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Immunohistochemical Analysis of Bin1/Amphiphysin II in Human Tissues: Diverse Sites of Nuclear Expression and Losses in Prostate Cancer

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Abstract The *Bin1/Amphiphysin II* gene encodes at least seven alternately spliced adapter proteins that have been implicated in membrane dynamics and nuclear processes. Nuclear localized Bin1 polypeptides have tumor suppressor and proapoptotic activities, suggesting that Bin1 may suppress cancer in tissues where nuclear expression may occur. One question is the extent to which human tissues express nuclear Bin1 isoforms. A secondary issue has been the need for a specific antibody that can detect all the splice isoforms expressed by the human, mouse, and rat *Bin1* genes. Using a novel mouse monoclonal antibody with these characteristics, we performed an immunohistochemical analysis of Bin1 expression in a panel of normal human tissues. We also compared the expression profile of Bin1 in normal or malignant tissues derived from human prostate, where *Bin1* is a candidate tumor suppressor gene. In brain, a distinct nuclear staining pattern overlapped with a cytosolic staining pattern present in certain layers of the cerebral cortex and cerebellum. Bone marrow cells displayed mainly nuclear localization whereas peripheral lymphoid cells exhibited mainly cytosolic localization. In several epithelial tissues, nuclear or nucleocytosolic staining patterns were displayed by basal cells in skin, breast, or prostate, whereas cytosolic or plasma membrane-associated staining patterns were noted in gastrointestinal cells. Interestingly, a striking gradient of expression was observed in gastrointestinal epithelia, particularly in the large intestine, with the strongest staining displayed by cells destined to undergo apoptosis at the villus tip. In prostate, Bin1 staining was frequently absent in cases of primary prostate adenocarcinoma. This study used a novel reagent to document the extent of expression of nuclear Bin1 isoforms, which exhibit cancer suppression and proapoptotic activity in human cells. J. Cell. Biochem. 88: 635–642, 2003.

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The human genome encodes at least five members of the Bin/Amphiphysin/Rvs (BAR) gene family, including Bin1, Bin2, Bin3, Amphiphysin, and KIAA1010. BAR genes encode a set of adapter proteins that are marked by a unique N-terminal domain of undetermined function, termed the BAR domain. The Bin1 and Bin3 genes are highly conserved in evolution, with homologs found in fruit flies, nematodes, and yeast. BAR adapter proteins have been implicated in diverse cellular processes, including endocytosis, actin organization, and apoptosis. In particular, there is a broad body of evidence that nuclear localized Bin1 adapter proteins can mediate cancer suppression and programmed cell death [Sakamuro et al., 1996; Ge et al., 1999; Elliott et al., 2000; Ge et al., 2000a,b; DuHadaway et al., 2001]. Bin1 studies are complicated by the fact that this gene encodes at least seven different splice isoforms that localize to different compartments of the cell [Sakamuro et al., 1996; Butler et al., 1997; Kadlec and Pendergast, 1997; Ramjaun et al.,

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1997; Tsutsui et al., 1997; Wechsler-Reya et al., 1997b; Wigge et al., 1997; Ramjaun and McPherson, 1998]. Tissue-specific isoforms expressed mainly in the central nervous system have been linked to synaptic vesicle recycling. These isoforms lack cancer suppression or cell death activities and their link to synaptic vesicle endocytosis is mediated through interactions with Amphiphysin, which is expressed predominantly in neurons [Wigge et al., 1997]. In contrast, muscle-specific and ubiquitous isoforms of Bin1 lack alternately spliced sequences that are required for interactions with key endocytotic proteins, and they do not affect endocytosis [Ramjaun and McPherson, 1998; Elliott et al., 2000]. Instead, these isoforms localize to the cytosol and nucleus, where they can interact with the nuclear c-Myc and c-Abl oncoproteins and mediate cancer suppression and cell death [Sakamuro et al., 1996; Kadlec and Pendergast, 1997; Elliott et al., 2000; Ge et al., 2000a; DuHadaway et al., 2001]. Thus, Bin1 appears to have at least two functions, perhaps distinguished in part by differential subcellular localization. Recent work on the homolog of Bin1 in fission yeast suggests that this gene participates in stress signaling processes [Routhier et al., 2002], rather than in the 'root' function of the diverse set of complexes in which it has been identified. To further investigate the patterns of expression and localization of Bin1 polypeptides in human tissues, we performed an immunohistochemical study using a novel monoclonal antibody that specifically recognizes all the splice isoforms encoded by the human, mouse, and rat *Bin1* genes.

MATERIALS AND METHODS

Monoclonal Antibody Generation

A glutathione-S-transferase (GST) chimeric protein that encoded the human Bin1 BAR domain was used to raise monoclonal antibodies in mice. Mice exhibiting a positive immune response in primary bleeds were boosted once before liver isolation and myeloma fusion essentially as described [Koprowski et al., 1979]. Supernatants from single cell hybridoma clones grown to confluency were screened for reactivity to GST–BAR or GST by ELISA. Eight clones exhibiting the strongest specific reactions were further analyzed. The supernatant from clone 2F11 was judged the most avid and specific in Western and immunohistochemical staining experiments. 2F11 is a 'pan-Bin1' antibody that recognizes all the Bin1 splice isoforms that have been described. This antibody recognizes human, mouse, and rat polypeptides. 2F11 does not crossreact with the related BAR adapter proteins amphiphysin or Bin2 despite the presence of Bin1-related sequences in those proteins.

Western Analysis

Cell extracts generated in NP40 lysis buffer were quantitated by Bradford assay. Equivalent amounts of cell extract per lane was fractionated by SDS–PAGE and gels were subjected to Western analysis with 2F11 or with 99D, an anti-Bin1 monoclonal antibody that is specific for exon 13-encoded splice isoforms that include the Myc-binding domain (MBD) [Wechsler-Reya et al., 1997a]. Gels blotted to nitrocellulose membrane were blocked by 30 min incubation in a blocking buffer composed of 3% solution of non-fat dry milk in PBS pH 7.2 containing 0.4% Tween 20 detergent. The concentration of Tween 20 used in the buffer is important to reduce non-specific binding of the 2F11 antibody. Membranes were then incubated in the same blocking buffer to which 2F11 was added at a concentration of 1 μg/ml (protein A-purified antibody) or as a 1:20 dilution of hybridoma culture supernatant. After incubation 1 h at room temperature, the membrane was washed 4×10 min in PBS containing 0.4% Tween 20. The secondary antibody used was a horseradish peroxidase (HRP)-conjugated anti-mouse antibody (Cell Signaling, cat. #7076) diluted 1:2,500 into blocking buffer. After incubation 1 h at room temperature, the membrane was washed again as before. Membranes were developed using a commercial chemiluminescence kit (Pierce, Rockford, IL).

Immunohistochemistry

Normal or malignant cases of human tissues were fixed, processed, and sectioned by standard methods. Tissue sections on glass slides were subjected typically to a sodium citrate (pH 6.0) antigen recovery process by treatment with steam heat for 20 min. Tissues were then blocked with 5% normal goat serum and stained with 2F11 as the primary antibody for 20–30 min at a concentration of 0.5 μg/ml. This antibody was detected with biotin-conjugated goat anti-mouse IgG antibodies (secondary antibody). Control experiments included staining

with another isotype-matched monoclonal antibody or with secondary antibody only. Endogenous peroxidases were blocked with 3% hydrogen peroxide and tissues were incubated with streptavidin–HRP for 10 min. Staining was visualized by incubation for 15 min in DAB chromogen. Tissues were counterstained 1 min by incubation in hematoxylin (blue nuclear stain) an permanently coverslipped. Ki67 antibody (Dako, Carpinteria, CA) was used at a 1:50 dilution with the same antigen recovery process.

RESULTS AND DISCUSSION

The specificity of Bin1 mAb 2F11 was examined using human and mouse cell extracts. C2C12 mouse myoblasts offer a benchmark for the two ubiquitous Bin1 isoforms Bin1-10 and Bin1-10-13, which are distinguished by alternately spliced exon 13 sequences that encode part of the MBD of Bin1 polypeptides [Wechsler-Reya et al., 1998]. The previously described Bin1 mAb 99D binds to an exon 13-encoded epitope [Wechsler-Reya et al., 1997a,b], so in C2C12 cells 99D recognizes the nuclear Bin1-10 isoform [Wechsler-Reya et al., 1998] but not the nucleocytosolic Bin1-10-13 isoform [Kadlec and Pendergast, 1997] (Fig. 1A). In WI-38 human fibroblasts, 99D recognizes cytosolic Bin1 + 12A isoforms [Elliott et al., 2000] that are expressed from aberrantly spliced messages including the neuron-specific exon 12A that are also generated in human malignant melanomas [Ge et al., 1999]. In LNCaP human prostate cancer cells, 99D recognizes mainly the Bin1-10 isoform (Fig. 1A). By comparison to 99D, the 'pan-Bin1' mAb 2F11 recognizes both the Bin1-10 and Bin1-10-13 isoforms in C2C12 cells (Fig. 1B). 2F11 reveals additional isoforms in WI-38 cells and it establishes clearly that Bin1-10-13 is not expressed in LNCaP cells (Fig. 1B). In established rat intestinal epithelial (RIE) cells, 2F11 establishes that Bin1-10 is the predominant isoform expressed, but lower levels of Bin1-10-13 and the aberrant Bin1 + 12A isoforms are also apparent (Fig. 1C). Transformation of RIE cells by activated *K-Ras*, *Rac1*, or *v-Src* led to distinct changes in the expression of distinct splice isoforms (Fig. 1C), illustrating the utility of 2F11 for studying cellular functions and regulation of *Bin1*. In additional experiments, we confirmed that 2F11 was tractable for immunoprecipitation and indirect cell immunofluorescence applications (data not shown). Using a set of BAR deletion mutants

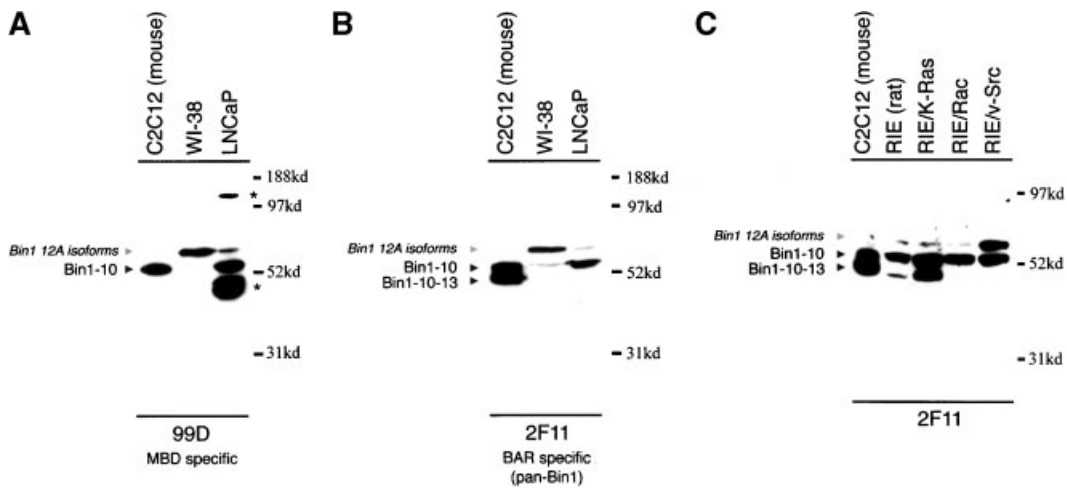

Fig. 1. Western analysis. **A**: mAb 99D. Mouse C2C12 cells express similar levels of the two ubiquitous isoforms of Bin1, termed Bin1-10 and Bin1-10-13. mAb 99D recognizes an alternately spliced epitope derived from exon 13 sequences, present only in the Bin1-10 isoform expressed in C2C12 and LNCaP cells but not in WI-38 cells [Wechsler-Reya et al., 1997a,b, 1998; Elliott et al., 2000; Ge et al., 2000b]. Bin1 + 12A isoforms result from missplicing of Bin1 in WI-38 and certain human tumor cell lines including certain LNCaP isolates [Ge et al., 1999; Elliott et al., 2000]. Non-specific bands in LNCaP cells are noted by asterisks. **B**: mAb 2F11. In the same cell extracts, mAb 2F11 recognizes both the Bin1-10 and Bin-10-13 isoforms that are expressed in C2C12 cells. This antibody reveals that Bin1-10-13 is undetectable in LNCaP cells and that WI-38 cells express low levels of a Bin1 isoform that includes exon 13-derived sequences. **C**: Splice regulation detected by 2F11. Parental RIE cells mainly express the Myc-interacting Bin1-10 isoform, with lower levels of Bin1-10-13 also present. Cell transformation by mutant *K-Ras-V12, Rac1-V12,* or *v-Src* oncogenes elicits differential expression of various splice isoforms, as revealed by the 'pan-Bin1' mAb 2F11.

[Elliott et al., 1999], the epitope recognized by mAb 2F11 was mapped to aa 179–207 of the human Bin1 sequence as defined by Sakamuro et al. [1996], encompassing the amino acid sequence TAKKKDEAKIAKAEEELIKAQKV-FEE (data not shown). This region of the BAR domain is a relatively less conserved region between Bin1 and other BAR family proteins, consistent with the observed specificity of 2F11 for the BAR domain of Bin1. Taken together, these observations illustrate the utility of 2F11 for applications aimed at studying the expression, function, and regulation of the *Bin1* gene in human, mouse, and rodent cells.

Based on previous work with 99D that established nuclear staining of Bin1 in normal breast ductal epithelial cells [Ge et al., 2000a], normal cases of breast tissue were used to confirm the utility of mAb 2F11 to detect Bin1 in tissue. This pattern of expression was confirmed and extended in immunohistochemical experiments with 2F11, which stained the cytosolic as well as the nuclear compartment of the same cells in normal mammary tissue (Fig. 2). Stromal cells stained were weakly if at all. It was notable that 99D detected only the Bin1-10 isoform and produced nuclear staining [Ge et al., 2000a], whereas 2F11 which additionally detected the Bin1-10-13 isoform stained both the cytosol and nucleus. Thus, one would infer that the Bin1-10 isoform, which includes an intact MBD was localized mainly to the nucleus of breast epithelial cells, whereas the Bin1-10-13 isoform which lacks an intact MBD was localized mainly to the cytosol of such cells.

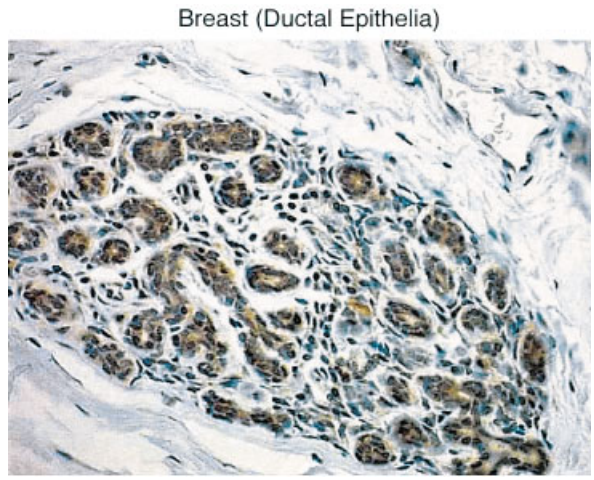

Fig. 2. Mammary gland. Strong Bin1 staining in the nucleus and cytosolic compartment of ductal epithelial cells in breast acini is apparent.

The results of an immunohistological analysis of Bin1 in normal human tissues define its diverse patterns of expression and subcellular localization in different cell types (Table I). Nuclear staining predominated in bone marrow cells, whereas cytosolic staining predominated in tonsil-derived lymphoid cells (Fig. 3). In the B cell-rich germinal centers in tonsils, staining was relatively light in comparison to the stronger staining of T cell-rich regions that surround the germinal centers. Staining for Ki67, a marker of proliferation, indicated an inverse relationship between the level of Bin1 expression and the extent of cell proliferation, which occurs mainly in germinal centers (Fig. 3, right panel).

TABLE I. Diverse Patterns of Bin1 Staining in Various Human Tissues

Tissue	Immunohistochemical staining pattern
Tonsil	Strong staining of T cell areas; only rare staining in B cell-rich germinal centers
Blood cells	No evident staining
Bone marrow	Light nuclear staining of a subset of cells
Cerebellum	Strong nuclear staining in some cells; light cytosolic staining throughout tissue
Cerebral cortex	Nuclear staining with general cellular staining prominent in certain layers
Skeletal muscle	Light to moderate grainy staining of muscle fibers
Heart	General light cytoplasmic grainy staining throughout tissue
Kidney	Moderate to strong cytoplasmic staining of a subset of tubules; light staining in other tubules; Bowman's capsule and glomeruli are positive
Liver	No evident staining
Lung	Light to moderate staining of macrophages in tissue
Large intestine	Graded staining of epithelial cells towards lumen with progressive increased in cortical/plasma membrane staining
Small intestine	Graded staining of epithelial cells towards lumen
Stomach	Strong cytoplasmic staining of epithelial cells at lumen
Prostate	Moderate nuclear staining of basal glandular epithelia
Breast	Moderate nuclear and cytoplasmic staining of ductal epithelia

The monoclonal antibody 2F11, which recognizes all known splice isoforms of Bin1, reveals diverse patterns of expression and localization of Bin1 in different cell types. Nuclear, cytosolic, and/or membrane-associated localizations was observed in various tissues as noted, in the first case in support of nuclear functions associated with tumor suppression that have been suggested for Bin1.

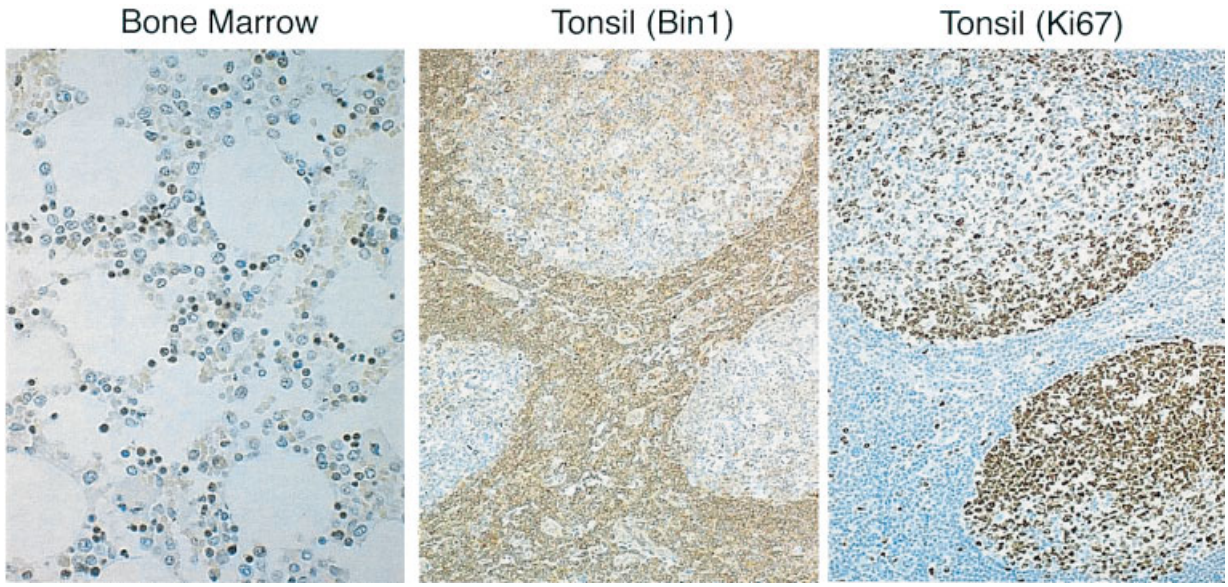

Fig. 3. Blood cells. Nuclear staining of Bin1 predominates in bone marrow-derived cells, many of which appear to be T cells (**left panel**), whereas cytosolic staining of Bin1 occurs in peripheral T and B cells in the tonsil (**middle panel**). Bin1 expression was elevated in T cell-rich sections of the tonsil that surround the B cell-rich germinal centers where most cell division occurs, as revealed by staining of an adjacent tissue section for the proliferation marker Ki67 (**right panel**).

In contrast to these observations, Bin1 polypeptides were not detected by 2F11 in peripheral blood smears (Table I), suggesting that stromal or cytokine cues dictate Bin1 expression in hematopoietic cells. Nuclear and cytosolic staining patterns were also apparent in brain cerebral cortex and cerebellum. In cerebral cortex, nuclear-stained cells were scattered throughout the tissue, occasionally amongst a broad background of cytosol-stained cells in particular cortical layers (Fig. 4). The nuclear pattern of Bin1 expression in brain contrasted sharply with the related BAR protein amphiphysin, which is expressed in neurons mainly at axon termini [Lichte et al., 1992; David et al., 1996]. The observations of nuclear Bin1 isoforms confirm evidence of a nuclear-based function for Bin1 that is non-amphiphysin-like, despite the structural relationship and interaction between Bin1 and amphiphysin proteins in brain.

Gastrointestinal epithelial cells exhibited strong staining of the cytosol and cortical regions of cells perhaps including the plasma membrane (Fig. 5). Interestingly, a gradient of expression was observed with highest staining in epithelial cells towards the lumen where differentiation and ultimately apoptosis occur. In contrast, little to no staining of non-epithelial stromal cells was detected. Stomach cells exhibited mainly cytosolic and cortical/plasma membrane staining, whereas small and large intestine cells displayed mainly only the cortical/plasma membrane staining. Interestingly, intestinal crypt cells, which stained lightly relatively to villus tip cells, exhibited a general cell staining; only the cytosol and cortical/plasma membrane region was stained in differentiating and/or apoptosing cells towards the upper crypts (Fig. 5, right panel). This staining

Fig. 4. Brain cerebral cortex. Strong nuclear staining of Bin1 in a small subset of neuronal cells was observed throughout different layers of the cerebral cortex of the brain. This pattern was overlaid with a generalized cytosolic staining of Bin1 in particular cortical layers as illustrated in the figure.

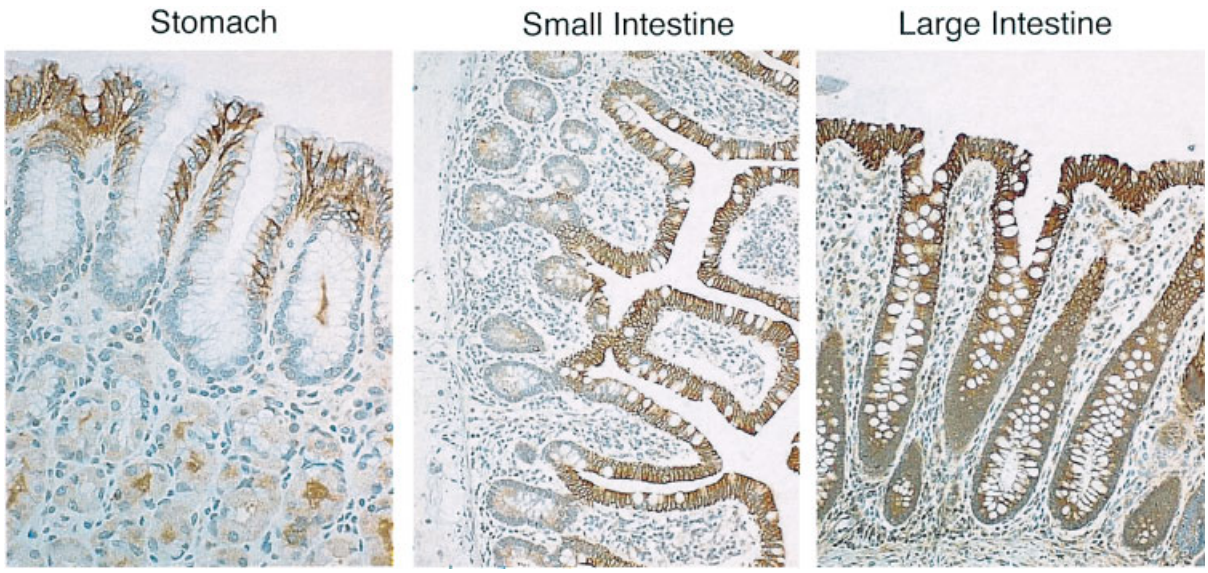

Fig. 5. Gastrointestinal tract. Strong cortical/plasma membrane-associated and cytosolic staining of Bin1 was observed in luminal cells of the gastrointestinal tract. Cytosolic and cortical/plasma membrane-associated staining of Bin1 predominated in luminal cells of the stomach whereas crypt cells are negative (**left panel**). In contrast, cortical staining of Bin1 predominated in luminal cells of the small and large intestine, particularly in cells located towards the lumen (**middle** and **right panels**). Staining of intestinal crypt cells, which was apparent in both nuclear and cytosolic compartments of these cells, is more robust in large compared to small intestine. Little to no staining of Bin1 in stromal cells was detected.

pattern was interesting given the maturation process of cells along intestinal epithelium: as proliferative crypt cells move up the epithelium they exit the cell division cycle, differentiate, and then apoptose and slough off at the villus tip. We noted a similar pattern of expression in skin keratinocytes, with dividing basal cells exhibiting mainly nucleocytosolic staining and differentiating squamous cells exhibiting mainly cytosolic staining (data not shown). Taken together, these observations suggested an association between cytosolic staining of Bin1 and differentiation and/or apoptotic commitment status.

Bin1 has features of a tumor suppressor gene in prostate cancer, with losses of expression observed with high frequency in metastatic tumors [Ge et al., 2000b]. A comparison of Bin1 staining patterns was performed in three cases of normal prostate and 50 cases of primary prostate adenocarcinoma. Strong staining of the nucleus and light staining of the cytosol of tubular and basal epithelial cells in the parenchyma was observed (Fig. 6, left panels). These findings confirmed previous observations of nuclear Bin1 staining revealed by mAb 99D in benign prostate tissue [Ge et al., 2000b], extending these observations by also identifying low level staining of cytosolic Bin1. The

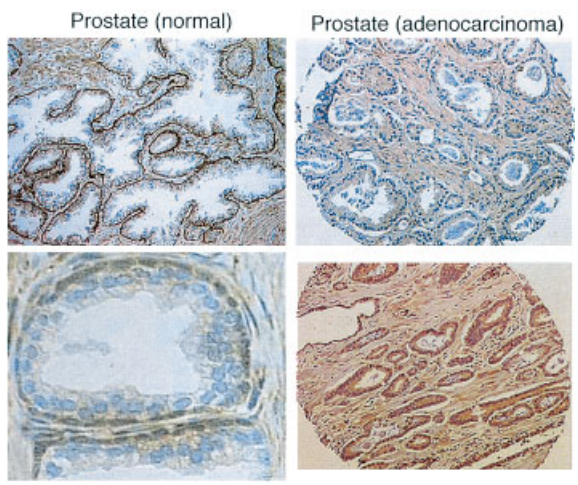

Fig. 6. Prostate. Strong nuclear staining of Bin1 was detected in basal epithelial cells of normal parenchyma whereas luminal columnar cells were uniformly negative (**left panel**). High magnification images reveal that staining is predominately nuclear in character. Two examples are shown of staining patterns that were observed in a tissue array of 50 cases of primary prostate adenocarcinoma (**right panels**). The major pattern observed (72% or 36/50 cases examined) was a general loss of staining in tumor cells. An alternate pattern observed in certain positive cases was a strong nucleocytosolic staining of Bin1 throughout both tumor and stromal cells (**bottom right panel**), possibly reflecting missplicing events in the tumor (see text).

staining pattern was similar to that observed in breast ductal epithelial cells, with an accentuation of the nuclear:cytosolic ratio of Bin1 expression in prostate cells. Differentiated columnar epithelial cells exposed to the lumen were uniformly negative. Stromal cells in normal prostate stained lightly by comparison. Taken together with the previous observations using 99D [Ge et al., 2000b], the observations suggested that the main isoform expressed in prostate epithelial cells was probably Bin1-10, which is predominantly nuclear. Tissue arrays were used to compare the Bin1 staining patterns in 50 graded cases of primary prostate adenocarcinoma. Notably, Bin1 staining was undetectable in 72% (36/50) of the cases examined (Fig. 6, top right panel). No apparent association of the losses with Gleason grade was noted. These results differed from a previous study, which documented reduction or loss of Bin1 expression by Northern analysis of primary tumors but more limited losses as detected by 99D immunohistochemistry [Ge et al., 2000b]. The observations using 2F11 might differ from the observations using 99D due to non-specific binding activity of the latter antibody, for example, as seen in the Western analysis of LNCaP cells (Fig. 1A, asterisks). In any case, the results obtained with 2F11 are consistent with the Northern analysis in the previous study. Of the primary tumors that were positive for 2F11 staining, an additional pattern was observed that was marked by strong nuclear and cytosolic staining of both tumor and stromal cells in the tumor (Fig. 6, bottom right panel). While the basis for this pattern was not established, it was reminiscent of that produced in malignant melanoma by misspliced Bin1 + 12A isoforms [Ge et al., 1999], which are expressed by the human prostate tumor cell lines PC3 and DU145 [Ge et al., 2000b]. Since these misspliced isoforms lack the tumor suppressor and proapoptotic activity of the wild-type Bin1 and Bin1-10 isoforms [Elliott et al., 2000], the altered staining pattern in some cases of prostate adenocarcinoma might reflect a loss-of-function similar to that documented previously in melanoma [Ge et al., 1999]. Loss of Bin1 expression in prostate adenocarcinoma might simply reflect a loss of basal cells in that disease; we are currently employing a knockout mouse model system developed recently in our laboratory to explore the significance of Bin1 loss to prostate tumor development and/or progression. In summary, the results presented here extended the findings of previous work, which indicated frequent losses of Bin1 expression and/or activity in prostate adenocarcinoma [Ge et al., 2000b].

We conducted an immunohistochemical analysis of Bin1 expression in human tissues using a novel and specific monoclonal antibody that recognizes all the major splice isoforms expressed by the *Bin1* gene. This reagent will be particularly useful for analysis of the diverse functions of this gene, which appear to be mediated to a significant degree by alternate mRNA splicing and differential subcellular localization. Robust expression was revealed in hematopoietic and epithelial cells that has not been previously described. An interesting association between expression levels and regions of differentiation and/or apoptosis was noted, particularly in gastrointestinal epithelial cells. Nuclear Bin1 isoforms that likely have non-endocytotic roles were observed in breast, prostate, skin, colonic crypt cells, bone marrow cells, and brain. Interestingly, nuclear Bin1 was confirmed in many cells in support of the reported interactions with the c-Myc oncoprotein that has been described [Sakamuro et al., 1996; Elliott et al., 1999; DuHadaway et al., 2001]. Based on the observation of nuclear Bin1 expression in the brain cerebral cortex and cerebellum, nuclear Bin1 isoforms may also have roles in non-proliferating cells. In contrast, cytosolic and cortical/plasma membrane-associated Bin1 predominated in cells with limited proliferative potential, for example, at sites of differentiation and apoptosis. In support of the hypothesis that nuclear isoforms have a function in tumor suppression, we observed that the predominant nuclear pattern of Bin1 expression in basal epithelial cells of normal prostate was frequently missing or altered in prostate adenocarcinoma cells. Additional work is needed to determine the significance of these observations to the development or progression of prostate cancer. Future work to dissect the non-endocytotic function of nuclear Bin1 proteins that have tumor suppression activity will be assisted by the definition of tissues that exhibit nuclear patterns of Bin1 expression.

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