A Novel Mr 32,000 Nuclear Phosphoprotein Is Selectively Expressed in Cells Competent for Self-Renewal

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ABSTRACT

We investigated the association between expression of a novel Mr 32,000 nuclear phosphoprotein (pp32) and cell proliferation in vivo using the well-characterized physiological model of androgen-dependent regeneration of prostate in orchietomized rats. pp32 is expressed at high levels in neoplastic cell lines and in certain anatomically defined stem cell compartments of normal human tissues such as intestinal crypt epithelial cells. Immunohistochemistry and in situ hybridization were used to monitor pp32 expression in rat ventral prostate epithelium following castration and androgen restoration. Castrated rats retained only 6% of prostate wet weight compared to intact controls but were capable of complete gland restoration upon androgen replacement. In intact controls, pp32 expression localized to small acini at the periphery of the gland and to rare basal cells in the central regions. Ten days following castration, there was a 3.5-fold enrichment in the frequency of pp32-positive cells with greater than 56% of remaining epithelial cells expressing pp32 protein. In situ hybridization showed that all remaining epithelial cells contained pp32 mRNA. Upon testosterone replacement, pp32 expression and localization returned to that of intact controls. In order to determine the association between pp32 expression and cell division, DNA synthesis was monitored by bromodeoxyuridine incorporation during prostate involution and regeneration. Bromodeoxyuridine incorporation peaked 3 days after androgen replacement and occurred diffusely throughout the gland. Thus, pp32-positive cells are anatomically distinguishable from the population of terminally differentiating cells undergoing rapid expansion.

Preliminary immunohistochemical studies of human prostatic neoplasia demonstrated increased expression of pp32 in human prostatic adenocarcinoma and prostatic intraepithelial neoplasia compared to benign prostatic hypertrophy and normal human prostate. The highest degree of expression occurred in the higher Gleason grades and 6% of prostatic intraepithelial neoplasia. This work suggests that pp32 is a nuclear protein which has a selective but presently undefined role in cells competent for self-renewal.

INTRODUCTION

pp32 is a Mr 32,000 nuclear phosphoprotein initially discovered as a constitutively expressed protein in neoplastic B-cells (1). Initial studies found that in contrast to the high levels of expression encountered in neoplastic cell lines, expression in normal tissues was highly restricted. The localization of pp32 to anatomically defined stem cell compartments such as intestinal crypts raised the possibility that pp32 expression may play a role in proliferative compartments. The pp32 structure also suggests a regulatory role for pp32 in the cell nucleus. The pp32 sequence is composed of two domains: an NH2-terminal amphipathic α-helical region containing a leucine zipper (2); and a COOH-terminal acidic region reminiscent of major centromere binding protein B (3) and transcriptional activating factors (4). While there are no close homologies, the overall organization of pp32 is similar to neurofilament triple helical L-protein at the ~25% homology level. Interestingly, the murine and human forms of pp32 are highly conserved with over 90% nucleic acid homology and over 95% protein-level homology. pp32 is phosphorylated in vivo by casein kinase II (1).

The expression pattern, structure, and phosphorylation of pp32 point toward a potential regulatory function in the control of cell proliferation. To define the relationship between pp32 and proliferation, we studied pp32 expression in rat prostate and seminal vesicle where epithelial cell mass can be negatively and positively regulated by orchietomy and androgen replacement. We report here that pp32 is expressed in the majority of epithelial cells remaining after prostate involution; these epithelial cells expand to repopulate the prostate under androgen stimulation. The total number of pp32-positive cells within the prostate is roughly equivalent between castrated rats and intact controls. During androgen-induced growth, the concentration of pp32-positive cells is diluted by pp32-negative, rapidly dividing, and terminally differentiating cells in the regenerating prostate gland. The pp32-positive cells of intact controls and androgen-treated castrated rats are located in an anatomically defined compartment. Extending the analysis to human prostatic neoplasia, we additionally report the preliminary finding that the proportion of pp32-positive cells increases with increasing Gleason grade in prostatic carcinoma and in PIN.

MATERIALS AND METHODS

Animals. For involution studies, three Sprague-Dawley rats were castrated at each time point. Ventral prostate and seminal vesicle were dissected and processed as indicated below. For androgen-dependent regeneration studies, five rats were used for each time point, except for four rats on days 0 and 14. All rats were castrated on day -43. On day 0, 5 rats received sham injections of 0.2 ml of sesame oil s.c., followed by an injection of BrdUrd, 50 mg/kg i.p., at 75 mg/ml in normal saline (5) and then were asphyxiated with CO2 30 min after the BrdUrd injection. All other animals received 2.0 mg of testosterone propionate in 0.2 ml of sesame oil s.c. daily and BrdUrd injections 30 min before euthanasia and tissue harvest. All tissues were fixed in 10% buffered formalin and processed into paraffin sections.

Immunohistochemistry. Paraffin sections were immunostained by previously described techniques (6) using biotinylated, affinity-purified secondary antibodies, avidin-peroxidase conjugate, and aminoethylcarbazole chromatogen. Rabbit anti-pp32 was raised to a purified Mr 21,000 COOH-terminal partial recombinant murine pp32 protein expressed in Escherichia coli using the pR vector. Specificity was confirmed by immunoblot using an A20 lysate and purified murine pp32 and pp35 proteins (Fig. 1); immunoblot procedures were as described (1) except that blots were developed with horseradish peroxidase-labeled donkey-anti-rabbit antibody and the chemiluminescent ECL detection system (Amersham) according to the manufacturer’s instructions, with exposure times ranging from 1 to 5 s. All pp32 staining used affinity-purified anti-pp32, except for the expression studies, which used high-titer antisemur. BrdUrd immunostaining used a commercial monoclonal anti-BrdUrd antibody (Calbiochem).

Quanti-fication of immunohistochemistry used a random grid selection technique (7). For the involution experiments, all epithelial cells falling within the grid in each of 3 randomly chosen fields were counted and scored for immunostaining, resulting in evaluation of 350–450 cells/animal in each organ.

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3 The abbreviations used are: pp32, Mr 32,000 nuclear phosphoprotein; BrdUrd, bromodeoxyuridine; PIN, prostatic intraepithelial neoplasia.

4 T-H. Chen et al., manuscript in preparation.

5 F.H. Chen and G. R. Pasternack, unpublished data.

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Androgen-dependent regeneration experiments were performed similarly, except that 5 randomly chosen fields were counted and scored, resulting in evaluation of 600-750 cells.

**RESULTS**

**Involution and Androgen-dependent Regeneration of Prostate and Seminal Vesicle.** Castration caused characteristic prostatic involution. Forty-three day castrates retained 6% of prostate and 15% of seminal vesicle wet weights. Complete gland restoration upon testosterone replacement occurred in 12 days, with prostate and seminal vesicle regenerating at rates of approximately 6.8 and 7.6 mg/day, respectively (Fig. 2A). The prostate rate of regeneration was calculated using the first 8 days of regeneration when weight gain is due to proliferation rather than secretion. These data are consistent with previous reports (10).

DNA synthesis during regeneration of the prostate and seminal vesicle was monitored by BrdUrd incorporation. In intact controls, approximately 0 and 3% of prostate and seminal vesicle epithelial cells, respectively, were positive for BrdUrd incorporation. Peaks in DNA synthesis appeared to occur in waves, with the first and largest peak in BrdUrd incorporation occurring at day 3 for ventral prostate and seminal vesicle with 14 and 26% positivity, respectively (Fig. 2B). These data are consistent with previous data obtained using tritiated thymidine incorporation (10), thus validating the model.

**In Situ Hybridization.** In the ventral prostate of the intact animal, pp32 mRNA was found primarily in small, peripheral glands (Fig. 3A, a). No message was found in larger, central glands except in occasional basal cells. In marked contrast, the castrated rats expressed pp32 mRNA diffusely, with only minor variation in hybridization signal intensity (Fig. 3A, b). The disruption of cell morphology that results from in situ hybridization conditions did not allow for direct quantitation of the percentage of epithelial cells containing mRNA; however, it did appear that nearly all cells were positive for pp32 mRNA. With androgen replacement, the mRNA signal intensified and the pattern began to return to the distribution found in the intact animal (Fig. 3A, c and d). In the seminal vesicle of the intact animal, pp32 mRNA had a basal distribution (Fig. 3B, a) but became uniform in the castrate (Fig. 3B, b). Upon androgen treatment, the mRNA signal intensified and the basal pattern progressively returned to the distribution found in the intact animal (Fig. 3B, c and d).

**Immunohistochemistry and Quantitation.** The ventral prostates of intact controls showed sparse staining with anti-pp32 antibody, with 16% of prostatic epithelial cells positive for pp32 (Fig. 4A). pp32-positive cells were predominantly localized to small acini at the periphery of the gland, although rare basal cells of central glands were also positive for protein. Fig. 4B, a shows the general absence of staining in central glands. By 10 days postcastration, 56% of the remaining epithelial cells were positive for protein (Fig. 4A) and positive cells were diffusely spread throughout the gland (Fig. 4B, b). Within 2 days of androgen replacement, the level of protein in cells expressing pp32 appeared to increase. The percentage of pp32-positive epithelial cells progressively returned to control levels and positivity was once again detected primarily in epithelial cells of smaller peripheral glands. In contrast, DNA synthesis monitored by BrdUrd incorporation of intact controls showed sparse staining with anti-pp32 antibody, with 16% of prostatic epithelial cells positive for pp32 (Fig. 4A). pp32-positive cells were predominantly localized to small acini at the periphery of the gland, although rare basal cells of central glands were also positive for protein. Fig. 4B, a shows the general absence of staining in central glands. By 10 days postcastration, 56% of the remaining epithelial cells were positive for protein (Fig. 4A) and positive cells were diffusely spread throughout the gland (Fig. 4B, b). Within 2 days of androgen replacement, the level of protein in cells expressing pp32 appeared to increase. The percentage of pp32-positive epithelial cells progressively returned to control levels and positivity was once again detected primarily in epithelial cells of smaller peripheral glands. In contrast, DNA synthesis monitored by BrdUrd incorporation of intact controls showed sparse staining with anti-pp32 antibody, with 16% of prostatic epithelial cells positive for pp32 (Fig. 4A). pp32-positive cells were predominantly localized to small acini at the periphery of the gland, although rare basal cells of central glands were also positive for protein. Fig. 4B, a shows the general absence of staining in central glands. By 10 days postcastration, 56% of the remaining epithelial cells were positive for protein (Fig. 4A) and positive cells were diffusely spread throughout the gland (Fig. 4B, b). Within 2 days of androgen replacement, the level of protein in cells expressing pp32 appeared to increase. The percentage of pp32-positive epithelial cells progressively returned to control levels and positivity was once again detected primarily in epithelial cells of smaller peripheral glands.
pp32 mRNA during involution and regeneration. Changes in distribution of pp32 mRNA which occur in prostate and seminal vesicle during involution and regeneration. A, prostate: a, intact; b, castrate; c, androgen day 2; d, androgen day 5. B, seminal vesicle: a, intact; b, castrate; c, androgen day 2; d, androgen day 6. A, d, × 160; all other magnifications, × 46.

In seminal vesicles, 37% of epithelial cells in intact controls were positive for pp32 (Fig. 4A), more than double the percentage found in prostate controls. pp32-positive cells in seminal vesicles tended to be more basally distributed (Fig. 4B, c). On day 10, castrated rats were 65% positive for protein (Fig. 4A) and positive cells were found diffusely throughout the gland (Fig. 4B, d). Upon androgen-induced regeneration, the percentage of pp32-positive epithelial cells returned to control levels and positivity was once again distributed basally. In contrast, BrdUrd incorporation occurred diffusely throughout the gland and was not restricted to a basal distribution following androgen replacement (data not shown).

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**pp32 Expression in Human Prostatic Neoplasia.** Paraffin sections of human prostate were stained with anti-pp32 or normal rabbit immunoglobulin. pp32 was expressed at low levels in benign prostatic hypertrophy (Fig. 5). In contrast, the percentage of cells expressing pp32, as well as the level of expression, appeared to increase with increasing Gleason grade in adenocarcinoma. PIN expressed pp32 in a manner similar to that for higher grade adenocarcinomas.

**DISCUSSION**

Our studies examine the association between the expression of pp32 and cell proliferation using the physiological model of androgen-dependent regeneration of involuted rat prostate and seminal vesicle.
Data from BrdUrd incorporation, *in situ* RNA hybridization, and immunohistochemical staining of both involuting and regenerating prostate and seminal vesicle support the following findings: (a) pp32 is expressed in those cells competent for self-renewal; (b) cells that express pp32 are localized to defined anatomic compartments; (c) regenerating ventral prostate contains cells expressing both pp32 mRNA and protein, cells expressing only message but no detectable protein, and cells expressing neither pp32 message nor protein; and (d) neoplastic prostatic epithelium expresses pp32 protein with increased frequency and level, correlating positively with increasing Gleason grade of human prostatic adenocarcinoma.

**pp32 Expression Occurs in Cells Competent for Self-Renewal.** Castration results in involution of rat ventral prostate and seminal vesicle. Without testosterone, terminally differentiated, androgen-de-
pp32 IN SELF-RENEWING PROSTATIC EPITHELIAL CELLS

Fig. 5. pp32 protein expression in human prostatic neoplasia. Immunohistochemical pp32 expression: A, benign prostatic hypertrophy; B, adenocarcinoma (Gleason 2+2); C, adenocarcinoma (Gleason 3+3); D and E, adenocarcinoma (Gleason 5+4), anti-pp32, and control with normal rabbit immunoglobulin; F, PIN. X 360.

pendent glandular cells die, leaving behind androgen-independent cells. When this occurs, nearly all of the remaining cells in prostate and seminal vesicle contain pp32 mRNA and more than one-half of the cells contain pp32 protein itself. This represents a profound enrichment for pp32-positive cells as compared to intact controls. The pp32-positive cells present following castration are likely the same cells present prior to castration, although it is possible that they represent an independent population of pp32-positive cells which arose separately. The pp32-positive cells that remain contain a population with the capability to regenerate the organs upon androgen replacement. Thus, there is an association between pp32 expression and the ability of cells to engage in extensive self-renewal. The mechanism whereby pp32 exerts its effects remains unknown. One possibility is that pp32 acts to prevent apoptosis, similar to bcl-2 in other systems (11), rendering cells resistant to androgen ablation.

Upon androgen-induced regeneration, the percentage of pp32-positive cells declines from 56% to 16%. Previous studies permit the numbers of prostatic epithelial cells to be estimated in intact and castrated rats (10). Using biomorphometric analysis of histological sections of rat ventral prostate, DeKlerk et al. determined the number of ventral prostatic epithelial cells in 400-g control Sprague-Dawley rats to be $5.62 \pm 0.3 \times 10^7$, whereas day 10 castrated rats contained $0.42 \pm 0.1 \times 10^7$ epithelial cells. This represents a 93% decline in the epithelial cell population after castration. Using these figures, we calculated that approximately $4.76 \times 10^7$ cells in intact controls are pp32-negative, whereas $0.84 \times 10^7$ cells are pp32 positive. In castrated rats, $0.19 \times 10^7$ cells are pp32 negative and $0.23 \times 10^7$ cells are pp32 positive. While subject to experimental error, these estimates of total cell number illustrate general trends in pp32 expression. The number of pp32-positive cells does not expand during involution but instead remains constant or declines slightly. The profound decline in the percentage of pp32-positive cells during regeneration seems to result from dilution of pp32-positive cells by pp32-negative cells, which repopulate and form the bulk of the regenerating gland. These cells are terminally differentiating cells that lose the capacity for self-renewal. Although, these cells incorporate BrdUrd and are thus
mitotically, they do not contain pp32. Therefore, cells which lack the capacity for indefinite self-renewal do not express pp32, even though they may transiently undergo mitosis.

Cells That Express pp32 Are Localized to a Defined Anatomic Compartment. In situ hybridization studies and immunohistochemical staining demonstrate that cells expressing pp32 are located in a discrete anatomic compartment. In the intact prostate, pp32-positive cells are found in acini of small luminal diameter located at the periphery of the gland. Rare basal cells found in central acini also stain positively for pp32. In the seminal vesicle, pp32-positive cells are basally distributed. In both prostate and seminal vesicles, the involved organs show diffuse staining for pp32 message and protein. In contrast, during androgen-induced regeneration, the distribution of pp32-positive cells returns to the anatomic compartments described for the intact animal. Whereas pp32 expression has a specific anatomic distribution in rat ventral prostate and seminal vesicles, BrdU incorporation occurs diffusely throughout the glands during androgen-induced regeneration. Cells that express pp32 are thus anatomically dissociated from cells that incorporate BrdU during proliferative regeneration. Whereas DNA synthesis occurs diffusely, pp32 expression is localized and specific to those cells competent for indefinite self-renewal. The pattern of compartmentalization in the seminal vesicle is strikingly similar to that found in colonic epithelium and suggests that pp32 may indeed be a stem cell marker. If pp32-positive cells are truly reserve cells or stem cells, their localization to small, peripheral acini in rat prostate supports morphological studies which suggest that the ductal system of the gland grows like an arborizing tree, with new growth occurring at the distal buds (12). Thus, the localization of cells containing a specific marker for competence in self-renewal is suggestive of a distribution for stem cells in the prostate and seminal vesicle glands of intact animals.

pp32 Distinguishes among Cell Populations in Rat Ventral Prostate. The prostate stem cell model of Isaacs and Coffey proposes three distinct cell populations: stem cells; amplifying cells; and transit cells (13). Stem cells do not require androgen for maintenance; nonetheless, they are responsive to androgen stimulation and are competent for extensive self-renewal. Amplifying cells are likewise androgen independent and androgen sensitive; they amplify the total number of cells through a more transient androgen-induced self-renewal and clonal expansion. Transit cells are androgen dependent, have very limited proliferative potential, and become terminally differentiated secretory cells. Three classes of cells can be defined on the basis of pp32 expression: those expressing detectable levels of both mRNA and protein; those in which only mRNA is detectable; and those in which neither can be found. pp32-negative cells may correspond to the terminally differentiated, transit cell population, whereas mRNA- and protein-positive cells may represent the stem cell population. It is tempting to speculate that the third population, the amplifying cells, are those cells expressing pp32 mRNA but little or no pp32 protein. Care must be taken in evaluating the message-positive, protein-negative population. The synthetic rates and half-lives of pp32 message and protein are not known. While either translational regulation or altered protein half-life could explain the presence of mRNA without detectable protein as a bona fide regulatory mechanism, it is also possible that cells with pp32 message but no detectable pp32 protein may not represent a discrete population. Such cells could be observed as a transient artifact of the relative kinetics of pp32 protein and mRNA synthesis. Whether or not the remaining cells after involution represent two populations, androgen-induced regeneration results in dilution of these cells by terminally differentiating cells that do not contain pp32 message or protein. We have definitively identified two cells types: those that express pp32 and are competent for extensive self-renewal; and those that do not express pp32 and ultimately lose the capacity to self-renew. Further study is required to determine whether there is an intermediate cell type that expresses pp32 message but does not produce protein. It is feasible, however, that this cell type could represent the intermediate condition between cells that actively synthesize protein from message and those that contain neither message nor protein.

Our data support the stem cell model of Isaacs and Coffey (13) and provide a scheme for cell differentiation in rat ventral prostate based on pp32 expression. We propose that stem cell competence for extensive self-renewal is associated with expression of pp32 message and protein. Stem cells which progress to become amplifying cells may cease making protein, although they may transiently continue to make message. Finally, cells which will become terminally differentiated may discontinue making message and protein and as a result lose the ability to self-renew. This hypothesis is illustrated in Fig. 6 in the context of the prostate organizational model of Isaacs and Coffey (13).

Recent experiments with ML-1 cells, a human myeloblastic leukemia cell line, support the association between pp32 and proliferative control. ML-1 cells grow in culture as a rapidly proliferating, immortalized cell line. When exposed to phorbol ester, ML-1 cells cease entry into mitosis, accumulate in G0 or G1, and express mature macrophage differentiation markers (14). pp32 mRNA is highly expressed in rapidly proliferating ML-1 cells but becomes nearly undetectable within 1 day after stimulation by phorbol ester.6 Thus, pp32 expression is intimately associated with the capacity for self-renewal in a completely independent system.

Human Prostatic Intraepithelial Neoplasia and Adenocarcinoma Express pp32 with Increased Frequency. Much diagnostic effort in cancer is aimed at evaluating parameters of cell proliferation in cancer. For example, tumor specimens are frequently evaluated for S-phase fraction by flow cytometry or for proliferating cell nuclear antigen. These techniques enumerate cycling cells but do not determine how many cells remain capable of entering the cell cycle, a population directly analogous to self-renewing cells in normal tissues. pp32 is expressed at low levels in normal human prostate and benign prostatic hypertrophy. Human PIN and adenocarcinoma show heightened pp32 expression. Increased pp32 expression is not an indicator of malignancy per se, since by definition PIN has not breached the basement membrane. It is presumed that other factors determine whether these cells will become invasive. The increased frequency and level of pp32 expression in PIN and adenocarcinoma suggest that

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6 Unpublished data.
pp32 identifies an increased fraction of cells competent for self-renewal in these neoplastic processes. This is consistent with our observations that tumors of increasing Gleason grade, with more rapid growth and self-renewal, have a higher level of pp32 expression. Assessment of pp32 expression in tumors measures a distinctive parameter, the proportion of cells with stem cell-like properties, and thus may find utility in cancer diagnosis. The role of pp32 in human cancer will become more fully understood as additional clinical studies are undertaken.

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