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Identification and Partial Characterization of a Novel Membrane-Associated Protein (MAP17) Up-Regulated in Human Carcinomas and Modulating Cell Replication and Tumor Growth

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Using the differential display technique, we have recently reported the identification of a novel gene originally designated DD96. As determined by Northern blot and in situ hybridization, DD96 was expressed at significant levels only in a single epithelial cell population, the proximal tubular epithelial cells of the kidney. However, it was diffusely expressed in various carcinomas originating from kidney, colon, lung, and breast. Using a specific polyclonal antibody, we have not determined that the DD96 protein product is a 17-kd membrane-associated protein, which we have therefore redesignated MAP17. In normal tissues, MAP17 is expressed in significant amounts only in the kidney, where it was localized to the brush border of proximal tubular epithelial cells. However, MAP17 is expressed abundantly in carcinomas arising from kidney, colon, lung, and breast, in some cases with a membrane-associated apical glandular distribution. In tissue culture, MAP17 was localized to the cell membrane in areas of cell-cell contact, ie, the distribution of cell-junction-associated proteins. Transfection of a full-length wild-type DD96 cDNA clone into a colon carcinoma cell line, HT-29, markedly decreased cell proliferation in vitro and tumor growth in vivo. Although the precise function of MAP17 remains to be determined, our findings suggest that this protein may play an important role in tumor biology. (Am J Pathol 1996, 149:493–500)

Many of the biological events involved in the development of neoplasia are still unknown or poorly understood. Therefore, it is increasingly important to identify new proteins that may be involved in oncogenesis.[1–3] A number of differential screening techniques have been developed in recent years in order to identify genes selectively modulated in carcinogenesis.[4–6] Using the differential display technique,[7–10] we have recently reported the identification of a novel, epithelial-specific gene, originally designated DD96, which was markedly up-regulated in a variety of human carcinomas as compared with normal epithelial cell populations.[11]

To study the distribution of the DD96 protein product, we raised a polyclonal antibody against the carboxyl-terminal peptide of the predicted protein sequence derived from a full-length cDNA clone. This antibody allowed us to characterize the DD96 protein product as a 17-kd membrane-associated protein, which we have now renamed MAP17. As predicted by earlier *in situ* hybridization studies, MAP17 is expressed at significant levels only in the proximal tubular epithelial cells of the normal kidney, where it is associated with the apical brush border. In addition, MAP17 is overexpressed in a variety of human carcinoma cells originating from kidney, colon, lung, and breast, compared with normal epithelial cell populations. To elucidate the function of DD96 and its protein product MAP17, we performed transfection experiments with a wild-type full-length DD96 cDNA clone isolated from a normal human kidney library transfected into the colon carcinoma HT-29 cell line. The results of these experiments suggest that DD96/MAP17 down-regulates cell replication *in vitro* and tumor growth *in vivo*.

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Materials and Methods

Generation of a Specific Antibody against the DD96 Gene Product and Western Blot Analysis

All studies were conducted with the approval of the Beth Israel Hospital Committee on Clinical Investigation. A polyclonal antibody was prepared against the 18-mer carboxyl-terminal peptide of the predicted protein sequence of DD96 (NAYENVPEEE-GKVRSTPM; Immuno-Dynamics, La Jolla, CA). A cysteine was added to the sequence at its amino terminus to conjugate the peptide to keyhole limpet hemocyanin. Rabbits were immunized, and the antibody was purified by affinity chromatography using the same peptide. For Western blot analysis, protein extracts were electrophoresed on sodium dodecyl sulfate polyacrylamide gel, transferred to Immobilon P (Millipore, Bedford, MA), and incubated with either the primary antibody against DD96 or normal rabbit IgG as a control at a concentration of 10 μg/ml and subsequently with an anti-rabbit IgG conjugated to horseradish peroxidase at a dilution of 1/500 (GIBCO BRL, Gaithersburg, MD). These incubations were followed by a diaminobenzidine reaction (Sigma Chemical Co., St. Louis, MO).

Immunoperoxidase and Immunofluorescence Studies

Tissues were collected in the operating room and fixed for 4 hours in 4% paraformaldehyde in phosphate-buffered saline, pH 7.4, at 4°C and then transferred to 30% sucrose in phosphate-buffered saline, pH 7.4, overnight at 4°C. Tissues were then frozen in OCT compound (Miles Diagnostics, Elkhart, IN) and stored in liquid nitrogen. A total of 28 tumors originating from kidney (4 carcinomas), colon (5 adenomas and carcinomas), lung (6 carcinomas), and breast (13 carcinomas) were examined. Immunoperoxidase studies were performed on 6-μm fixed-frozen tissue sections using either the affinity-purified primary antibody against the DD96 carboxyl-terminal protein sequence or rabbit IgG as a control at a concentration of 5 μg/ml. The sections were then incubated with a biotinylated anti-rabbit IgG using a 1/200 dilution (Vector Laboratories, Burlingame, CA) and subsequently treated with the Vectastain ABC reagents (Vector) and diaminobenzidine (Research Genetics, Huntsville, AL), according to the manufacturer's protocol.

For immunofluorescence studies, HT-29 and 184B5 cell lines were obtained from the American Type Culture Collection (Rockville, MD). Cultured cells were grown on gelatin-coated Nunc chamber glass slides (Nunc, Naperville, IL) in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% fetal calf serum (GIBCO BRL). After fixation, either with 4% paraformaldehyde for 20 minutes at 4°C followed by 0.5% Triton X-100 permeabilization for 15 minutes at room temperature or with methanol for 5 minutes at −20°C, cells were incubated either with the primary antibody or with normal rabbit IgG at a concentration of 25 μg/ml and then with a fluorescein-isothiocyanate-conjugated anti-rabbit IgG at a dilution of 1/40 (Kirkegaard & Perry Laboratories, Gaithersburg, MD).

Northern Blot Hybridization

For RNA extraction, cells were scraped from petri dishes using a rubber policeman and homogenized in an extraction buffer containing 4.5 mol/L guanidine thiocyanate, 50 mmol/L EDTA, 25 mmol/L sodium citrate, pH 7.0, 0.1 mol/L 2-β-mercaptoethanol, and 2% sodium-*N*-sarcosine using a syringe equipped with a 25-gauge needle. RNA was purified by ultracentrifugation through a cushion of 5.7 mol/L CsCl as previously described.[12] For Northern blot hybridization, RNAs (20 μg per lane) was denatured with formaldehyde and electrophoresed in 1% agarose formaldehyde gels containing 0.5 μg/ml ethidium bromide, examined under ultraviolet light, and transferred to Biotrans filters (ICN, Costa Mesa, CA). After blotting, filters were ultraviolet cross-linked using a Stratalinker (Stratagene, La Jolla, CA). Northern blots were hybridized with the DD96 cDNA probe isolated from a human kidney library labeled with [^{32}P]dCTP (New England Nuclear, Boston, MA) using a random primer labeling kit from Amersham (Arlington Heights, IL).

Transfection Studies

The 930-bp DD96 cDNA clone containing the complete amino acid sequence was subcloned into the *Eco*RI site of the pcDNA3 expression vector (Invitrogen, San Diego, CA). The orientation of the DD96 cDNA clone was verified by restriction enzyme digestion and DNA sequence analysis.[13]

The HT29 colon carcinoma cell line was used for transfection experiments. A total of 10^6 cells were plated in 60-mm tissue culture dishes and transfected using lipofectin (GIBCO BRL) with 5 μg of pCDNA3 plasmid containing either no insert or the DD96 clone in the sense orientation. Selection of G418-resistant cells was begun 48 hours after trans-

fection with 900 μg/ml G418 (GIBCO BRL). Transfected HT-29 cells were cloned using glass cloning rings (Bellco, Vineland, NJ), and the level of expression of the transfected gene was determined by Northern hybridization. HT-29 clones expressing high levels of the transfected gene as well as HT-29 control clones transfected with pcDNA3 were selected for additional experiments.

Proliferation Assays and in Vivo *Tumor Growth Assays*

For cell proliferation assays, cloned HT-29 cells transfected with either control pcDNA3 (three clones) or pcDNA3 containing the DD96 cDNA insert (four clones) were plated in 24-well dishes at a concentration of 1.7×10^4 cells/ml in DMEM supplemented with 10% fetal calf serum. Experiments were performed in triplicate. Cells were counted at 1, 3, 5, 7, and 9 days after plating and growth curves were generated.

To assay for tumorigenicity, cloned HT-29-transfected cells were resuspended in serum-free DMEM and 2×10^6 cells were injected subcutaneously (two separate sites in the flanks of 6-week-old athymic Ncr nude mice (Taconic, Germantown, NY). Three separate control pcDNA3-transfected HT-29 clones and four DD96-pcDNA3-transfected HT-29 clones were evaluated. Four individual tumors were generated from each different clone, totaling twelve control and sixteen DD96-transfected tumors. Tumors were harvested 3 weeks after injection, weighed, and processed for histology.

Results

Generation of a Specific Antibody against the DD96 Protein Sequence

Hydropathic analysis of the predicted protein sequence derived from the DD96 cDNA clone revealed that the amino terminus of the protein was markedly hydrophobic, containing a signal peptide and a transmembrane domain, whereas the carboxy terminus was hydrophilic.[11] Therefore, an 18-mer peptide representing the carboxyl-terminal protein sequence was used to generate a polyclonal antibody that was affinity purified.

Western blotting was used to determine the molecular weight of the DD96 protein product. We previously reported that normal kidney was the only normal tissue that expressed significant amounts of DD96 mRNA. Therefore, we used extracts of normal kidney as a source of antigen. A single band corresponding to 17 kd was detected on Western blots prepared under both nonreducing and reducing conditions (Figure 1). Because of its molecular weight and its likely association with the cell membrane, we have designated the DD96 gene produce membrane-associated protein-17 (MAP17).

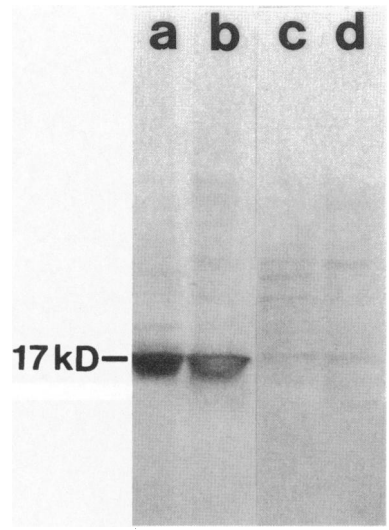

Figure 1. *Western blot analysis of reduced* (**a** *and* **c**) *and nonreduced* (**b** *and* **d**) *normal human kidney protein extracts incubated with the affinity-purified antibody against MAP17* (**a** *and* **b**) *or rabbit IgG* (**c** *and* **d**). *A single band of 17 kd is observed in both reduced and nonreduced samples incubated with the MAP17 antibody.*

Distribution of MAP17 in Normal Human Tissues and Carcinomas

Using the MAP17 affinity-purified antibody, we performed immunohistochemistry on human kidney, colon, lung, and breast tissue samples as well as carcinomas arising from these organs (Figure 2). In normal tissue samples, no or minimal staining was detected using the MAP17 antibody, except for normal kidney where the antibody strongly labeled the brush border of the proximal tubular epithelium (Figure 2A). Renal cell carcinomas showed positive staining of variable intensity in three of four of the cases studied (Figure 2B).

Both adenomas and adenocarcinomas arising in the colon (Figure 2, G–I) stained for MAP17 in contrast to normal colonic mucosa (Figure 2F). Interesting, staining was predominantly associated with the apical membrane of glandular structures in both adenomas and adenocarcinomas.

Strong staining was also observed in 3 of 6 pulmonary adenocarcinomas and in 9 of 13 infiltrating breast carcinomas, compared with corresponding

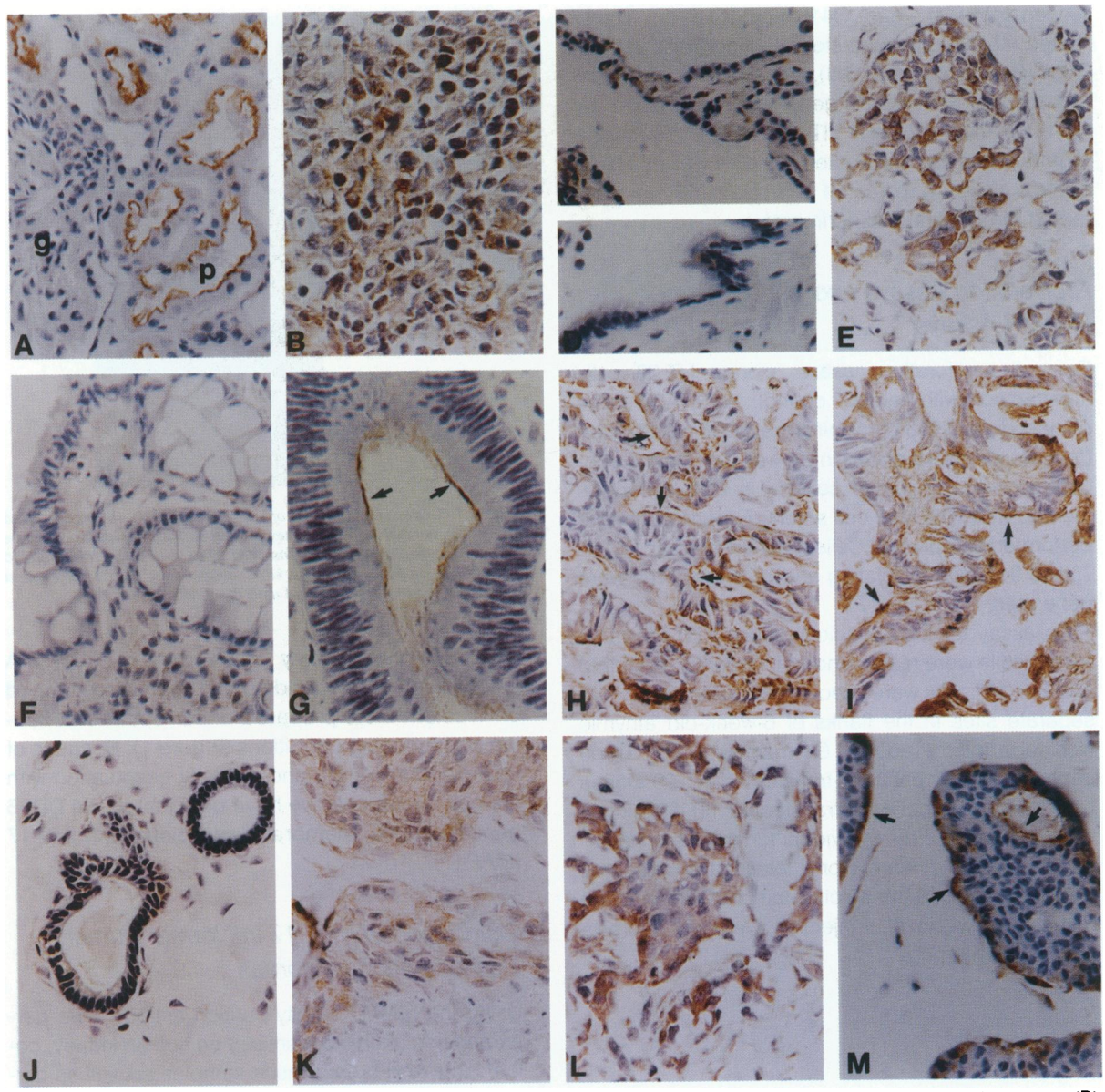

Figure 2. *Immunoperoxidase staining of normal renal cortex* (A), *renal cell carcinoma* (B), *normal lung* (C), *normal bronchia mucosa* (D), *lung carcinoma* (E), *normal colonic mucosa* (F), *colonic adenoma* (G), *colonic adenocarcinoma* (H *and* I), *normal breast* (J), *two breast ducts involved with ductal carcinoma* in situ, *one of them with comedo necrosis* (K), *infiltrating ductal carcinomas* (L), *and mucinous carcinoma* (M) *of the breast using the MAP17 antibody. In normal kidney* (A), *the staining is limited to the brush border of proximal epithelial cells* (*p*) *whereas glomeruli* (*g*) *are not labeled. The renal cell carcinoma shows strong staining* (B). *The intensity of staining is markedly increased in lung carcinoma* (E), *colonic adenoma and carcinomas* (G *to* I), *and breast* in situ *and infiltrating carcinomas* (K *to* M), *compared with normal tissue samples* (C, D, F *and* J). *Note the apical glandular staining pattern in colonic tumors* (G *to* I) *and in the breast mucinous carcinoma* (M) (arrows). *Magnification,* ×*200.*

normal tissue samples (Figure 2, C–E and J–M). A breast carcinoma of the mucinous type showed a glandular staining pattern associated with the apical aspect of tumor cells, very similar to that of colonic adenomas and adenocarcinomas (Figure 2M). In addition, preinvasive ductal carcinoma *in situ* arising in breast showed increased staining compared with normal breast tissue (Figure 2K).

Cellular Localization of the DD96 Gene Product MAP17 in Cultured Cell Lines

To determine the cellular localization of the MAP17 in cultured cell lines, we selected two cell lines known to express DD96 mRNA,[11] namely, HT-29, a colon carcinoma cell line, and 184B5, an immortalized breast epithelial cell line.[14] Immunofluorescence

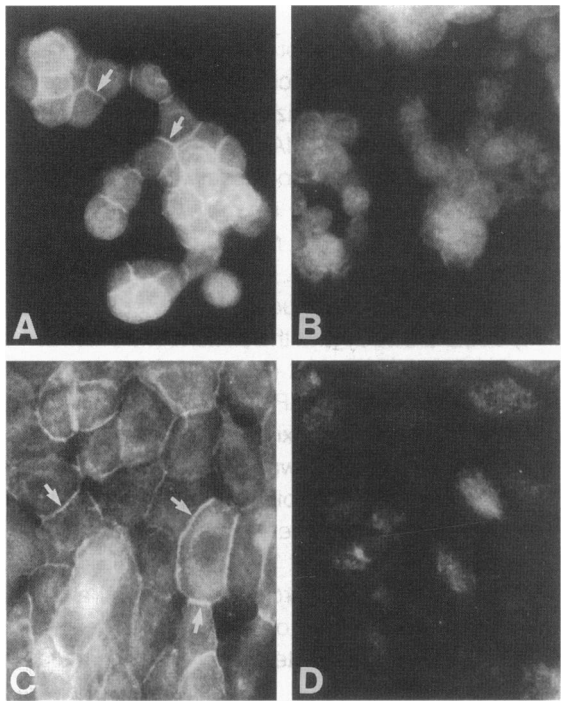

Figure 3. *Immunofluorescence staining of cultured HT-29* (**A** *and* **B**) *and 184B5 cells* (**C** *and* **D**) *using the MAP17 antibody* (**A** *and* **C**) *or control rabbit IgG* (**B** *and* **D**). *In both HT-29* (**A**) *and 184B5* (**C**) *cells, the staining is associated with the cell-cell contact areas of the cytoplasmic membrane* (**arrows**). *Magnification,* ×*350.*

staining was confined to cell membranes in areas of cell-cell contacts (Figure 3). Staining was observed only in permeabilized cells, suggesting that MAP17 is associated with the cytoplasmic aspect of the cell membrane. This pattern of staining was not observed in cell populations that did not express DD96 mRNA (data not shown).

Transfection Experiments

To obtain clues as to MAP17 function, we transfected a full-length wild-type DD96 cDNA previously isolated from a human kidney library into the colon carcinoma cell line HT-29. The purpose of these experiments was to overexpress DD96 in the HT-29 cell line, which constitutively expresses only low levels of DD96 mRNA. All experiments were performed on cloned cell populations, either transfected with DD96 subcloned into the pcDNA3 vector or transfected with the pcDNA3 vector alone as a control.

RNA was extracted from cloned transfected cells to determine the level of DD96 mRNA expression. HT-29 cells transfected with pcDNA3 expressed the 1.0-kb DD96 mRNA as previously described.[11] HT-29 cells transfected with the DD96-pcDNA3 construct expressed, in addition to the 1.0-kb DD96 mRNA, a larger 1.4-kb mRNA corresponding to the transfected DD96 gene product (Figure 4). The difference in size is due to the addition of polyadenylation sequences to the transcripts promoted by the presence of bovine growth hormone polyadenylation signal sequences in the pcDNA3 vector. Three control and four DD96-transfected HT-29 clones were selected for *in vitro* proliferation studies.

Proliferation studies were carried out over a 9-day period. The number of cells were counted 1, 3, 5, 7, and 9 days after seeding. Cell numbers obtained from control pcDNA3-transfected HT-29 or DD96-pcDNA3-transfected HT-29 cells were averaged and growth curves were generated (Figure 5). DD96-pcDNA3-transfected HT-29 cells divided significantly more slowly than control cells; after 9 days, the number of DD96-pcDNA3-transfected HT-29 cells was only 47% of the control pcDNA3-transfected HT-29 cell number ($P < 0.01$).

Tumor Growth in Nude Mice

Three control pcDNA3-transfected and four DD96-pcDNA3-transfected HT-29 clones were injected subcutaneously into athymic Ncr nude mice. Tumors were allowed to grow for 3 weeks and were then recovered and weighed. DD96-pcDNA3-transfected HT-29 tumors were significantly smaller (69 ± 9 mg) than those produced by control pcDNA3-transfected HT-29 (155 ± 36 mg; $P < 0.05$). The histologies of control and DD96-transfected tumors were not significantly different, both appearing as poorly differentiated adenocarcinomas.

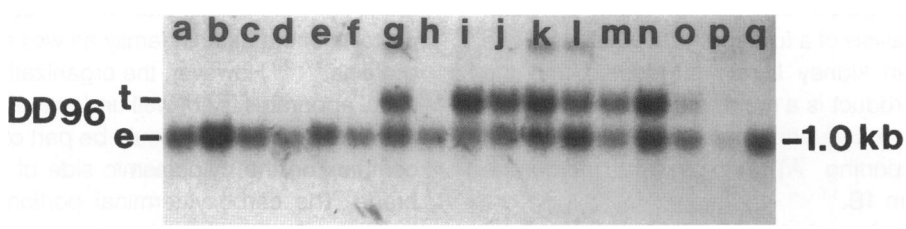

Figure 4. *Northern blot hybridization of total RNA from cloned cultured HT-29 cells transfected with either control pcDNA3* (**a** *to* **f**) *or DD96-pcDNA3* (**g** *to* **q**). *The 1.0-kb hybridization band* (**e**) *corresponds to the endogenous DD96 mRNA, whereas the 1.4-kb band* (**t**) *corresponds to the transfected mRNA. Control clones* **a**, **b**, *and* **c** *and DD96-transfected clones* **i**, **j**, **m**, *and* **n** *were selected for additional experiments.*

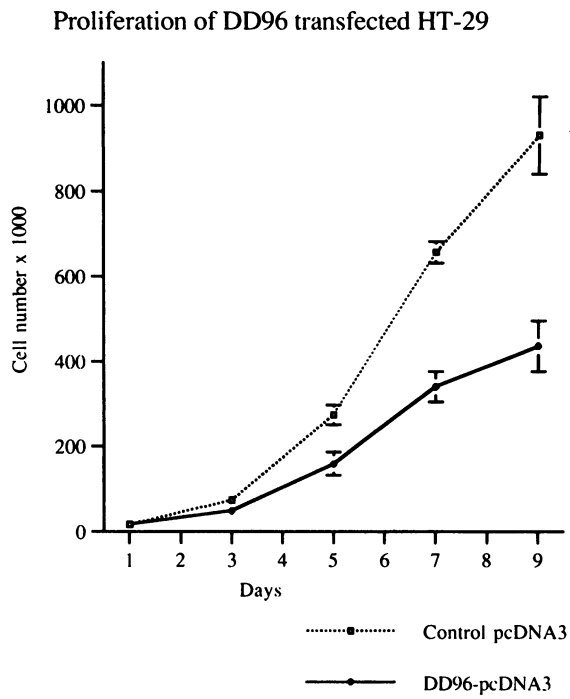

Figure 5. *Growth curves generated from proliferation studies comparing control pcDNA3- and sense DD96-pcDNA3-transfected HT29 cells. Over a 9-day period, cell growth of DD96-pcDNA3-transfected cells was only 47% of that of control pcDNA3-transfected cells.*

Discussion

Malignant transformation is a multistep process that is far from being completely understood. Therefore, it is increasingly important to identify new genes and proteins that may play a role in this process. We recently identified a novel gene, designated DD96, which we originally isolated from a human renal cell carcinoma using the differential display technique. As demonstrated by *in situ* hybridization and Northern blot analysis, DD96 was expressed in normal adult tissues at significant levels only in proximal tubular epithelial cells of the kidney. Very low levels of expression were also observed in a few other epithelial tissues such as pancreas and stomach. DD96 mRNA was not detected in cells of mesenchymal origin. The expression of DD96 was up-regulated in a variety of carcinomas and premalignant lesions as compared with normal tissue samples. Sequence analysis of a full-length DD96 cDNA clone from a human kidney library suggested that the DD96 gene product is a membrane-associated protein with partial sequence homology to the human calcium-transporting ATPase plasma membrane protein isoform 1B.[11]

To begin to characterize the DD96 gene product, we prepared a polyclonal antibody against the carboxyl-terminal peptide corresponding to the predicted protein sequence derived from a full-length cDNA clone. Western blot analysis revealed that the antibody reacted specifically with a single band of approximately 17 kd, a size consistent with that predicted by the DD96 cDNA protein sequence.[11] Because of its putative association with the cell membrane (see below) and its molecular weight, the DD96 gene product was named membrane-associated protein 17 (MAP17).

Immunoperoxidase studies confirmed the specificity of the antibody and the pattern of distribution of DD96 previously studied by *in situ* hybridization. In normal adult tissues, MAP17 was expressed at significant levels only in proximal tubular epithelial cells of the kidney, where it was localized to the apical brush border. A similar apical membrane-associated distribution was also observed in the glandular cells of a number of different tumors, including colonic adenomas and adenocarcinomas, as well as mucinous adenocarcinomas of the breast but was not seen in poorly differentiated carcinomas that did not form glandular structures. The apical pattern of staining distribution in adenocarcinomas is similar to that previously reported for other cancer-associated antigens including carcinoembryonic antigen and the tumor-associated glycoprotein DF3.[15,16] In control experiments, the immunoperoxidase staining was completely abrogated using the 18-mer peptide against which the MAP17 antibody was generated, confirming the specificity of the antibody.

In cultured cell lines, MAP17 showed a somewhat different pattern of membrane staining, being associated with cell-cell membrane contact areas, ie, in a pattern very similar to that of junction-associated proteins such as cadherins. However, this staining pattern could be demonstrated only when cultured cells were permeabilized, indicating that MAP17 is an intracellular protein that is associated with the cytoplasmic aspect of the plasma membrane.

It is interesting that, similarly to MAP17, cadherins, depending on the cell population, display either a polar distribution in gland-forming epithelia or a membrane-associated circumferential distribution in other types of epithelia.[17] On the cytoplasmic aspect of the cell membrane, cadherins associated with the proteins of the catenin family as well as cytoskeletal proteins.[17–20] However, the organization of the junctional apparatus is not well understood, and one can speculate that MAP17 could be part of the junctional complex on the cytoplasmic side of the cell membrane. The carboxyl-terminal portion of cadherins interact with intracytoplasmic proteins. When the carboxy terminus is truncated, the cell adhesion properties of cadherins are lost.[21] Several studies

have suggested that disturbance of protein interaction at the level of cell junctions may occur during malignant transformation and result in decreased cell adhesion.[21]

Overexpression of the wild-type DD96 cDNA clone in the HT-29 colon cancer cell line led to a marked reduction of cell proliferation *in vitro* and reduced tumor growth *in vivo*, without significantly changing the morphology of cultured cells or tumor histology. The mechanisms by which DD96 transfection decreased cell proliferation have not yet been defined. Possibilities include altered molecular transport or modulation of cell-cell adhesion, as suggested by the tissue and cellular distribution of MAP17. It is well recognized that proper organization of the cell-cell adhesion system is essential not only for the spatial arrangement of epithelial cells but also for the control of their growth.[22] It may appear paradoxical that a gene selectively overexpressed in cancer decreases cell proliferation as a result of transfection. However, in many cases, cancer cells do not possess a growth advantage over normal cell populations.[23] This is particularly true for colonic carcinoma cells, which tend to divide more slowly than normal colonic mucosa epithelial cells.[23] Indeed, the percentage of cells in S phase varies between 3 and 15% in a wide variety of solid tumors. This is lower than normal epithelia with high cellular turnover, such as in the intestine, where the percentage of cells in S phase is approximately 16%.[24] In addition, studies have demonstrated that there is no significant difference between the growth of normal and cancerous breast tissue.[25] Tumor growth results from an imbalance between cell proliferation and cell death, cell production being greater than cell loss in tumors, whereas both are equal in adult normal tissues.[24] One could also speculate that overexpression of MAP17 is an attempt of cells to counteract the transformation process during the course of oncogenesis. The transfection experiments reported here were performed using a wild-type DD96 gene isolated from a normal kidney cDNA library, and we have yet to determine whether similar results would be obtained using a tumor-derived DD96 cDNA. Comparable results have previously been described for members of the cadherin and cyclin families, where a reduction of cell proliferation was observed after transfection of the wild-type gene into tumor cells.[22,26]

Several questions remain to be answered to define the role of MAP17 in oncogenesis. The identification of molecules associated with MAP17 in the cytoplasmic membrane could be crucial in determining its function in normal tissues such as the proximal tubule of the kidney, the mechanisms by which it modulates cell replication in tumor cells, and its role in cancer biology.

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