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AZU-1: A Candidate Breast Tumor Suppressor and Biomarker for Tumor Progression

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To identify genes misregulated in the final stages of breast carcinogenesis, we performed differential display to compare the gene expression patterns of the human tumorigenic mammary epithelial cells, HMT-3522-T4-2, with those of their immediate premalignant progenitors, HMT-3522-S2. We identified a novel gene, called anti-zuai-1 (AZU-1), that was abundantly expressed in non- and premalignant cells and tissues but was appreciably reduced in breast tumor cell types and in primary tumors. The AZU-1 gene encodes an acidic 571-amino-acid protein containing at least two structurally distinct domains with potential protein-binding functions: an N-terminal serine and proline-rich domain with a predicted immunoglobulin-like fold and a C-terminal coiled-coil domain. In HMT-3522 cells, the bulk of AZU-1 protein resided in a detergent-extractable cytoplasmic pool and was present at much lower levels in tumorigenic T4-2 cells than in their nonmalignant counterparts. Reversion of the tumorigenic phenotype of T4-2 cells, by means described previously, was accompanied by the up-regulation of AZU-1. In addition, reexpression of AZU-1 in T4-2 cells, using viral vectors, was sufficient to reduce their malignant phenotype substantially, both in culture and in vivo. These results indicate that AZU-1 is a candidate breast tumor suppressor that may exert its effects by promoting correct tissue morphogenesis.

INTRODUCTION

Significant advances in breast cancer research have been gained from studies of disease-linked genetic mutations. The identification of genes such as BRCA-1 and BRCA-2 confirms that inherited genetic lesions can influence tumorigenic conversion of breast epithelial cells, either by activating oncogenes or inactivating tumor suppressors (Haber and Harlow, 1997). Increasingly studies indicate that, along with predisposing chromosomal abnormalities, misexpression of genes with otherwise wild-type sequences also contributes to the process of tumorigenesis (Sager, 1997; Zhang *et al.*, 1998). For example, growth factor receptors ErbB1 and ErbB2 are overexpressed in breast tumor tissue in vivo with little evidence of mutation (Alroy and Yarden, 1997). Yet they have become accepted prognostic indicators for breast cancer diagnosis and treatment (Pinkas-Kramarski *et al.*, 1997). Therapies aimed at reducing their levels are now in clinical trials. Thus, comparison of gene expression patterns in normal and tumor cells is a promising strategy for discovering gene function and for eventually understanding, diagnosing, and treating cancers of the breast.

The results of comparative gene expression studies, although continuing to demonstrate the importance of growth regulators and transcription factors in cancer progression, have also implicated other cancer-related genes with surprisingly diverse functions. In the case of breast cancer, these include proteases and protease inhibitors (Zou *et al.*, 1994; Sternlicht *et al.*, 1999), extracellular matrix components and their receptors (Weaver *et al.*, 1997; Zhang *et al.*, 1998), and cytoskeletal elements (Sager, 1997; Mielnicki *et al.*, 1999). Such gene misregulation can be due to defects in the breast epithelial cells themselves or can be due to the effects of neighboring cells, such as myoepithelial or stromal cells, that could indirectly influence the behavior of the epithelial cells (Zou *et al.*, 1994; Lochter *et al.*, 1997; Thomasset *et al.*, 1998).

A recently developed human epithelial breast cell model, the HMT-3522 progression series, is proving to be a useful

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[§] Corresponding author. E-mail address: MJBissell@lbl.gov. Abbreviations used: AZU-1, anti-zuai-1; CCD, coiled-coil domain; EGFR, epidermal growth factor receptor; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; Ig, immunoglobulin; NLS, nuclear localization sequence; RACE, rapid amplification of cDNA ends; SPAZ domain, serine- and proline-rich AZU-1 domain; TACC, transforming acidic coiled coil; 3D rBM, three-dimensional reconstituted basement membrane.

system for studies of breast tumor progression. Serial culture of the HMT-3522 cells, which originated from primary breast epithelial cells of a woman diagnosed with fibrocystic breast disease, allowed for the generation of a continuum of genetically related cell populations that range in phenotype from nonmalignant (S1) to premalignant (S2) to tumorigenic (T4-2) (Briand *et al*., 1987, 1996). Because these cell lines share common genetic origins, observed differences in gene expression patterns between these cells are likely indicative of changes that influence tumorigenic progression rather than differences in genetic backgrounds.

To identify genes misexpressed upon tumorigenic conversion in the breast, we used a differential display strategy to compare the gene expression profiles of tumorigenic T4-2 cells with their premalignant S2 progenitors. Here, we report the identification and characterization of a novel gene we refer to as AZU-1, which is expressed abundantly in nonmalignant (both primary and immortalized) and premalignant breast epithelial cells but is dramatically down-regulated in a number of breast tumor cell lines and primary tumors. Restoration of normal AZU-1 expression levels in T4-2 cells was sufficient to reduce tumor formation in vivo and resulted in phenotypic reversion in culture (Weaver *et al*., 1997). Collectively, our results suggest that AZU-1 may protect nonmalignant cells from tumorigenic conversion by promoting proper cellular organization and tissue morphogenesis.

An abstract of this work has appeared previously (Chen *et al*., 1998).

MATERIALS AND METHODS

Cell Culture

HMT-3522 human mammary epithelial cells (S1, S2, and T4-2) and MCF10A cells were grown in chemically defined medium (Briand *et al*., 1987, 1996; Soule *et al*., 1990). HMT-3909 and MCF-7 cells were cultured on type I collagen-coated dishes in Dulbecco's modified Eagle's medium/F-12 medium supplemented with 1.4×10^{-6} M hydrocortisone and 2 mM glutamine, respectively. Primary human breast epithelial cells were purified and cultured as previously described (Petersen and van Deurs, 1987). Protein extracts were prepared from monolayer cultures using established protocols (Wang *et al*., 1998).

Three-dimensional reconstituted basement membrane (3D rBM) cultures were generated as described previously (Petersen *et al*., 1992; Weaver *et al*., 1997) using a commercially prepared rBM (Matrigel; Collaborative Research, Waltham, MA). 3D rBM assays were evaluated by phase-contrast microscopy and by measuring colony diameter using an eye piece equipped with a micrometer spindle. Cellular polarity was determined by immunostaining for the basal markers collagen IV and β4 integrin (Weaver *et al*., 1997). Reversion assays, using the β1 integrin function-blocking antibody mAb AIIB2 and Tyrphostin AG 1478 (Calbiochem, San Diego, CA), were performed as described previously (Weaver *et al*., 1997; Wang *et al*., 1998).

RNA Extraction and Northern Blot Analysis

Total RNA was extracted from cells and tissues using TRIzol reagent (Life Technologies, Grand Island, NY). For Northern blots, total RNA (20 μg/lane) was resolved on denaturing agarose gels and transferred to Hybond-N^+ membranes (Amersham, Cleveland, OH). Resulting blots were hybridized with ^{32}P-labeled cDNA probes and analyzed by autoradiography. A glyceraldehyde-3-phosphate dehydrogenase (GAPDH) probe was used to control for sample loading. Relative band intensities were quantified by densitometric analysis.

Differential Display

Differential display was performed using the RNAimage kit as per the manufacturer's instructions (GenHunter, Nashville, TN). Briefly, total RNA (DNA-free) from S2 and T4-2 cells was reverse transcribed, and the cDNA products were amplified by PCR using the anchored (H-T_{11}M, M=A,C,G) and arbitrary (H-AP-1) primers provided in the kit and α[^{33}P]dATP. PCR products were resolved on denaturing gels, and differential expression was evaluated by autoradiography. Confirmation of the expression pattern of a 180-bp cDNA was achieved by subjecting the fragment to a second PCR amplification and by analyzing the products on agarose gels.

AZU-1 Cloning Strategy

The sequence of the 180-bp differential display cDNA fragment was compared with existing GenBank sequences and was found to be identical to three expressed sequence tags (*Homo sapiens* cDNA clones N57107, R38679, and H23488). All three clones contained the 180 bp plus additional 5′ and/or 3′ sequences. Two of these clones exhibited polyadenylation sites, and none displayed apparent open reading frames. Rapid amplification of cDNA ends (5′ RACE; Life Technologies) was performed to characterize the 5′ sequence of the identified gene. Primers corresponding to the 180-bp differential display fragment were used to initiate the 5′ RACE procedure according to the manufacturer's instructions. The protocol was repeated 12 times to obtain 3.8 kb of sequence; in each cycle, 500–800 bp of additional 5′ sequence were obtained. Sequencing was conducted using cycle sequencing (Amersham). The 3.8-kb sequence contained a candidate translation start codon (consistent with the Kozak consensus rules; Kozak, 1984) and a downstream in-frame stop codon.

To confirm the accuracy of the 3.8-kb AZU-1 sequence and to generate a composite AZU-1 cDNA, primers corresponding to the AZU-1 5′ and 3′ ends were used in PCRs. In two independent experiments, each using distinct pools of total S1 cellular cDNA as a template, the resulting PCR products were identical in composition to the sequence obtained using 5′ RACE. We call the isolated gene AZU-1 (GenBank accession number AF176646). Full-length AZU-1 cDNAs were subcloned into pCR 2.1 (pCR2.1-AZU-1; Invitrogen, Carlsbad, CA) for further amplification and use. The pI of AZU-1 was determined using Genetics Computer Group (Madison, WI) software.

AZU-1 Constructs

To subclone AZU-1 coding sequences into pET-28a (Novagen, Madison, WI), PCR was performed using pCR2.1-AZU-1 as a template and primers supplemented with *Sac*I and *Sal*I restriction sites (forward primer, 5′-CTGAGCTCATGCCCCTGAGGAGGCCAAAGAT-3′; reverse primer, 5′-GCGTCGACTTTAGCTTTTCCCCATTTTGGCAATCAGTTC-3′). pCIneo-AZU-1 and pLXSN-AZU-1 constructs were generated by subcloning *Nhe*I–*Xho*I and *Eco*RI–*Xho*I cDNA fragments from pET-28a-AZU-1 into pCIneo (Promega, Madison, WI) and pLXSN (Clontech, Palo Alto, CA), respectively.

In Vitro Transcription and Translation

In vitro transcription and translation reactions, programmed with the pCIneo-AZU-1 construct, were performed using the TNT coupled reticulocyte lysate kit (Promega) as per the manufacturer's instructions. Luciferase cDNA (molecular mass, 61 kDa) was used as a positive control. ^{35}S-Labeled AZU-1 produced in the in vitro transcription and translation was immunoprecipitated in radioimmunoprecipitation assay buffer in the presence of 1 μl of whole rabbit serum, either preimmune or AZU-1 specific, as described previously (Weaver *et al*., 1997). The molecular mass of AZU-1 was determined using ChemiImager software (Alpha Innotech, San Leandro, CA).

AZU-1 Antibody Production and Western Immunoblots

A polyclonal antibody was generated against a 20-amino-acid N-terminal AZU-1 peptide supplemented with a C-terminal cysteine (MPLRRPKMKKTPEKLDNTPAC; ImmunoVision Technologies, Daly City, CA). Preimmune and immune sera were used as probes in Western blots at a dilution of 1:250. Primary antibody binding was detected using an HRP-conjugated goat anti-rabbit secondary antibody followed by chemiluminescent detection.

Indirect Immunostaining and Image Acquisition

Cells were fixed directly in 2% paraformaldehyde ("intact cells") or were permeabilized in situ with 0.5% Triton X-100 before fixation as described previously (Lelièvre *et al.*, 1998). After blocking, cells were incubated with equivalent amounts (24 μg/ml) of affinity-purified AZU-1 antibody or nonimmune rabbit immunoglobulin Gs (IgGs) (Weaver *et al.*, 1997). Primary antibodies were detected with an FITC-conjugated anti-rabbit antibody (Jackson Immunoresearch, West Grove, PA). F-actin was detected in parallel samples using FITC-phalloidin. Cells were visualized using a Bio-Rad (Hercules, CA) MRC 1024 laser scanning confocal microscope attached to a Nikon (Melville, NY) Diaphot 200 microscope. All immunofluorescence images were recorded at 120× magnification.

Expressing AZU-1 by Retroviral Infection

AZU-1 expression in T4-2 cells was achieved using the Retro-X viral gene delivery system (Clontech) according to the manufacturer's protocols. The studies performed here were done on pooled populations of T4-2 cells that were stably infected with the vector alone (pLXSN) or with AZU-1 sequences (pLXSN-AZU-1). The AZU-1 transgene comigrates with the endogenous AZU-1 message at 4.4 kb. Northern blots probed with sequences from the AZU-1 $3'$ untranslated region show no increase in endogenous AZU-1 expression in AZU-1-overexpressing cells (our unpublished results). Thus, the increased AZU-1 expression observed in the T4-2-AZU-1 cells was entirely attributable to expression from the AZU-1 transgene.

Assays of Tumor Phenotype

For soft agar assays, cells were seeded at 1×10^5 cells per well in 0.35% soft agar in 12-well plates. After 4 wk, colonies >40 μm were scored as positive for growth (Wang *et al.*, 1998). Invasion assays were performed as described previously (Lochter *et al.*, 1997). The data are expressed as the number of cells per field at 200× magnification. Tumorigenic potential was assessed by subcutaneous injection of 2.5×10^6 cells into flanks of 4- to 6-wk-old BALB/c nu/nu female mice. Tumor nodules were measured using a caliper 6–8 wk after injection.

RESULTS

Identifying Putative Determinants of Tumorigenic Conversion by Differential Display

We used a PCR-based differential display strategy to screen for genes that were variably expressed in S2 and T4-2 cells. We detected a 180-bp cDNA that was present at higher levels in the S2 cells than in their T4-2 counterparts. The cDNA fragment was isolated, amplified, and used as a probe in Northern blots of total RNA from these cells. In S2 cells, the probe hybridized with an abundant 4.4-kb message and two minor transcripts of $\sim$7.5 and 9.5 kb (Figure 1A). The

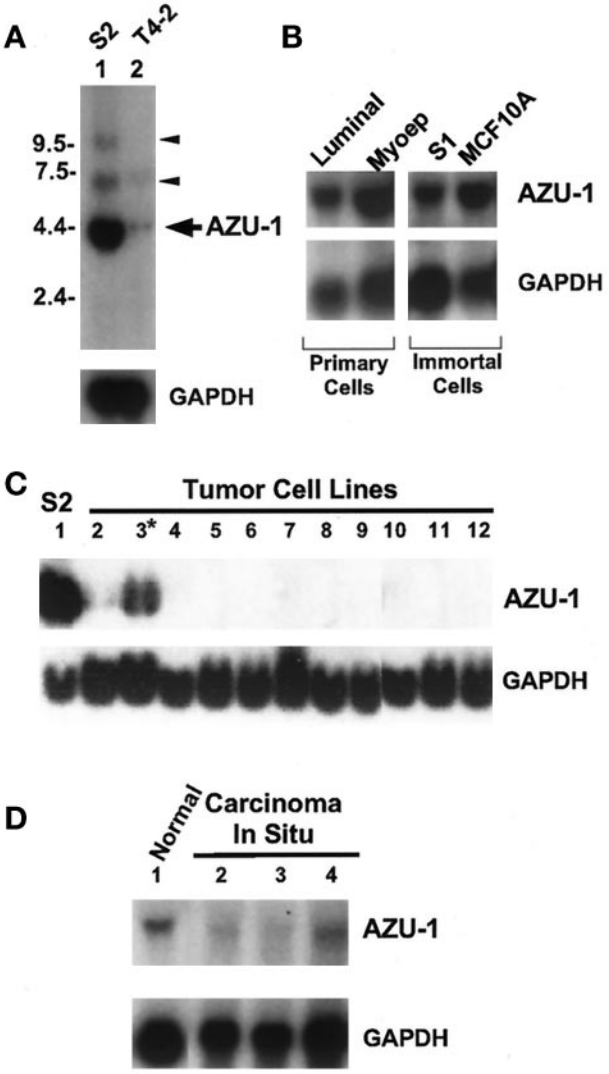

Figure 1. The AZU-1 gene is differentially expressed in nonmalignant and tumorigenic human breast cells. Northern blot analysis was performed on total RNA (20 μg/lane) from breast cell and tissue extracts using ^{32}P-labeled AZU-1-specific probes. (A) Comparison of AZU-1 expression in S2 and T4-2 cells detected with the 180-bp differential display cDNA probe. Two lower-abundance transcripts are indicated by small arrows; the presence of these bands was not always reproducible. (B) AZU-1 expression in normal primary luminal epithelial and myoepithelial cells and in nonmalignant breast cell lines HMT-3522-S1 and MCF10A. (C) Compared with S2 cells (lane 1), AZU-1 expression is reduced in a number of breast carcinoma cell lines: lane 2, T4-2; lane 3, HMT-3909; lane 4, MCF-7; lane 5, CAMA-1; lane 6, BT-20; lane 7, MDA-MB-468; lane 8, SKBR-3; lane 9, T47D; lane 10, MDA-MB-231; lane 11, Hs578T; and lane 12, BT549. *, HMT-3909 cells display partial myoepithelial differentiation (O.W. Petersen, unpublished result). (D) AZU-1 expression in tissues derived from normal breast (lane 1) and three carcinomas in situ (lanes 2–4). For B and C, an AZU-1 coding region probe was used; in all cases, a GAPDH probe was used as a loading control.

T4-2 cells displayed a dramatic reduction in the expression of the 4.4-kb message in comparison with S2 cells.

Northern blots using probes derived from the full-length cDNA sequence (see below) confirmed the expression pattern of the 4.4-kb gene product. We detected an abundant and specific message not only in the nonmalignant human epithelial cell lines, HMT-3522-S1 and MCF10A, but also in primary cultures of human luminal epithelial and myoepithelial cells (Figure 1B). Expression of the 4.4-kb message was significantly reduced in 10 of the 11 breast carcinoma cell lines examined (Figure 1C). Likewise, two of three carcinomas showed reduced AZU-1 expression when compared with normal tissue (Figure 1D). Based on these observations and the functional studies described below, we have named this gene product anti-zuai-1 (AZU-1), with "zuai" meaning "breast cancer" in Chinese.

AZU-1 Protein Expression and Sequence Analysis

We used 5′ RACE to recover a full-length AZU-1 cDNA and found that the AZU-1 sequence did not correspond to any previously published gene. The AZU-1 gene encodes a protein of 571 amino acids with an estimated pI of 5.1 (Figure 2A). Although predicted to have a molecular mass of 64 kDa, the full-length AZU-1 protein, when produced in vitro, displays a significantly higher relative mobility of 80 kDa when resolved on denaturing gels (Figure 3A). This aberrant migration may be due to the proline-rich composition of the protein's N-terminal 361 amino acids (>11% proline; see the predicted amino acid sequence in Figure 2A) (Ollo and Maniatis, 1987; Sadler *et al.*, 1992). An AZU-1-specific antibody recognized both the in vitro-translated AZU-1 protein (Figure 3A) and a protein of identical size in HMT-3522 cell extracts (Figure 3B). Like the transcript, AZU-1 protein levels were significantly reduced in T4-2 cells; on average, AZU-1 protein levels were threefold lower in T4-2 cells in comparison with their nonmalignant S1 counterparts (mean, 3.0 ± 0.85; n = 11). AZU-1 protein expression is basically absent in MDA-MB-231 breast carcinoma cells (our unpublished results).

Using BLAST analysis (Altschul *et al.*, 1997), we found that AZU-1 shares significant similarity (particularly at its N and C termini) with three sequences deposited in GenBank, called TACC1 (Still *et al.*, 1999a), TACC2 and TACC3 (Still *et al.*, 1999b) (TACC = transforming acidic coiled coil; GenBank loci AF049910, AF095791, and AF0935 and 43, respectively). TACC2 is most similar to AZU-1 and is likely to be an AZU-1 splice variant, because, apart from two small insertions (4 and 47 amino acids long) and a single amino acid change, it is identical to AZU-1 at both the nucleic acid and protein levels. The second most closely related gene to AZU-1 is TACC1, a gene cloned from the breast cancer amplicon 8p11 (Still *et al.*, 1999a). TACC3, although more distantly related to AZU-1 than TACC1, is also similar to AZU-1 with respect to both its domain organization and amino acid sequence. These genes may thus represent a new superfamily.

Alignment of AZU-1 with TACC1 and TACC3 suggests four AZU-1 protein domains (Figure 2B). At its N terminus, AZU-1 exhibits a domain of 83 amino acids that we call a "SPAZ" domain (for serine- and proline-rich AZU-1 domain; Figure 2C). The combined serine-proline content of this domain is 36%. SPAZ domains are found in AZU-1 (or TACC2), TACC1, TACC3, and the *Saccharomyces cerevisiae* gene product BCK1, a member of the MAPK kinase kinase family of serine/threonine kinases (Lee and Levin, 1992). In all of these gene products, two serine residues in the domain are invariant.

The central domains of AZU-1, called region I and region II, are defined by virtue of their relationship to TACC1. Region I shows some sequence identity (20%) with the corresponding region of TACC1. One particular sequence motif common to both AZU-1 and TACC1 in region I (HATDEEKLA; highlighted in Figure 2A) is not conserved in TACC3. Region II corresponds to the segment in AZU-1 that is absent from TACC1 (and present only partially in TACC3). PSORT predictions (Nakai and Horton, 1999) indicate that AZU-1 contains two putative nuclear localization sequences (NLSs), one at its N terminus and one at amino acid 122 (Figure 2A).

The fourth and C-terminal region of AZU-1 displays a series of heptad repeats consistent with the presence of an extensive, but discontinuous, coiled-coil domain (Figure 2D). The seven structural positions of each heptad repeat are named a–g; positions a and d (capital letters in Figure 2D) are occupied by hydrophobic residues and are predicted to form a nonpolar helix interface, whereas the remaining residues are hydrophilic and form the solvent-exposed part of the helix surface (Lupas, 1996, 1997).

Although most homologous to TACC1 and TACC3, the AZU-1 coiled-coil domain is also similar to that of the human SB1.8/DXS423E protein, a putative homologue of the *S. cerevisiae* SMC1 protein that is essential for proper chromosomal segregation during mitosis (Protein Information Resource locus I54383) (Rocques *et al.*, 1995). Alignments indicate three major regions where the characteristic heptad repeats fall into register in all four proteins (Figure 2D). The MultiCoil program predicts that all of these domains are likely to form dimers ($p > 0.90$) (Wolf *et al.*, 1997).

AZU-1 Subcellular Localization

To gain insight into the cellular function of the AZU-1 gene product, we performed immunolocalization studies in HMT-3522 cell monolayers by confocal microscopy using an affinity-purified version of the anti-AZU-1 antibody described above (Figure 4). In intact nonmalignant S1 cells, the majority of AZU-1 protein appears to be uniformly distributed throughout the cytoplasm; above-background staining is observed also throughout the nucleus and in round, subnuclear dots (Figure 4A). S1 cells probed in parallel with an equivalent amount of nonimmune rabbit IgG antibody did not exhibit significant staining (Figure 4C), indicating that the localization pattern observed with the AZU-1 antibody is specific. AZU-1 localization in tumorigenic T4-2 cells showed a subcellular distribution similar to that observed with S1 cells in both the cytoplasmic and nuclear compartments (Figure 4D). In comparison to S1 cells, however, T4-2 cells generally exhibited a diminished AZU-1 staining intensity. T4-2 cells with higher levels of AZU-1 were occasionally observed; the significance of this heterogeneity is unknown.

Coiled-coil domains are observed in a variety of cytoskeleton-associated structural proteins, including actin-associated myosin and cytokeratins (Lupas, 1996). Given the prominent C-terminal coiled-coil domain of AZU-1, it

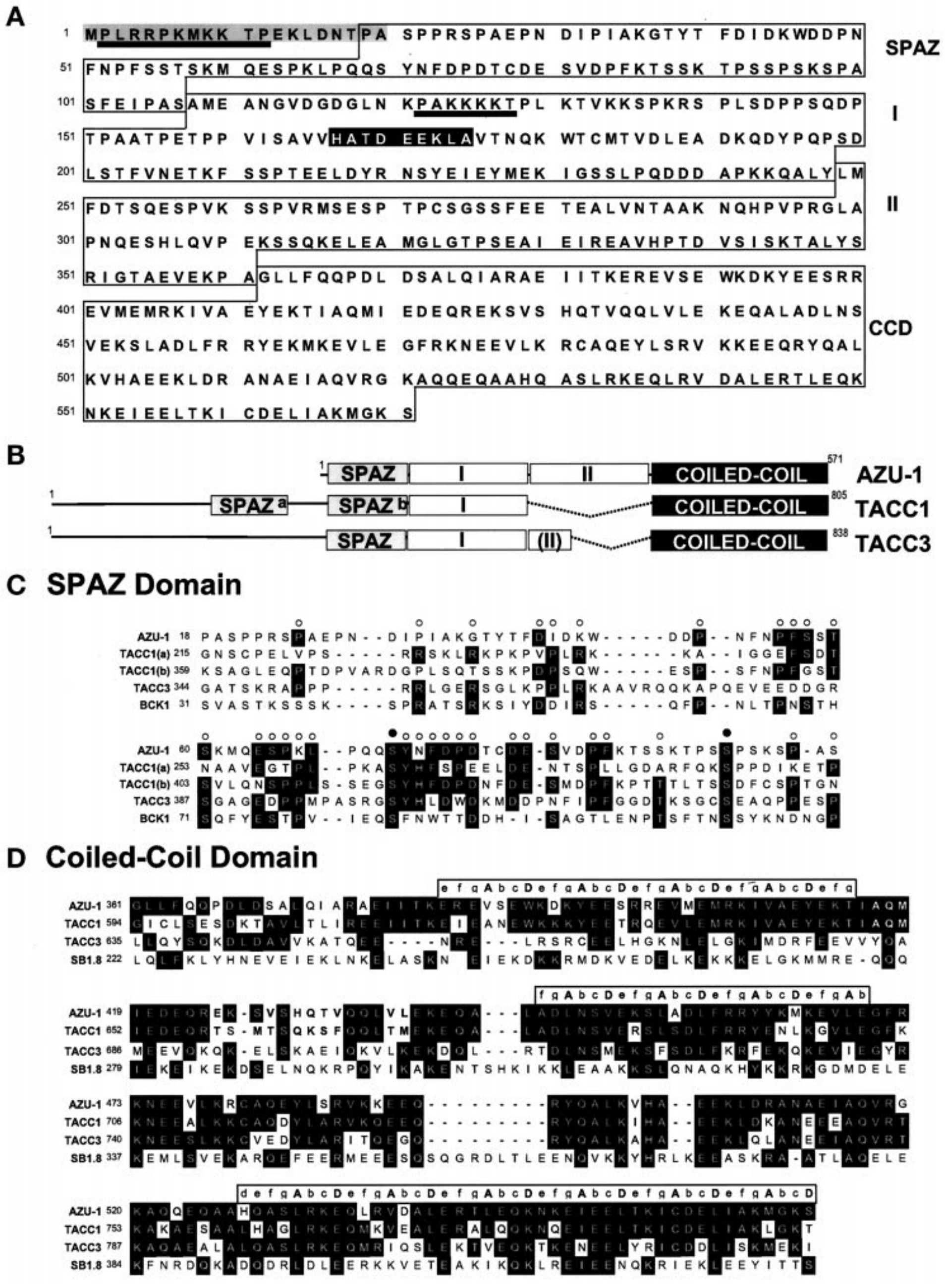

Figure 2. Sequence and structure of AZU-1. (A) Deduced amino acid sequence of the AZU-1 571-amino-acid open reading frame. Four structural domains, labeled SPAZ, region I, region II, and CCD, are boxed, and two predicted NLS motifs are underlined. The N-terminal peptide used to generate the AZU-1 antibody is highlighted in gray. The HATDEEKLA sequence, a peptide conserved between AZU-1 and TACC1, appears in black. (B) Domain organization of AZU-1 and two AZU-1-related genes, TACC1 and TACC3. Based on its similarity with TACC1 and TACC3, AZU-1 can be partitioned into four domains: 1) the N-terminal SPAZ domain, 2) region I, a region that shares a moderate sequence similarity with TACC1 and to a lesser extent with TACC3, 3) region II, which is totally absent in TACC1 and partially removed from TACC3, and 4) the C-terminal coiled-coil domain. (C) Sequence alignments of SPAZ domains from AZU-1, TACC1 (2 copies, a and b), TACC3, and BCK1 from *S. cerevisiae*. Residues that are conserved in three or more of these sequences appear in black; the corresponding columns are marked with open circles. Two invariant serine residues are indicated by filled circles. Fold recognition analyses predict that SPAZ domains adopt Ig-like folds. (D) CCD sequence alignments of AZU-1, TACC1, TACC3, and SB1.8/DXS423E. Amino acid identities observed in two or more of the aligned sequences are indicated in black; in cases in which two pairs of identical amino acids are observed in the alignment, AZU-1-like sequences are preferentially highlighted. The CCD heptad repeat positions, a–g, are indicated in brackets above the three regions where all four proteins fall into register. Positions a and d, often occupied by hydrophobic residues, are indicated in capital letters. Sequence identities among all four proteins in this region are most notable in the second half of the CCD.

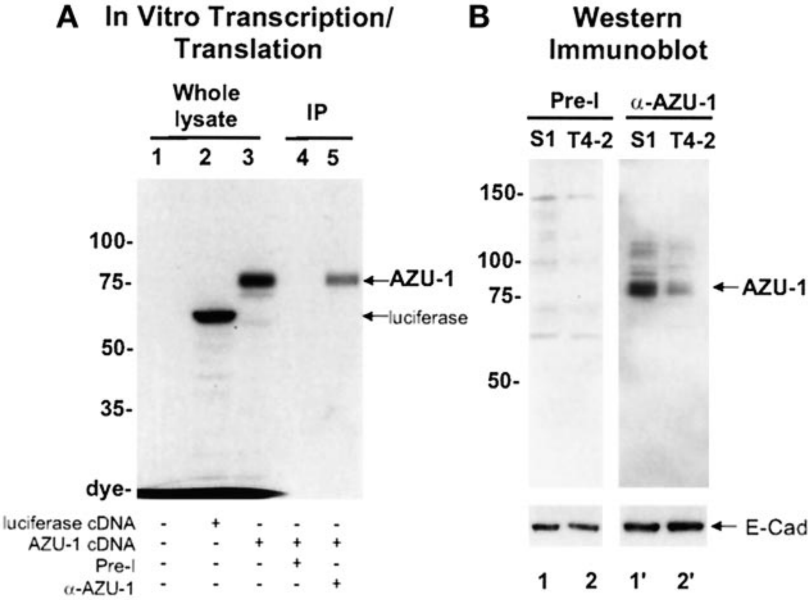

Figure 3. The AZU-1 protein migrates with an apparent molecular mass of 80 kDa on SDS-polyacrylamide gels. (A) In vitro transcription and translation reactions were performed with [^{35}S]methionine in the absence (lane 1) or presence of luciferase cDNA (lane 2, positive control at 61 kDa) or AZU-1 cDNA (lanes 3–5). In lanes 1–3, 5 μl of whole lysate were loaded in each lane. The remaining AZU-1 lysate was immunoprecipitated with either preimmune (lane 4) or AZU-1-specific (lane 5) rabbit sera, and the precipitated samples were loaded into adjacent wells. The resolved protein products were analyzed by autoradioagraphy. The AZU-1 cDNA gives rise to a single predominant protein with an apparent molecular mass of 80 kDa. (B) Protein extracts from S1 and T4-2 monolayer cultures (20 μg/lane) were analyzed by Western immunoblotting using preimmune (lanes 1 and 2) or anti-AZU-1 (lanes 1′ and 2′) rabbit sera. E-cadherin antibodies were used to control for protein loading. Like the in vitro-translated protein, cellular AZU-1 migrates with an apparent molecular mass of 80 kDa by SDS-PAGE. On average, T4-2 cells exhibit a threefold reduction in AZU-1 protein levels in comparison with nonmalignant S1 cells.

seemed plausible that AZU-1 might also associate with the cellular cytoskeleton. We tested this possibility by introducing a differential detergent extraction step in the immunostaining protocol. Cell monolayers were permeabilized with 0.5% Triton X-100 before fixation and stained for AZU-1 (Figure 4, E and H) or cytoskeletal F-actin (Figure 4F). Our results demonstrate that detergent extraction of HMT-3522 cells depleted the cytoplasmic pools of AZU-1, leaving only nuclear immunostaining behind. Given that insoluble F-actin was not depleted in detergent-treated cells, these results indicate that the cytoplasmic AZU-1 resides in a detergent-sensitive cellular subcompartment.

Assays of AZU-1 Tumor Suppressor Function In Vivo and in Culture

Reduced expression of AZU-1 in a high percentage of tumorigenic cell lines suggested that the loss of AZU-1 may play a role in tumorigenic conversion. To test this hypothesis, we asked whether reexpression of AZU-1 in T4-2 cells is sufficient to attenuate their tumorigenic phenotype. Using a viral-mediated gene transfer system, we introduced a full-length AZU-1 transgene into T4-2 cells. Pooled populations of stably infected cells were screened for AZU-1 expression and were shown to contain AZU-1 message and protein levels comparable with those observed in S1 cells (Figure 5, A and B). These levels were approximately two- to threefold higher than AZU-1 expression in the vector-infected T4-2 cells.

To test the potential tumor suppressor function of the AZU-1 gene product, assays of anchorage-independent growth and invasive potential were performed (Figure 5, C and D, respectively). S1 and T4-2 cells displayed expected behaviors in these assays: S1 cells did not support growth in soft agar and were noninvasive, whereas T4-2 cells (uninfected or vector-infected) gave positive responses in both assays. T4-2-AZU-1 cells showed a significantly diminished tumor phenotype in soft agar and invasion assays, with behavioral responses that were 25% (for soft agar assays) and 15% (for invasion assays) of those displayed by the vector-infected T4-2 cells.

S1 and T4-2 cells and their corresponding AZU-1 transfectants were also examined for tumorigenicity in vivo (Table 1). As reported previously (Briand *et al.*, 1987, 1996; Weaver *et al.*, 1997), S1 cells did not give rise to tumors when injected into nude mice, whereas the T4-2 cells produced tumors in ~90% of the injected sites. Mice injected with T4-2-AZU-1 cells gave a significantly reduced tumorigenic response with only four of the 32 inoculated sites (13%) producing detectable tumors. Furthermore, these tumors were ~7-fold smaller than those formed by control T4-2 cells (Table 1).

Restored AZU-1 Levels Promote Normal Tissue Architecture in Tumorigenic Breast Cells in Culture

We have demonstrated previously that normal and tumorigenic breast cell phenotypes can be effectively distinguished in the context of 3D rBM assays (Petersen *et al.*, 1992; Weaver *et al.*, 1997). In 3D rBM assays, S1 cells form polarized, growth-arrested, acinar structures, characterized by polarized β4 integrin localization and basal deposition of an endogenous BM. T4-2 cells, cultured under the same conditions, form large, growing and unpolarized colonies with higher, but disorganized, β4 integrin and collagen IV deposition. In the presence of inhibitors of β1 integrin or epidermal growth factor receptor (EGFR), T4-2 cells undergo "phenotypic reversion" to form near-normal growth-arrested acini similar to those formed by S1 cells (Weaver *et al.*, 1997; Wang *et al.*, 1998). Thus, culturing cells in 3D rBM provides a simple, yet informative, assay that allows for the evaluation of tissue polarity and architecture as well as cellular growth.

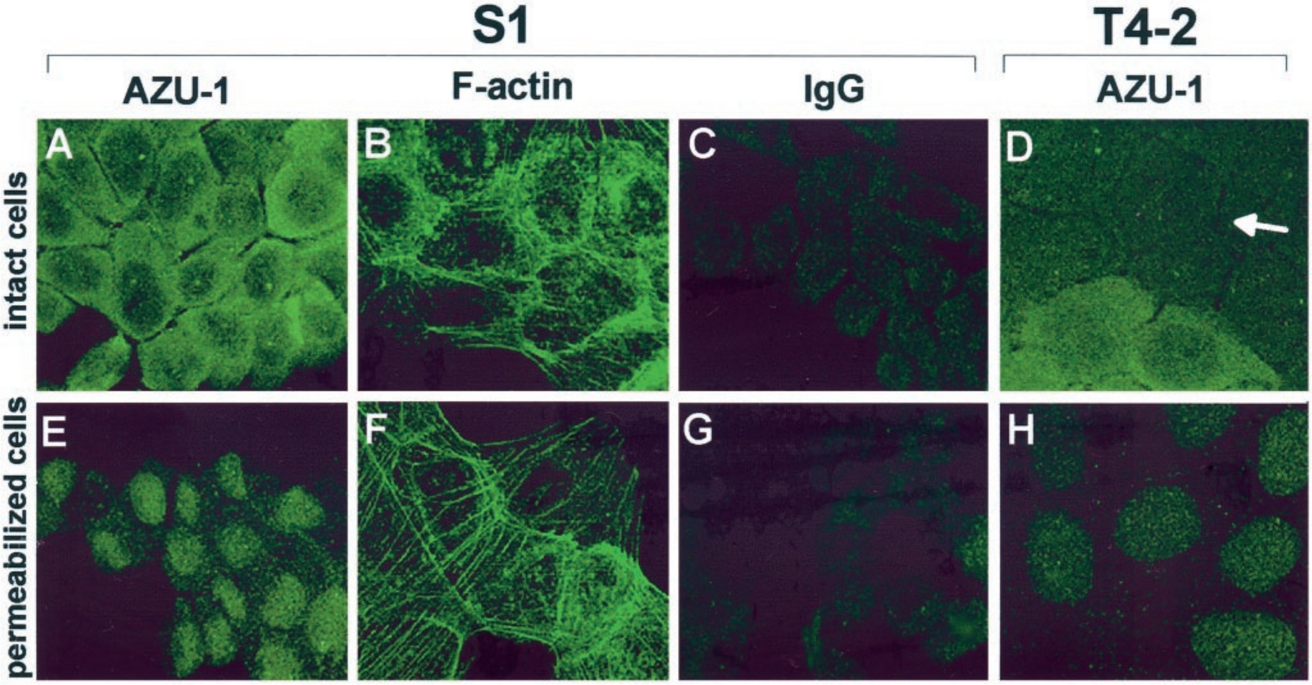

Figure 4. AZU-1 is a predominantly cytoplasmic protein in S1 and T4-2 cells. After 4 d in culture, cell monolayers were either directly fixed with 2% paraformaldehyde (A–D) or permeabilized with Triton X-100 before fixation (E and F). Cells were immunostained with affinity-purified anti-AZU-1 polyclonal antibody (A, D, E, and H) or with an equivalent amount of purified rabbit IgG (B and F). Primary antibodies were detected using an FITC-conjugated secondary antibody. F-actin was visualized in S1 cells using FITC-phalloidin. Confocal images in A, C–E, G, and H show a 0.4-μm optical section through the center of the cell nuclei. In both S1 and T4-2 cells, AZU-1 is found primarily in the cell cytoplasm, albeit at generally lower levels in the T4-2 cells (see arrow in D for typical T4-2 expression pattern). In both cells, the cytoplasmic pool of AZU-1 is detergent extractable, indicating that AZU-1 is not likely to be tightly associated with the insoluble cytoskeleton. (F-actin was monitored as a positive indicator of detergent resistance.) A minor, detergent-resistant pool of AZU-1 is found throughout nuclei in dim speckles as well as in distinct subnuclear foci. All images were recorded at 120× magnification.

We asked whether reexpression of AZU-1 would be sufficient to cause phenotypic reversion of T4-2 cells in the 3D rBM assay. AZU-1-overexpressing T4-2 and control cells were embedded in 3D rBM gels. After 10 d, S1 cells formed small, uniform, typical multicellular spheres with organized basement membranes and basally localized β4 integrin (Figure 6A; Weaver *et al.*, 1997). T4-2 colonies (both unmodified and vector infected) continued to grow and formed large, irregular, unpolarized colonies (Figure 6A). In contrast, T4-2-AZU-1 cells underwent phenotypic reversion, forming S1-like colonies that displayed appropriate cellular polarity. These results indicate that reexpression of AZU-1 at levels comparable with nonmalignant cells is sufficient not only to reduce the growth capacity of the tumor colonies but also to reinstate the polarized phenotype typical of normal breast epithelial acini.

Phenotypic reversion of T4-2 cells requires bidirectional cross-talk between at least two signaling pathways (β1 integrin and EGFR) (Wang *et al.*, 1998). We showed previously that inhibition of either pathway reduced the signaling activity of the other and resulted in the reduction of total β1 integrin and EGFR protein levels. Given the ability of AZU-1 to revert the T4-2 phenotype, we reasoned that it might be part of the orchestrated signaling events. If so, then its expression might be expected to be up-regulated during reversion. To test this hypothesis, we measured the AZU-1 mRNA levels in T4-2 cells treated with or without inhibitors of either β1 integrin (mAb AIIB2) or EGFR (tyrphostin AG1478) functions (Figure 6B, panel a). We found that AZU-1 expression was significantly higher in T4-2 cultures treated with the β1 integrin or EGFR antagonist (Figure 6B, panel b). AZU-1 up-regulation was not seen in two-dimensional T4-2 monolayers treated with either of the functional inhibitors (our unpublished results). These findings suggest that AZU-1 expression is coupled to β1 integrin and EGFR signaling pathways in HMT-3522 cells cultured in a three-dimensional context.

DISCUSSION

AZU-1 as a Tumor Suppressor

Using the genetically paired HMT-3522 human breast progression series, we have identified a novel gene, AZU-1, that is expressed abundantly in phenotypically normal and premalignant mammary epithelial cells (both primary and immortalized) but is dramatically down-regulated in a variety of breast carcinoma cell lines and carcinomas in situ. Restoration of AZU-1 expression to levels comparable with those seen in nonmalignant S1 cells is sufficient to reduce the tumorigenic phenotype of T4-2 tumor cells and to restore their ability to form normal tissue structures in 3D assays,

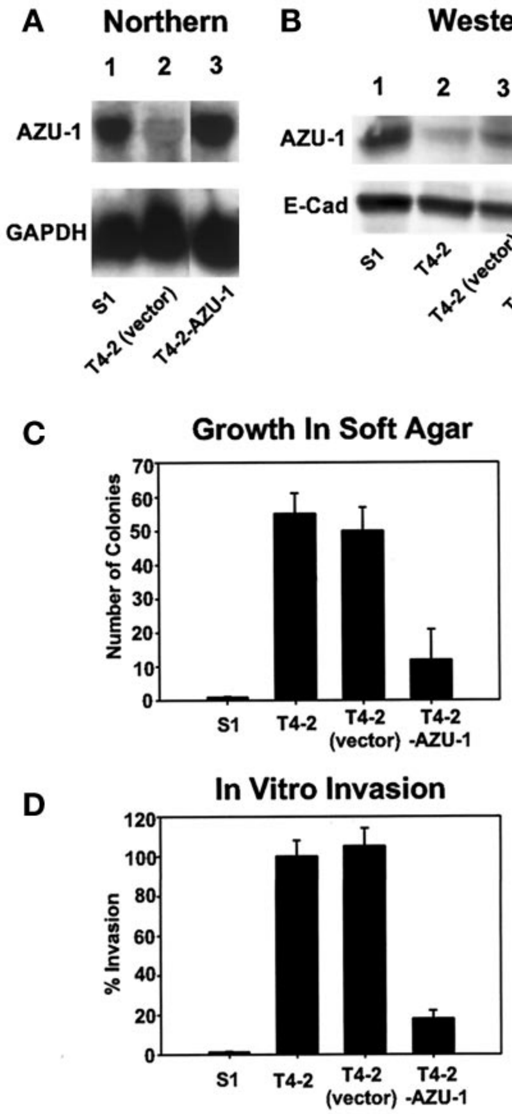

Figure 5. Reexpression of AZU-1 in T4-2 cell reduces their tumorigenicity in vitro. Northern (A) and Western (B) blot analyses were performed to monitor AZU-1 levels in S1, T4-2 control cells, and AZU-1-infected T4-2 cells. AZU-1 expression is increased at both the RNA and protein levels upon introduction of the AZU-1 transgene into T4-2 cells (in both cases approximately two- to threefold). A GAPDH probe and an E-cadherin antibody were used as loading controls in Northern and Western blots, respectively. In vitro tumorigenicity of the various HMT-3522 cells was measured in soft agar assays (C) and in invasion assays (D). In both cases, overexpression of AZU-1 in T4-2 cells gave rise to reduced tumorigenic behavior (i.e., reduced anchorage-independent growth and reduced capacity to migrate through a basement membrane-like gel). The data presented here represent the averages of three independent experiments and correspond to the mean activity of triplicate measurements ± SE.

Table 1. In vivo tumorigenicity of HMT-3522 cell lines

Cell type	No. of injection sites[a]	No. of sites with tumors[b]	Mean tumor size (mm^3) ± SE (n)
S1	32	0	0 (0)
T4-2	32	28	250 ± 80 (28)
T4-2 + vector	32	28	265 ± 95 (28)
T4-2 + AZU-1	32	4	38 ± 19 (4)

[a] Two injection sites per mouse; 16 mice in each group.

[b] Lump >10 mm^3.

using a reconstituted basement membrane. Our findings suggest that AZU-1 can be classified as a class II tumor suppressor, a wild-type gene that exerts phenotypic effects through altered gene expression (Sager, 1997; Zhang *et al.*, 1998). Although we have not yet analyzed actual breast tumors for possible mutations, our finding that AZU-1 transcripts are effectively reexpressed in phenotypically reverted T4-2 cells indicates that these particular tumor cells have not incurred any gross genetic mutations that would inactivate the endogenous AZU-1 message. Interestingly, another previously identified class II tumor suppressor gene, maspin (a serine protease inhibitor or serpin) also is expressed in both luminal epithelial and myoepithelial cells (Zou *et al.*, 1994; Sager *et al.*, 1997). The multicellular expression patterns of both of these gene products underscore the potential role of myoepithelial cells themselves in regulating tumor progression.

Given the functions of many class II tumor supressors in cell adhesion and cell structure (Sager *et al.*, 1993; Sager, 1997; Alford and Taylor-Papadimitriou, 1996; Hirschi *et al.*, 1996; Weaver *et al.*, 1997; Mielnicki *et al.*, 1999), we were intrigued by the possibility that AZU-1 might also play a structural role in cells. We reasoned that such a finding would explain why high levels of AZU-1 expression not only inhibit tumor cell proliferation but also enable tissue reorganization. However, our immunolocalization studies suggest that AZU-1 is not tightly associated with cytoskeletal networks or the cell membrane. Rather, the majority of AZU-1 appears to reside in a "soluble" fraction of the cytoplasm. Two AZU-1-specific monoclonal antibodies also show prominent cytoplasmic staining in HMT-3522 cells (our unpublished results). Using the polyclonal antibody, a subpopulation of AZU-1 is present also in the nuclei of both nonmalignant and tumorigenic cells, a reasonable finding given the two putative NLSs encoded in AZU-1. Although the functional significance of the observed nuclear staining is still unclear, a potential centrosomal function was recently reported for the AZU-1-related *Drosophila* gene dTACC (Gergely *et al.*, 1999).

AZU-1-related Genes

AZU-1 shares overall sequence similarity with three genes called TACC1 (a putative oncogene cloned from the 8p11 breast cancer amplicon) (Still *et al.*, 1999a), TACC2, and TACC3 (Still *et al.*, 1999b). Comparison of AZU-1 and TACC2 sequences reveals that these two gene products, with the exception of two insertions and one amino acid substitution, are identical. Moreover, the AZU-1 gene maps to chromosome 10q26 (in collaboration with W.L. Kuo and

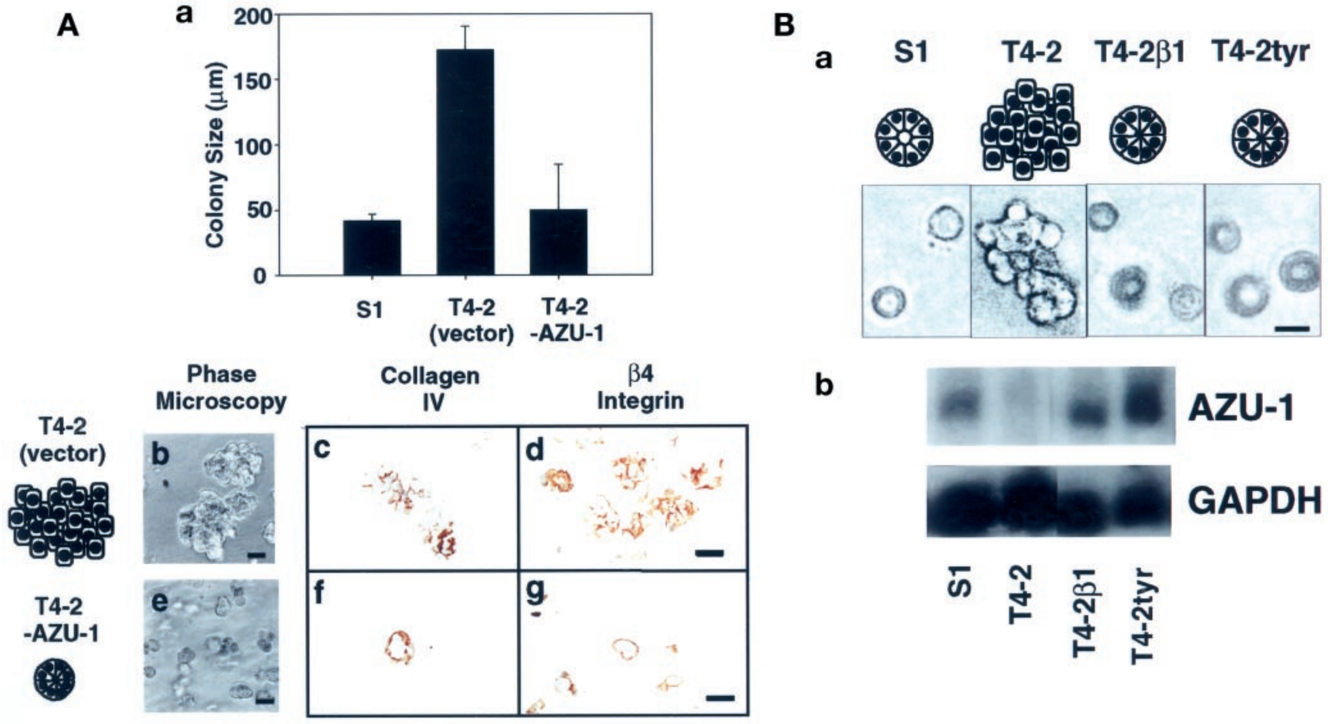

Figure 6. Increased AZU-1 expression levels correlate with phenotypic reversion in 3D rBM assays. (A) AZU-1 induces phenotypic reversion. S1, T4-2 (vector-infected), and T4-2-AZU-1 cells were embedded as single cells in 3D rBM assays. After 10 d in culture, the colonies were measured (expressed as colony diameter in micrometers ± SE) and imaged using phase microscopy (a, b, and e). Cultures were immunostained with antibodies specific for collagen IV (c and f) or β4 integrin (d and g). (B) AZU-1 is reexpressed upon EGFR- and β1 integrin-induced phenotypic reversion. (a) S1 and T4-2 cells were cultured in 3D rBM assays in the absence or presence of functional inhibitors of β1 integrin (T4-2β1) or EGFR (T4-2tyr; tyr, tyrphostin). Unlike control cells, inhibitor-treated T4-2 cells exhibit an S1-like, acinar phenotype in 3D cultures. (b) Total RNA harvested from these cultures was analyzed in Northern blots using an AZU-1-specific probe. GAPDH was used as a loading control. AZU-1 expression is restored to S1-like levels in T4-2 cells that have undergone phenotypic reversion in the 3D rBM assay. Bars, 50 μm.

J.W. Gray, unpublished results), a site analogous to the one reported for the TACC2 gene (Still *et al.*, 1999b). Whether the differences between AZU-1 and TACC2 sequences are due to differential splicing or to variations in cloning procedures is not clear. However, it is unlikely that the additional sequences found in TACC2 are required for the AZU-1 tumor suppressor function because the cDNAs used in our studies were sufficient to reduce the tumorigenic phenotype. Based on our results showing a tumor-suppressive, rather than a cell-transforming, effect on cells, we propose that the name AZU-1 be adopted as the preferred nomenclature for this gene. It is tempting to speculate that, similar to p53, the wild-type AZU-1 may function as a tumor suppressor but that its aberrant overexpression in normal cells may play a role in tumorigenicity.

A Potential Role for AZU-1 in Protein–Protein Interactions

Of the four predicted protein domains of AZU-1, two show structural conservation with previously characterized protein-binding motifs. The N terminus of the protein contains a protein element we refer to as a SPAZ domain. Two invariant serines, found in all four SPAZ domains identified to date, may be important kinase recognition sites and thus targets for regulation through phosphorylation. Fold recognition studies, using the GenTHREADER program (Jones, 1999), indicate that the SPAZ domain is likely to possess an Ig-like β-sandwich fold. Based on these sequence predictions and evidence demonstrating a role for Ig-like domains in protein binding (Givol and Yayon, 1992; Smith and Xue, 1997; Improta *et al.*, 1998), the SPAZ domain is possibly a new member of the Ig superfamily and as such may function as a protein-binding interface.

A coiled-coil domain (CCD) is predicted at the C terminus of AZU-1. CCDs form amphipathic helices that associate with other CCDs to form superhelical bundles of two to five protein subunits (Lupas, 1996, 1997). Our predictions indicate that the coiled-coil region of AZU-1 is best suited for the formation of dimers. Conceivably, this region may support the formation of AZU-1 homodimers or possibly heterodimers with similarly proportioned coiled-coil domains, such as those found in TACC1 or TACC3. Given that overexpression of TACC1 in normal cells results in cell transformation (Still *et al.*, 1999a), although reexpression of AZU-1 at endogenous levels in malignant cells suppresses tumor growth, it seems plausible that dimerization of these two molecules may be required for properly regulated cell growth and tissue morphogenesis.

The CCD of AZU-1 also shares notable similarity with the human gene SB1.8 (DXS423E), a human homologue of the SMC1 protein of *S. cerevisiae* (Rocques *et al*., 1995). SMC1 belongs to a family of myosin-like genes, called cohesins, that regulate chromosome segregation during mitosis; mutations in SMC1 give rise to chromosomal nondisjunction or total chromosome loss, both of which could contribute to genome instability and perhaps tumor progression (Michaelis *et al*., 1997). Although it is still unclear whether AZU-1 functions cooperatively with the SB1.8 gene product in HMT-3522 cells, mutations in D-TACC cause defects in chromosomal segregation during mitosis in *Drosophila* embryos (Gergely *et al*., 2000).

Coupling AZU-1 Expression with β1 Integrin and EGFR Activities

We have demonstrated that inhibition of either β1 integrin or EGFR function was sufficient to promote phenotypic reversion of T4-2 cells in 3D rBM assays (Weaver *et al*., 1997; Wang *et al*., 1998). Regardless of the inhibitory agent used, phenotypic reversion was accompanied by down-regulation of both β1 integrin and EGFR proteins to levels observed in nonmalignant cells. Evidence presented here suggests that AZU-1 mRNA is also coordinately regulated by β1 integrin and EGFR function (as observed with inhibitor-treated cells in 3D rBM assays). The fact that AZU-1 was not up-regulated in T4-2 cell monolayers treated with inhibitors suggests that the coordinate modulation is dependent on the formation of tissue-like structures in the 3D rBM assays (Wang *et al*., 1998). Given that overexpression of AZU-1 is also sufficient to cause phenotypic reversion of T4-2 cells, it is possible that AZU-1 engages in an integrated cross-talk with the cell surface receptors β1 integrin and EGFR. Thus, the tumorigenic conversion of the HMT-3522 cells would require the collective disruption of all of these coordinately regulated elements. As such, AZU-1 may be an important regulator of breast unit structure and function.

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