Identification of Sequences Required for Inhibition of Oncogene-mediated Transformation by pp32*

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Oncogenic potential in prostate cancer is modulated in part by alternative use of genes of the pp32 family. This family includes the tumor suppressor pp32, expressed in normal tissue, and the pro-oncogenic genes pp32r1 and pp32r2 that are found principally in neoplastic cells. At the protein level, pp32, pp32r1, and pp32r2 are approximately 90% identical, yet they subsume opposite functions. In this study, we identify the region of pp32 associated with the ability to inhibit oncogene-mediated transformation in a rat embryo fibroblast system, an in vitro correlate of tumor-suppressive activity. Deletion and truncation analysis define a region spanning pp32 amino acids 150–174 as absolutely required for inhibition of transformed foci elicited by RAS and MYC. Comparison of pp32 with the pp32r1 sequence by moving averages of sequence identity reveals divergence over this region; pp32r2 also differs in this region through truncation after pp32 amino acid 131. The deletion experiments and the experiments of nature therefore converge to demonstrate that tumor-suppressive functions of pp32 reside in amino acids 150–174. Identification of this minimal tumor-suppressive region should help elaborate the pathways and mechanisms through which pp32 family members exert their functions.

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pp32 is a member of a closely related family of nuclear phosphoproteins that are alternatively expressed in normal tissues and in human prostate cancer (1–6). Whereas pp32 is a tumor suppressor that is expressed in stem-like and long-lived cells in normal tissues in vivo, pp32r1 and pp32r2 are tumorigenic molecules that are expressed in prostate and breast cancers immediately adjacent to normal tissues that continue to express pp32. The pronounced difference in function of these molecules is striking in view of the high degree of structural conservation. pp32r1 is a 234 amino acid protein that is 87.6% identical to the 249 amino acid pp32; pp32r2, although C-terminally truncated after amino acid 131, is 89.3% identical to pp32 over the length of the predicted protein sequence. Since pp32 inhibits transformation in vitro and tumorigenesis in vivo (1–6), it is reasonable to hypothesize that these functions will map to one or more discrete regions of the pp32 molecule and that pp32 will differ in sequence from pp32r1 and pp32r2 in regions that relate to its tumor suppressor function. In this paper, we localize the pp32 function of inhibition of transformation in vitro and analyze the resultant information in the context of the tumor-derived pp32r1 and pp32r2 sequences.

MATERIALS AND METHODS

Molecular Biology Reagents and Generation of Expression Constructs—All reagents were purchased from Life Technologies, Inc. except for calf intestinal alkaline phosphatase, which was purchased from Roche Molecular Biochemicals. pp32 truncation constructs were generated via polymerase chain reaction amplification of desired pp32 sequences. All constructs utilized a common upstream primer designed to include the 5′-untranslated region from base 32 onward, including the endogenous translational start site of human pp32 cDNA (2). All downstream primers included at least one stop codon in the proper reading frame. To facilitate subcloning, both upstream and downstream polymerase chain reaction primers included BamHI sites at their 5′ ends. Primer pairs were optimized using Oligo version 4.1. Following amplification from a previously described cDNA clone of human pp32 (Ref. 6, GenBank™ accession number HSU73477), amplified segments were gel-purified, digested with BamHI, and subcloned into the BamHI site of a pCMV expression vector. All constructs were checked for proper orientation by restriction digest. Clone Δ201–360 was constructed in two stages. In the first stage, bases 32–202 were amplified and cloned into pCMV; the downstream primer introduced a translationally silent XhoI site at bases 196–202. Sequence analysis confirmed the correct orientation. In the second stage, bases 361–987 were amplified and cloned into the XhoI site by a strategy analogous to that used for the other constructs. All constructs were purified by double-banding in cesium chloride gradients.

Transformation Assays—Constructs were analyzed for their ability to inhibit formation of transformed foci of rat embryo fibroblasts in vitro and tumorigenicity in vivo. MYC and RAS+MYC constructs were evaluated previously with the following experiments were performed, consisting in aggregate of six replicates for each construct 1–666, 1–543, 1–350, and Δ201–360, and of nine replicates for each constructs 1–484 and 1–618. For each individual experiment, the mean and S.E. was determined for the RAS + MYC + neo (blank vector) controls; experiments were discarded as technically inadequate where the S.E. equaled or exceeded 10% of the mean number of foci obtained in this control. Each data point avg. 1 represents 100 times the quotient of the number of foci obtained in an individual flask divded by the mean of the corresponding control for the corresponding experiment.

Analysis of Expression of pp32 Constructs by Transient Transfection and Immunohistochemistry—Approximately 5 × 10⁴ rat embryo fibroblasts cells were plated on two-well, 4-cm² growth area plastic Permanox Lab-Tek chamber slides (Nunc), incubated for 1 day, and transfected with either full-length pp32 or construct 1–543 by the same method used for the transformation assays. Cells transfected with blank pCMV vector and untransfected rat embryo fibroblasts served as negative controls. Adherent cells were fixed 48 h after transfection in ice-cold methanol for 30 min at 4 °C and air-dried. All steps were carried out at room temperature in a humidified box with the chambers attached to the slide. Before staining, cells were rehydrated in TBS (100 mM Tris-HCl, pH 7.5, 150 mM NaCl). Endogenous peroxidase activity was blocked by incubation in 0.3% H₂O₂ for 20 min in the dark followed by two washes in TBS. Cells were permeabilized by washing in TBS containing 0.01% Tween 20 (TBS-T) for 3 min. Nonspecific antibody binding was blocked by incubation in 10% normal swine serum (Dako Corp.) in TBS-T for 1 h. Cells were sequentially incubated in a 1:5 dilution of affinity-purified polyclonal rabbit anti-human recombinant pp32 (8) in TBS-T for 18 h, washed three times for 10 min each in TBS-T to remove excess antibody, and then incubated in a 1:100 dilution of biotinylated swine anti-rabbit IgG (Dako) in TBS-T for 3 h. Following

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three subsequent washes in TBS-T, cells were incubated in an avidin-biotin-peroxidase complex (Dako) for 3 h, washed three times in TBS-T, then incubated with diaminobenzidine chromagen and H2O2 (DAB kit, Vector Laboratories). After adequate color development the chambers were removed, and the slides were rinsed in water and coverslipped with an aqueous glycerol-gelatin mounting medium. Basal levels of pp32 expression were defined as those found in untransfected rat embryo fibroblasts; cells showing greater than basal amounts of nuclear staining were counted as described in the legend to Table I.

Sequence Analysis—The pp32 and pp32r1 sequences were compared using the Plotsimilarity program (Genetics Computer Group, Inc., Wisconsin Package, version 9.1) using a window of 25 amino acids with the program option set to show percent identity.

RESULTS AND DISCUSSION

pp32 Deletion Constructs—The amino acid sequence of pp32 predicts a generally α-helical N-terminal domain and a C-terminal acidic domain (6). The α-helical domain contains several motifs, including a leucine zipper, two putative nuclear localization signals comprised of clusters of basic residues, and potential casein kinase II phosphorylation sites. The purpose of these experiments was to use systematic truncation and deletion of pp32 to determine the role of these structural features in the functional ability of pp32 to suppress oncogene-mediated transformation (1, 6), and to relate the resultant information to the structures of tumor-derived pp32r1 and pp32r2, which are tumorigenic.

Each of the series of constructs used to analyze pp32 function included the 5′-untranslated region, the Kozak sequence, and the translational start site of pp32 cDNA as well as at least one of the putative nuclear localization signals. Truncations terminated with an in-frame stop codon. Fig. 1 shows that the process of systematic truncation and deletion generated a series of constructs sequentially altering regions encoding each of the major structural features of pp32. Constructs 1–666 and 1–618 fully maintained the α-helical domain while deleting the acidic tail. Construct 1–543 deleted the acidic tail and a small portion of the α-helical domain. Constructs 1–501 and 1–366 deleted additional portions of the α-helical domain while preserving the leucine zipper, whereas D201–360 selectively deleted the portion of the α-helical domain containing the leucine zipper.

Localization of Transformation-inhibiting Activity within pp32—Full-length pp32 suppresses transformation of rat embryo fibroblasts by a number of disparate oncogene pairs including RAS in combination with MYC, mutant p53, c-JUN, or E1a as well as E6 in combination with E7 (6). The assay analyzed pp32 function by co-transfecting rat embryo fibroblasts with pp32 in the pCMV expression vector along with...
expression vectors containing the test oncogene pairs. Since pp32 behaved similarly with each of the previously tested oncogene pairs, we analyzed the pp32 deletion constructs in the well characterized RAS-MYC system (7).

Several features emerge when pp32 structure is correlated with the transformation inhibition function. First of all, the acidic domain is not required to inhibit formation of oncogene-induced foci. Constructs 1–666 and 1–618 each inhibited focus formation to a degree similar to full-length pp32. Likewise, Δ201–360, which deletes the leucine zipper, also maintained full suppressive activity. In contrast, deletion of the additional 25 amino acids that distinguish construct 1–618 from 1–543 abolished suppressive activity. Fig. 1 identifies two regions as indeterminate. Since construct 1–543 was already inactive, this analysis cannot determine whether sequence encoded between bases 366 and 543 also contributes to transformation inhibition. Likewise, the critical Kozak, translational start, and nuclear localization sequences encoded between bases 1 and 201 precluded functional analysis.

Expression and Nuclear Localization of pp32 Constructs—The loss of activity observed when bases 543–618 were deleted discretey identifies pp32 amino acids 150–174 as required for transformation inhibition. The assignment of function to this region, however, critically depends upon demonstrating that construct 1–543 is expressed in rat embryo fibroblasts at levels comparable with full-length pp32 and that the expressed product localizes to the nucleus. Fig. 2 shows the results of an immunohistochemical analysis of transiently transfected cells. The figure clearly shows that construct 1–543 is expressed and that it localizes to the cell nucleus. Moreover, staining intensity is comparable between cells transfected with full-length pp32 and those receiving the construct 1–543. Examined quantitatively, as shown in Table I, the data show increased expression in 27.0% of cells receiving full-length pp32, and 29.7% of cells receiving construct 1–543. Therefore, the data clearly indicate that the loss of functional activity accompanying the deletion of bases 543–666 cannot be explained by failures of expression or localization of the protein encoded by the 1–543 construct.

Comparison with pp32r1 and pp32r2—pp32 differs significantly from pp32r1 and pp32r2 in precisely the region identified as functionally significant by deletion analysis. The moving average of the identity score, shown in Fig. 3, identifies three regions where the two sequences diverge. The N-terminal region, spanning approximately amino acids 45–85 does not participate in transformation inhibition, since deletion of amino acids 53–88 failed to abolish activity. Similarly, deletion of the acidic tail had no effect. In contrast, deletion of pp32 amino acids 150–174 abolished activity, indicating that the transformation function overlies, at least in part, the area of divergence approximately spanning pp32 residues 140–180. It is also significant that pp32r2, which is also tumorigenic, does not include this region, since it truncates at residue 131. Thus independent analyses of pp32 deletions and of functionally divergent members of the pp32 family converge and underscore the identification of this region as functionally important for a tumor suppressor-related activity of pp32.

The question of precisely what molecular activity lies in pp32 amino acids 140–180 remains open. A search of GenBank™ with this region yielded no informative homologies, and the region contains no significant protein motifs. Although pp32 is a known inhibitor of the heterogeneous protein phosphatase 2A class of phosphatases (9), our preliminary data¹ indicate that this activity does not map to residues 140–180; however, these data do not preclude interaction with a larger complex that might also contain such a phosphatase. It is likely that residues 140–180 form a site of interaction with molecules of a signaling or other pathway that leads to resistance to transformation. The linkage of this sequence to this tumor suppressive function through two convergent and independent lines of evidence provides an important insight that will ultimately lead to identification of other constituents of the clinically important pathway of pp32-mediated tumor suppression.

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