Modulation of oncogenic potential by alternative gene use in human prostate cancer

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Only a small percentage of primary prostate cancers have genetic changes. In contrast, nearly 90% of clinically significant human prostate cancers seems to express high levels of the nuclear phosphoprotein pp32 by *in situ* hybridization. Because pp32 inhibits oncogene-mediated transformation, we investigated its paradoxical expression in cancer by comparing the sequence and function of pp32 species from paired benign prostate tissue and adjacent prostatic carcinoma from three patients. Here we demonstrate that pp32 is expressed in benign prostatic tissue, but pp32r1 and pp32r2, closely-related genes located on different chromosomes, are expressed in prostate cancer. Although pp32 is a tumor suppressor, pp32r1 and pp32r2 are tumorigenic. Alternative use of the pp32, pp32r1 and pp32r2 genes may modulate the oncogenic potential of human prostate cancer.

In human prostate cancer, high-level expression of pp32 RNA occurs in nearly 90% of clinically significant prostate cancers, in contrast to the substantially lower frequencies of alterations of other oncogenes and tumor suppressors[1–3]. This highly conserved nuclear phosphoprotein may act as a tumor suppressor. Functionally, pp32 inhibits transformation *in vitro* by a wide variety of oncogene pairs, including ras and myc, ras and mutant p53, ras and E1a, ras and jun, and human papilloma virus E6 and E7 (refs. 4–7). It also inhibits the growth of transformed cells in soft agar[4]. Ras-transfected NIH3T3 cells previously stably transfected to overexpress normal human pp32 do not form foci *in vitro* and do not form tumors in nude mice, unlike control cells. In contrast, reduction of endogenous pp32 in the same system by an antisense pp32 expression construct augments tumorigenesis considerably (J.B. *et al.*, unpublished observations). Here we have addressed the paradox of the apparent expression of high levels of an anti-oncogenic protein by prostate cancers. We compared the sequence and function of pp32 species from paired benign prostate tissue and adjacent prostatic carcinoma from three patients, and found that prostate cancers express little or no pp32, but do express other members of the pp32 family encoded by genes on separate chromosomes. The alternative pp32 genes expressed in prostate cancer are tumorigenic, unlike pp32.

pp32 mRNA in benign prostate tissue and prostate cancer

In benign prostate tissue, basal cells express pp32, whereas *pp32* mRNA is not detectable by *in situ* hybridization in differentiated glandular cells (Fig. 1*a*). In contrast, there is strong *in situ* hybridization to *pp32* probes in nearly all clinically significant human prostatic adenocarcinomas. Strongly hybridizing tumors show intense immunopositivity with antibodies to pp32, indicating that they express pp32 or immunologically related proteins (Fig. 1*a* and *b*). The restricted expression of *pp32* mRNA in benign prostate is consistent with observations that pp32 has a distinct pattern of expression *in vivo*[4–6]. The expression of *pp32* mRNA in normal peripheral tissues is restricted to 'stem-like' cell populations, such as crypt epithelial cells in the gut and basal epithelium in the skin; cerebral cortical neurons and Purkinje cells also express pp32.

Molecular analysis of *pp32*-related transcripts

Given the localization data (Fig. 1*a* and *b*), it would seem that pp32 is expressed in both normal and neoplastic prostatic epithelial cells, despite its ability to inhibit neoplastic functions such as transformation. The explanation for this apparent 'discordant' expression is that prostate tumors do not usually express pp32; rather, they express variant pp32 species that promote transformation, instead of inhibiting it. RT–PCR of paired benign prostate tissue and prostatic adenocarcinoma from three patients yielded amplification products ranging from 889 to 907 bp (Fig. 2*a*). For this RT–PCR, consensus primers capable of amplifying the full-length coding sequence from *pp32* and the two closely-related intronless genomic sequences *pp32r1* (GenBank AF008216) and *pp32r2* (GenBank U71084) were used. The only difference between the samples was a lower amplicon yield from benign tissue than from neoplastic tissue. Four human prostatic adenocarcinoma cell lines, DU-145, LNCaP, PC-3 and TSUPR-1, also yielded products of a similar size (data not shown). Qualitative differences between normal and neoplastic tissue were demonstrated when the RT–PCR products were subcloned and analyzed by cleavase fragment length polymorphism analysis (CFLP) and sequence analysis. All clones of the RT–PCR products from benign prostate tissue produced a normal CFLP pattern that corresponded precisely to that obtained from previously cloned *pp32* cDNA template (Fig. 2*b*). Prostatic adenocarcinomas yielded four distinct CFLP patterns, of which three were unique and one was like the normal pp32 pattern.

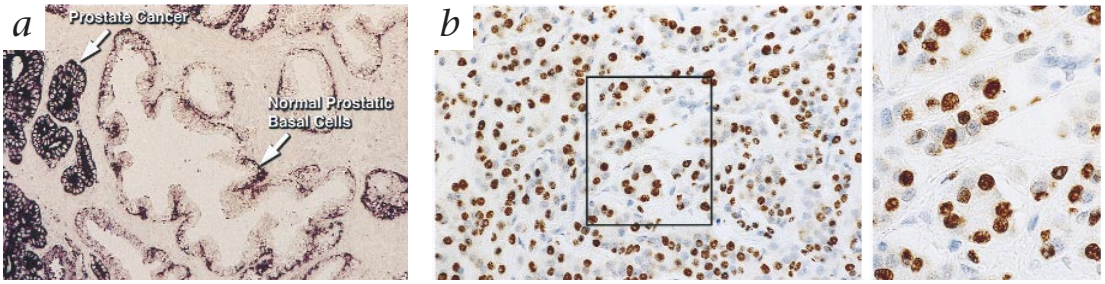

Fig. 1 ***a***, Detection of pp32-related mRNA in benign prostate tissue and prostate cancer. The pp32 mRNA was detected by stringent *in situ* hybridization with a pp32 probe; the signal is cytoplasmic, as mRNA and not protein is detected in this assay. Normal prostatic basal cells are positive, whereas the clear, differentiated glandular cells are negative. In contrast, prostatic adenocarcinoma (left arrow) is very positive. ***b***, Prostate cancers stain intensely with antibody against pp32. High-grade human prostate cancer stained with affinity-purified rabbit polyclonal antibody against pp32 (ref. 17). Left, a representative field (original magnification, ×250); rectangle, the area shown in computer-generated detail (right).

In contrast to benign prostate samples, which yielded only *pp32* transcripts by sequence analysis, adjacent prostate cancers expressed little or no *pp32*. Instead, four *pp32*-related transcripts with distinct sequences encoding open reading frames were obtained from the adjacent prostate cancers, varying from 92.4% to 95.9% nucleotide identity to normal *pp32* cDNA. Of these, two transcripts corresponding to the products of the *pp32*-related genes *pp32r1* and *pp32r2* were obtained repeatedly from patient samples; thus, we studied them further. We compared the properties of the *pp32r1* and *pp32r2* transcripts obtained from prostatic adenocarcinomas (Table 1). The identical *pp32r1* sequences obtained from two patients differed by four nucleotides from the *pp32r1* genomic sequence. The *pp32r2* sequences obtained from two patients were also identical and differed by three nucleotides from the *pp32r2* genomic sequence. Both sets of differences are considered consistent with polymorphic variation.

Multiple pairwise alignment of the predicted protein sequences[8] demonstrated that pp32, pp32r1 and pp32r2 have sequence changes along their entire lengths (Fig. 3). The 'pile-up' and pairwise alignments show that there is a high degree of sequence conservation at the predicted amino acid levels, corresponding to an underlying conservation of nucleotide sequence; and that the sequence differences are distributed throughout the length of the sequence without obvious clustering, 'hotspots' or segmentation of sequence differences. No straightforward process of somatic mutation or alternate splicing could explain these results. Instead, given the correspondence of the variant sequences with previously identified genes on chromosomes 4 and 12, the data are consistent with alternative gene expression.

Oncogenic potential of pp32, pp32r1 and pp32r2

A switch in the oncogenic potential of the expressed pp32 family members accompanies the expression of alternative pp32 genes in prostate cancer. Expressed pp32r1 and pp32r2 often fail to inhibit or, indeed, sometimes stimulate transformed focus formation when co-transfected with ras and myc, compared with the number of foci obtained when ras and myc are transfected with control vector (Fig. 4). In contrast, normal pp32 consistently suppresses transformation. Similarly, both pp32r1 and pp32r2 are tumorigenic when stably transfected into NIH3T3 cells, in contrast to pp32, which is non-tumorigenic (Tables 1 and 2).

Table 1 Characteristics of the pp32 family

Property	pp32	pp32r1	pp32r2
Localization in prostate	Normal prostatic basal cells; rare in prostate cancer	Prostate cancer; none in normal tissue	Prostate cancer; none in normal tissue
Effect on *in vitro* transformation	Inhibits	Variably stimulates	Variably stimulates
Effect on tumorigenesis	Inhibits	Tumorigenic	Tumorigenic
Chromosomal Localization	Chromosome 15	Chromosome 4	Chromosome 12
Protein identity to pp32	-	87.6%	89.3%
Nucleic acid identity to pp32	-	94.4%	92.4%

Discussion

Species of pp32 have been found in a variety of biologic contexts, although the relationship to neoplasia has not always been appreciated. An essentially equivalent molecule, PHAPI, was cloned from an EBV-transformed human B-lymphoblastoid cell line[9]; PHAPII, cloned by the same strategy, is unrelated to pp32. PHAPI was identified through its association in solution with human HLA class II protein, and was localized to the plasma membrane, cytoplasm and nucleus. The *PHAPI* gene putatively localizes to chromosome 15q22.3-q23, as shown by fluorescent *in situ* hybridization[10]. (*PHAPI* and pp32 are equivalent). The protein pp32 has been variously identified as I1PP2a, an inhibitor of protein phosphatase 2a (ref. 11; I2PP2a is unrelated to pp32); a cytoskeletally-associated cytosolic protein in CHO cells[12] (perhaps due to a difference in system, or perhaps pp32 can localize to the cytoplasm under certain circumstances); and LANP, a leucine-rich nuclear protein in the central nervous system[13]. There are reports of gene products with less homology to pp32 as well. PHAPI2a (EMBL HSPHAP12A) and PHAPI2b (EMBL HSPHAP12B) were also cloned from an EBV-transformed human B-lymphoblastoid cell line. These variant pp32 sequences, different from the sequences reported here, represent APRIL (acidic protein rich in leucines; ref. 14; EMBL HSAPRIL), a protein cloned from human pancreas that is shorter than PHAPI2a by two N-terminal amino acids; PHAPI2b is identical to a subset of APRIL. Silver-stainable protein SSP294 (GenBank HSU70439) was cloned from HeLa cells and is identical to PHAPI2a.

There are probably *pp32* genes other than the three with known chromosomal localization studied here; however, their characterization remains incomplete and their precise number is not known. We obtained single isolates of tumor cDNA encoding two additional tumorigenic pp32 variants that are undergoing

Table 2 Tumorigenicity in nude mice of NIH3T3 cells transfected with pp32 and pp32 variants

pp32 Species	Clone	Tumors/mice	Average tumor weight ± s.d. (grams)
pp32r1	1	3/3	10.5 ± 2.8
	2	3/3	3.8 ± 2.1
pp32r2	1	3/3[a]	1.3 ± 0.9
	2	3/3	13.8 ± 3.3
pp32	5[b]	0/3	
	6[b]	0/3	
Vector control	2[b]	0/3	
	3[c]	0/3	

[a]One tumor in this group, weighing 0.5 gm, was detected only on postmortem dissection. [b]pp32, clones 5 and 6, and vector control, clone 2 were tested in separate groups of animals simultaneously receiving tumorigenic pp32 variants derived from PC-3 cells on the contralateral side. [c]Vector control, clone 3 was tested in a group of animals simultaneously receiving a tumorigenic pp32 variant derived from a patient tumor on the contralateral sides of a single group of animals. s.d., standard deviation

further characterization. The previously mentioned cell lines, DU-145, PC-3, and TSUPr-1, yielded pp32r1 and pp32r2, but also yielded single isolates of additional tumorigenic variants of unknown chromosomal origin that are undergoing further analysis.

There seem to be at least four genes in the *pp32* family in rodents, which is consistent with the existence of a gene family of comparable size in humans. This is a minimum estimate, as the expressed sequence tags detected so far may not represent the complete extent of the *pp32* gene family. For example, a murine pp32 (ref. 4) (GenBank U73478) has 89% amino acid identity with pp32, but less identity with pp32r1 and APRIL. We have also identified expressed sequence tags predicted to encode pp32-related proteins in *Caenorhabditis elegans*, schistosomes, zebrafish and Drosophila (data not shown), indicating that pp32 family members effect fundamental functions subject to phylogenetic conservation.

The human *pp32* gene has been mapped to chromosome 15q22.3-q23 by fluorescence *in situ* hybridization[10]. A Unigene entry for *pp32* (Hs. 76689; HLA-DR associated protein I) lists 93 expressed sequence tags corresponding to this gene, 12 of which contain a mapped sequence-tagged site. These sequence-tagged sites all map to chromosome 15, as do many of the *pp32* expressed sequence tags (http://www.ncbi.nlm.nih.gov). *APRIL* was also mapped to chromosome 15q25 (ref. 14; GenBank Y07969). The *pp32r1* gene maps to chromosome 4, as determined by PCR of the NIGMS monochromosomal panel 2 (National Institute of General Medical Sciences human genetic mutant cell repository; ref. 15) followed by sequencing of the PCR product. The full sequence of *pp32r1*, including 4,364 nucleotides of sequence upstream of the putative translational start site , had more than 400 matches in a 'Blastn' search of the nonredundant GenBank database. These matches were to two short regions of about 278 and 252 base pairs (nucleotides 674–952 and 2542–2794) that represent repeats in opposite orientations. The repeats are related to elements on many chromosomes.

The data reported here indicate that the alternative use of pp32 genes is a common feature of human prostate cancer, and that this alternative gene use is accompanied by a change in oncogenic potential. Of prostate cancers of Gleason Score 5 and above, 87% express pp32 or closely related transcripts[3], which is in contrast to the much lower frequency of molecular alterations in other widely studied oncogenes and tumor suppressor genes. For example, myc overexpression[2] occurs in about 60% of cases, and p53 is abnormal in only about 25% of primary tumors[1]. In contrast to these oncogenes and tumor suppressors, the high frequency of pp32 variant expression indicates that alternative expression of variant pp32 species may be involved in the etiology of human prostate cancer. These findings may have important diagnostic and prognostic implications.

Modulation of oncogenic potential by alternative gene use has interesting implications. Preliminary comparison of structures predicted by energy minimization programs for pp32 and variant pp32 species indicates considerable structural differences that might form the basis for interaction with different mechanistic pathways by pp32 and the variant pp32 species. It is not yet apparent how early in the neoplastic process the use of alternative genes of the *pp32* family occurs. Because alternative *pp32* gene use in prostate cancer is common, it could be an early, important event in tumorigenesis.

Alternative gene use is potentially reversible, which has additional clinical and mechanistic implications. The malignant potential of tumor cells might eventually be modified by manip-

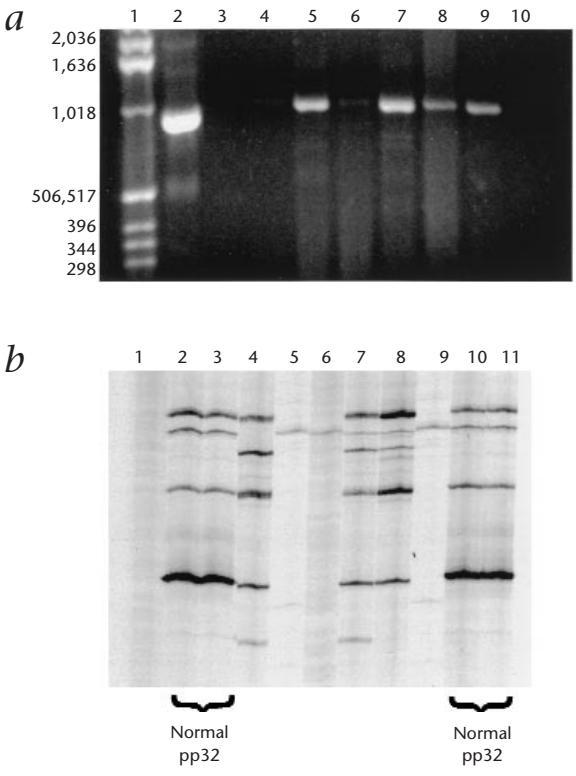

Fig. 2 ***a***, Amplification of pp32 and pp32 variants from human prostate cancer. Lanes: 1, 1-kb DNA 'ladder' (left margin, size in kb); 2, pCMV32; 3, FT-1, without reverse transcription; 4, FN-1; 5, FT-1; 6, FN-2; 7, FT-2; 8, FN-3; 9, FT-3; 10, negative control with template omitted. FN, frozen benign prostate; FT, frozen prostatic adenocarcinoma; numbers indicates patient designations. ***b***, Cleavase fragment length polymorphism (CFLP) analysis of pp32 detects variant pp32 transcripts in human prostate cancer. CFLP analysis of cloned cDNA amplified by RT–PCR from human prostatic adenocarcinoma and adjacent benign prostate tissue, using primers derived from the normal pp32 cDNA sequence. Lanes: 1, undigested normal pp32 cDNA; 2 and 3, normal pp32 cDNA; 4, FT1.11; 5, FT1.7; 6, FT2.2; 7, FT2.4; 8, FT3.18; 9, FT3.3;10, FN3.17; 11, FN2.1. FT, frozen prostate cancer; FN, frozen benign prostate. The band shifts correspond to sequence differences.

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          1                                                  50
  pp32    MEMGRRIHLE LRNRTPSDVK ELVLDNSRSN EGKLEGLTDE FEELEFLSTI
pp32r1            s       a                        a            k
pp32r2         kw                   f    q                   l n

          51                                                 100
  pp32    NVGLTSIANL PKLNKLKKLE LSDNRVSGGL EVLAEKCPNL THLNLSGNKI
pp32r1     g     sd          r         k                     y
pp32r2     i                           a v

          101                                                150
  pp32    KDLSTIEPLK KLENLKSLDL FNCEVTNLND YRENVFKLLP QLTYLDGYDR
pp32r1              q                        g       l        scyw
pp32r2                    e       t        n  ~~~~~~~~~ ~~~~~~~~~~

          151                                                200
  pp32    DDKEAPDSDA EGYVEGLDDE EEDEDEEEYD EDAQVVEDEE DEDEEEEGEE
pp32r1     h     y  i  dh           g h                    g e
pp32r2    ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~

          201                                                249
  pp32    EDVSGEEEED EEGYNDGEVD DEEDEEELGE EERGQKRKRE PEDEGEDDD
pp32r1         gd              g                        ~~ ~~~~~~~~~
pp32r2    ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~~ ~~~~~~~~~
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Fig. 3 Alignment of pp32 with human prostate tumor-derived pp32r1 and pp32r2 sequences. Differences from the pp32 sequence are indicated with lower-case letters; agreement with the pp32 sequence is indicated as a blank. The variant pp32r2 encodes a truncated protein (wavy lines indicate the truncated region). The GCG Pileup and Pretty programs were used[9].

ulating the pattern of expression of pp32 family members in a form of endogenous gene therapy. Gene therapy by pharmacological manipulation of the differential expression of pp32 family members must await characterization of the switching mechanism, which is now unknown. Mutation and loss of heterozygosity of the *pp32* locus at 15q22.3-q23 could theoretically lead to increased expression of *pp32r1* and *pp32r2*; however, loss of heterozygosity is unlikely, as chromosomal loss of 15q22.3-q23 is not a common feature of prostate cancer. More likely possibilities involve regulatory aberrations. Epigenetic regulation should be explored, as it could lead to inactivation of the *pp32* gene and concomitant activation of the *pp32r1* and *pp32r2* genes by means such as methylation and demethylation. Regulation by one or more transcription factors that act differentially to repress *pp32* while inducing *pp32r1* and *pp32r2* is possible. Finally, post-transcriptional mechanisms could also be invoked, whereby differential expression would be regulated by changes in mRNA or protein stability. For carcinogenesis, all of the mechanisms involving regulatory aberrations would contribute to tumorigenesis through a plastic, potentially reversible regulatory change rather than an irreversible structural change in the genome. Thus, members of the pp32 family may eventually be used as targets for pharmacologic chemopreventive and therapeutic strategies in prostate cancer.

Methods

***In situ* hybridization.** Bases 1–298 of the *pp32* cDNA sequence (GenBank HSU73477) were subcloned into the Bluescript vector by standard techniques. Digoxigenin-labeled antisense and sense RNA probes were generated using a commercially available kit (Boehringer). Vector DNA linearized with *Bam*HI and *Xho*I served as template for the generation of antisense and sense probes, respectively. DNA was transcribed *in vitro* for 2 h at 37 °C in a final volume of 20 µl containing 1 µg of template DNA; 2 U/µl of either T3 or T7 RNA polymerase; 1 U/µl ribonuclease inhibitor; 1 mM each of ATP, CTP and GTP; 0.65 mM UTP; 0.35 mM digoxigenin-11-UTP; 40 mM Tris-HCl, pH 8.0; 10 mM NaCl; 10 mM DTT; 6 mM MgCl2; and 2 mM spermidine. The addition of 2 µl of 0.2M EDTA, pH 8.0 stopped the reaction, and subsequent incubation for 30 min at –70 °C with 2.2 µl of 4 M LiCl and 75 µl of pre-chilled ethanol precipitated the synthesized transcripts. RNA was pelleted by centrifugation, washed with 80% ethanol, partially dried, and dissolved in 100 µl of DEPC-treated water. Yields of labeled probe were determined by an enzyme linked immunoassay using a commercially available kit (Boehringer). Nonradioactive *in situ* hybridization used anti-sense and sense *pp32* RNA probes generated by *in vitro* transcription[3]. The signal is cytoplasmic, as mRNA and not protein is detected in this assay.

Immunohistochemistry. Formalin-fixed, paraffin-embedded tissue was cut into sections 4 µM in thickness, deparaffinized, hydrated, processed for heat-induced antigen retrieval at 95 °C in 0.01 M citrate buffer, pH 6.0, for 20 min (ref. 16), then incubated overnight at room temperature with a 1:20 dilution of antibody against pp32. After being washed, slides were sequentially developed with biotinylated swine antibody against rabbit IgG at a diltuion of 1:100 (Dako, Carpinteria, California), streptardin peroxidase (Dako, Carpinteria, California) and diaminobenzidine.

RT–PCR and CFLP. Sequences were 'reverse-transcribed' and amplified using bases 32–52 of HSU73477 as a forward primer and bases 919–938 of the same sequence as a reverse primer in conjunction with the Titan One-Tube RT–PCR kit (Boehringer). Reverse transcription was done at 50 °C for 45 min followed by incubation at 94 °C for 2 min; the subsequent PCR comprised 45 cycles of 92 °C for 45 s, 55 °C for 45 s and 68 °C for 1 min with a final extension at 68 °C for 10 min in a PTC 100 thermocycler (MJ Research, Watertown, Massachusetts). Template RNA was isolated from cell lines or frozen tumor samples using RNAzol B (Tel-Test, Friendswood, Texas) according to the manufacturer's instructions, then digested with RNAse-free DNAse I (Boehringer). The plasmid pCMV32 was used as a positive control without reverse transcription. After RT–PCR, amplicons were cloned into pCR2.1 or pCR3.1 (Invitrogen, Carlsbad, California) for further analysis. Cloned inserts were analyzed by cleavase fragment length polymorphism (CFLP) assay according to the manufacturer's specifications (Life Technologies), with digestion at 55 °C for 10 min in 0.2 mM $MnCl_2$, and were electrophoresed on a 6% denaturing polyacrylamide sequencing gel. Figure 2*b*, lane 1 is an undigested control whose band migrated substantially slower than the digestion products; samples in all other lanes were digested with cleavase as described.

Sequence analysis. Some comparisons of sequences to determine nucleotide identity were done using the Wisconsin Package, version 9.1 (1997; Genetics Computer Group, Madison, Wisconsin).

Transformation assay

Rat embryo fibroblasts were transfected with constructs as described[4] and transformed foci were counted. For each experiment, approximately 1×10^6 cells were plated per 75-cm^2 flask and incubated for 2–3 d before transfection, to achieve approximately 40% confluency. For each flask of primary rat embryo fibroblasts, the following amount of plasmid was

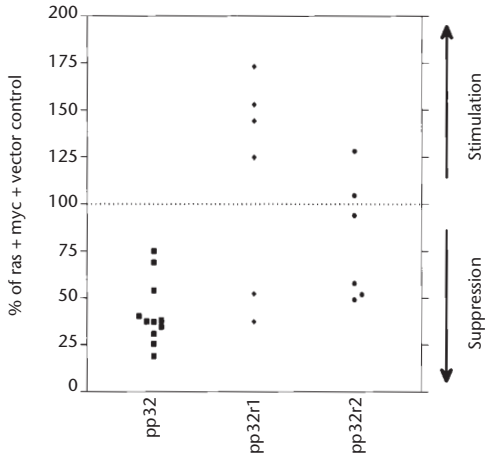

Fig. 4 Effect of variant pp32 species on transformation: altered oncogenic potential of pp32r1 and pp32r2. Each data point represents the results from an individual flask expressed as the percent foci obtained in the contemporaneous control of ras+myc+vector. Data represent four separate experiments.

added: pEJ-ras, 5 μg; pMLV-c-myc, pCMV32, pCMVneo or variant pp32 constructs in pCR3.1 (Invitrogen, Carlsbad, California), 10 μg. Plasmids were prepared in two volumes of lipofectin (2 μl lipofectin per μg DNA; Life Technologies), then gently mixed by inversion in 1.5 ml OPTIMEM (Life Technologies) 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, and then incubated in 6 ml of OPTIMEM and 1.5 ml of the DNA/lipofectin mix. After overnight incubation, the cells were grown in standard media and supplied with fresh media twice weekly. Foci were counted 14 days after transfection.

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