Mukherjee *et al.*, 1992; Yehiely *et al.*, 1992; Resar *et al.*, 1993; van den Heuvel *et al.*, 1993). There is no easily discernible common feature among the oncogenes inhibited by pp32. Although pp32 can reverse myc-mediated phenotypic changes in at least one system, its ability to inhibit transformation may be relative rather than absolute. Circumstantial evidence indicates that pp32 is unable to cause complete reversion of an established transformed state, since high levels of pp32 are seen in clinical specimens of human tumors and cell lines of various species (Malek *et al.*, 1990; Walensky *et al.*, 1993).

pp32 appears to act at a discrete point of mechanistic convergence of a number of important nuclear oncogenes. Because of the potentially significant implications for pp32 ligands, much of our current effort is directed toward a search for pp32 ligands using both biochemical means and molecular ones in the form of the two-hybrid system. Although recent studies (Li *et al.*, 1996) suggest that pp32 may inhibit a specific protein phosphatase, the relationship to transformation inhibition is not yet clear. The relationship should be clarified by experiments that will determine whether these activities reside on the same or different portions of the pp32 molecule.

The high levels of pp32 expression seen in human cancer are a puzzling feature. It is not presently known whether the pp32 found in cancer is the same as in normal tissues. Studies are in progress to resolve these issues through characterization of pp32 in paired normal and tumor tissue samples. It is entirely possible that functional differences will be uncovered between wild-type pp32 and tumor pp32, differences which affect pp32 function, confer a selective advantage, and thus contribute to tumorigenesis.

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