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Characterization of human N8 protein

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We have shown before that the N8 mRNA is expressed at higher levels in lung tumor and lung tumor-derived cell lines than normal lung cells. In this paper, we have characterized the N8 protein, and studied its properties. The N8 gene encodes a major 24 kDa protein and its expression correlates well with the N8 mRNA expression pattern observed in different cell lines. N8 protein is capable of forming a homodimer or multimeter *in vitro*. It is a phosphorylated cytoplasmic protein and phosphorylation occurs mainly at serine residues. N8 protein is expressed at higher levels in epithelial cells than in mesenchymal cells. N8 protein expression is induced in a fibroblast cell line expressing adenoviral E1a protein, which acquired epithelial-like characteristics. Furthermore, ectopic expression of N8 protein in NIH3T3 cells converts them into a spheroid form. These spheroids also have some of the characteristic features of epithelial cells. Taken together, these results suggest that the N8 protein may be associated with the development or maintenance of epithelial cell phenotype.

Keywords: leucine zipper protein; homodimerization; phosphorylation; cytoplasmic protein; epithelial cells

Introduction

In a quest to identify genes that are differentially expressed between normal and tumor cells, we and others have isolated a novel gene, N8 (also known as D52), which is expressed in multiple cancers (Chen *et al*., 1996; Byrne *et al*., 1995). We have shown that the N8 mRNA is expressed at high levels in tumor-derived cell lines from lung, breast, colon, pancreas, prostate and kidney cancers. However, the N8 gene is also expressed at high levels in normal human adult brain, kidney, prostate, pancreas and intestine and at low levels in lung, liver, hematopoietic cells and gonads. The expression pattern of N8 mRNA is qualitatively similar in normal adult mice and humans. During murine embryonic development, N8 mRNA is expressed in the epithelium of the intestine, stomach, olfactory epithelium, neuronal layers of retina, kidney and salivary gland (Chen *et al*., 1996). The N8 gene is differentially expressed in fetal and adult human tissues, as well in murine embryos, newborns and adults, indicating that its expression may be regulated during development.

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We have shown previously that the N8 gene is capable of coding multiple mRNAs. However, very little is known about N8 proteins. In this paper, we describe the characterization of the N8 protein and studied its properties. For this purpose, we have expressed N8 protein as a His-tagged fusion protein. Purified recombinant soluble N8 protein was used to raise antisera. Affinity-purified antisera was used to characterize cellular N8 protein. Our results demonstrate that the antisera works well in immunoprecipitation, Western blot and immunohistochemical assays. Furthermore, we show that a major 24 kDa protein is specifically recognized by the N8 antisera and its expression pattern co-relates well with the N8 mRNA expression in different cell types. Expression of N8 open reading frame containing cDNA in NIH3T3 cells also generates a 24 kDa protein. Consistent with the high mRNA expression in tumor tissues and cell lines, N8 protein is also expressed at high levels in lung tumor tissues, and in cell lines derived from lung, breast, colon, kidney and pancreatic tumors as well as B, T and myeloid leukemic cells. N8 protein is capable of forming homodimers or multimeters *in vitro*. It is a phosphorylated cytoplasmic protein and phosphorylation occurs mainly at serine residues.

N8 protein is also expressed at a higher level in epithelial cell lines than mesenchymal-derived cells. Immunohistochemical analysis of N8 protein in the colon indicated that the N8 protein is expressed at higher levels in epithelial cells than in surrounding lamina propria, mucosal and sub-mucosal layers of mesenchymal origin. Fibroblasts infected with retrovirus containing the 12S form of adenovirus-5 E1a acquired epithelial-like characteristics and also expressed elevated amounts of N8 protein. Furthermore, ectopic expression of N8 protein in NIH3T3 cells converts these cells into a spheroid form which has been shown to have some of the characteristic features of epithelial cells. Taken together, these results show that the N8 gene encodes a major 24 kDa protein and may be associated with the development or maintenance of epithelial cells.

Results

Preparation and characterization of N8 antisera

We and others have shown before that N8 mRNA is expressed in carcinoma cell lines derived from multiple tumors (Chen *et al*., 1996; Byrne *et al*., 1995;). In order to identify cellular N8 proteins, we have expressed human N8 cDNA in *E. coli* as a His-tagged protein

under the control of T7 promoter and lac operator, which can be induced by the addition of IPTG (Dubendorff and Studier, 1991). As shown in Figure 1, a 32 kDa protein is induced only after IPTG addition (lane 2) and its apparent molecular size (32 kDa) is in agreement with the calculated size of His-tagged N8 protein. As expected, 32 kDa protein binds to His-bind resin and eluted at higher concentrations of imidazole (Figure 1, lane 3). The 32 kDa protein is soluble (in agreement with its hydrophilic nature) and N-terminal amino acid sequence analysis (data not shown) further confirmed that it contains protein species encoded by human N8 cDNA.

Proteins eluted from His-bind resin column were used to raise antisera in rabbits (Figure 1, lane 3). Since the antigen used contained minor contaminating proteins, antisera was further purified by affinity chromatography using SDS–PAGE purified 32 kDa N8 recombinant protein as described in Materials and methods (Figure 1, lane 4). The purified antisera was used to examine if it can recognize N8 protein by radioimmunoprecipitation and Western blot analyses. N8 protein was synthesized in a rabbit reticulocyte *in vitro* and translation products (TP) were immunoprecipitated using purified N8 antisera. Results shown in Figure 2 demonstrates that the 24 kDa N8 protein was immunoprecipitated from TP by N8 antisera but not by preimmune sera (Figure 2a, lanes 2 and 3). Furthermore, N8 protein immunoprecipitation was blocked by an excess of purified 32 kDa His-tagged N8 protein (Figure 2a, lane 3 *versus* 4 and 5). In addition, N8 antisera did not recognize ETS-1, Jun, Fos, Max and Myc proteins made in reticulocyte lysate *in vitro* (data not shown). These results demonstrate that the N8 antisera specifically recognizes the N8 protein.

Identification of cellular N8 protein

To identify cellular N8 protein, cell extracts were prepared from cell lines expressing either very low (CCD8Lu) or high levels of N8 mRNA (NCI-H209) and N8 protein was detected by Western blot analysis. A 24 kDa protein was recognized by N8 antisera in NCI-H209 extracts expressing high levels of N8 mRNA but not in CCD8Lu cells expressing very low levels of N8 mRNA (Figure 2b, lanes 1 and 2). The elevated levels of 24 kDa protein in NCI-H209 is not due to different amounts of cell extracts used, because similar levels of actin proteins were observed in both CCD8Lu and H209 cell extracts (Figure 2b, lanes 5–8). Furthermore, the 24 kDa protein has identical SDS–PAGE mobility as N8 protein made in the reticulocyte lysate. The ability of the antiserum to recognize the 24 kDa protein on Western blots was effectively blocked by an excess of purified 32 kDa His-tagged N8 protein (Figure 2b, compare lanes 2 and 4). However, the 32 kDa N8 protein did not block binding of actin antisera to actin (Figure 2b, lanes 5–8),

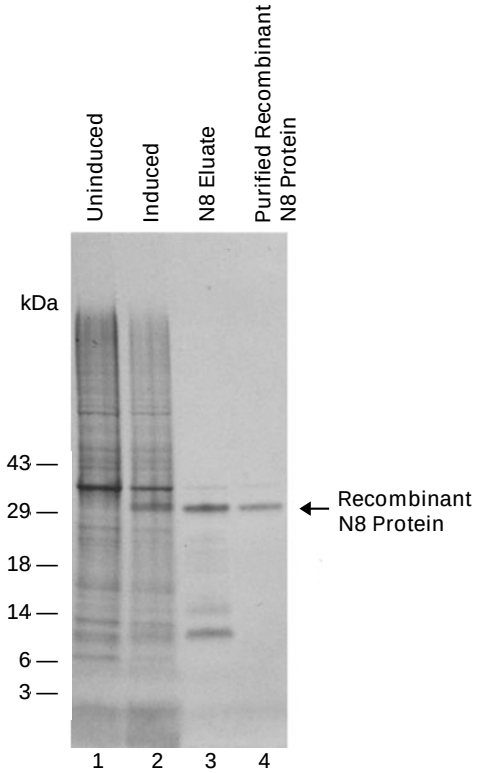

Figure 1 The preparation and purification of recombinant N8 protein. His-tagged N8 proteins were purified as described in Materials and methods. Proteins were electrophoretically separated in 10–20% gradient tricine gel and stained with Coomassie Blue. Lane 1, cell extract of uninduced *E. coli* host bacteria. Lane 2, cell extracts of induced *E. coli* host bacteria with 1 mM IPTG for 3 h. Lane 3, recombinant N8 protein eluted from His-bind resin column. Lane 4, purified recombinant N8 protein isolated from SDS–PAGE

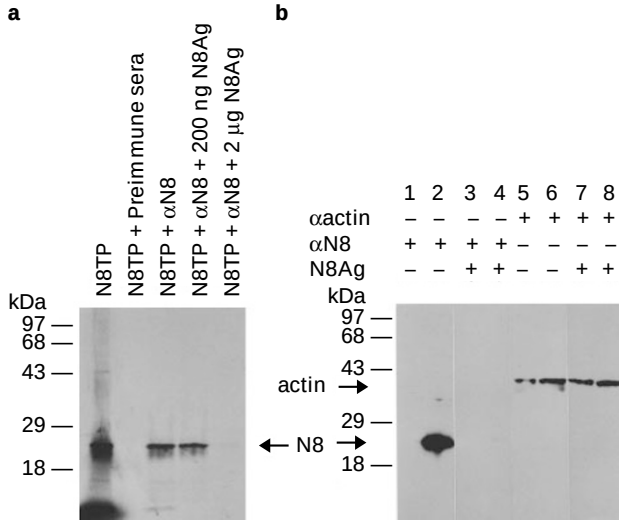

Figure 2 N8 antisera recognizes a 24 kDa protein. (**a**) N8 total translation products (TP) were immunoprecipitated using rabbit preimmune sera or N8 antisera. On lanes 4 and 5, 200 ng and 2 μg of SDS–PAGE purified N8 protein was mixed with N8 antisera for 5 min before immunoprecipitation. Immunoprecipitated products were separated on 12% tris-glycine gel and visualized by fluorography as described in Materials and methods. (**b**) Western blot analysis: Cell extracts isolated from CCD-8Lu (lanes 1, 3, 5, 7) and NCI-H209 (lanes 2, 4, 6, 8) were separated on 12% tris-glycine gel and transferred to nitrocellulose membrane and processed for Western blot analysis as described in Materials and methods. αactin, actin antisera. αN8, N8 antisera. N8 Ag, N8 antigen. Actin and N8 antisera were used at 1 : 5000 dilution. Sheep anti-mouse Ig and donkey anti-rabbit Ig antibody conjugated with horseradish peroxidase were used at 1 : 2500 as second antibodies for αactin and αN8, respectively

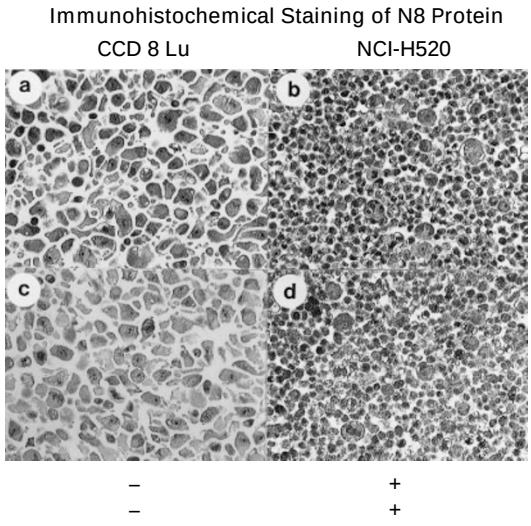

Figure 3 Immunohistochemical staining of N8 protein. Cells were stained with N8 antisera at 1 : 500 dilution using VECTASTAIN ABC kit. (**a** and **c**) CCD-8Lu cells; (**b** and **d**) NCI-H209 cells; (**a** and **b**) were stained with H & E (**c** and **d**) were stained with αN8

indicating that the neutralizing activity of recombinant N8 protein is specific and not caused by impurities or denaturants present in the antigen preparation. We have also used large panel of cell lines expressing either high or very low levels of N8 mRNA and the levels of 24 kDa protein correlated well with the levels of N8 mRNA found in these cell lines (Figure 8a). Taken together, these results suggest that the 24 kDa protein is encoded by the N8 gene.

To find out if N8 antisera recognizes the protein *in situ*, we performed immunohistochemical studies of CCD8Lu and NCI-H520 cells using N8 antisera. Results shown in Figure 3 demonstrate that a high level of staining was observed in the NCI-H520 cell line (Figure 3d). However, N8 antisera did not stain CCD8Lu cells. These results are consistent with the high levels of N8 mRNA and proteins made in NCI-H520, but not in CCD8Lu cell lines. These results further support the notion that the 24 kDa protein is the major translation product of the N8 gene and demonstrate that the antisera is specific, and can recognize N8 proteins in immunoprecipitation, Western blot and immunohistochemical analyses.

N8 protein expression in normal and tumor tissues, and tumor cell lines

We have previously shown that the N8 mRNA is expressed at high levels in carcinoma cells derived from different types of tumor tissues and in normal tissues rich in epithelial cells (kidney, prostate, intestine and salivary gland). The 24 kDa N8 protein is expressed at a high level in human lung tumor tissue, as well as cell lines derived from lung, breast, prostate, colon and kidney tumors, leukemia and Burkitt's lymphoma cell lines (Tables 1 and 2). N8 protein is also expressed at high levels in human normal kidney, brain and liver, but at low levels in muscle, lung and heart tissues (Table 2). N8 protein was not detected in cell lines derived from human normal lung tissues and the mouse NIH3T3 fibroblast cell line. The protein data are consistent with N8 mRNA expression pattern. In addition to a major 24 kDa protein, we have also observed other minor protein species in tumor cell lines and tissues. These minor proteins are specifically recognized by N8 antisera and are not observed in cell lines in which N8 mRNA is not expressed. They could represent the translation products of minor N8 mRNA species. More detailed analyses are needed to identify their origin. Collectively, these results further confirm that the 24 kDa protein is the major translation product of the N8 gene.

Table 1 The presence of N8 mRNA and protein in several different cell lines and tissues

Human Cell Lines	*RNA*	*Protein*
Normal Lung		
W138, CCD8Lu, CCD11Lu CCD19Lu	–	–
Lung Carcinoma		
NCI-H69, H209, H82, H146, H446, H460, H520	+	+
Breast Carcinoma		
BT-474, Hs 578T, MCF7, MDA-MB-231	ND	+
BT-20	+	+
Colon Carcinoma		
colo 320	+	+
Kidney Carcinoma		
CaKi-1	ND	+
CaKi-2	+	+
ACHN	–	+
Prostate Carcinoma		
FNC 267 B1, FNC 267 B1/Ki, HPV-18, HPV-18/Ki, MLC SV40	ND	+
PC3	+	+
Leukemia		
Jurkat, Molt4, K562, RS411, H260, CEM	ND	+
Burkitt Lymphoma		
Dandi, p3HR-1, Raji	ND	+
Mouse		
NIH3T3	–	–

Total cell extracts were isolated from different cell lines and proteins were size fractionated by SDS–PAGE and transferred to nitrocellulose membranes. The membranes were probed with N8 antisera (1:5000) and donkey anti-rabbit Ig (Amersham, 1:2500), as described in Materials and methods. the protein data was compared to RNA data and Northern blot analysis of NIH3T3 cells were as described previously (Chen *et al*., 1996). ND = not determined

Table 2

	Tissue	*RNA*	*Protein*
Human, normal (MTW blot)	Heart	+/–	+
	Brain	++	+++
	Placenta	++	ND
	Lung	+	+
	Liver	+	++
	Skeletal muscle	+	+
	Kidney	+++	+++
Matched	lung tumor	+++	+++
	normal lung	+	+

Total cell extracts from matched tumor and normal lung tissues were prepared as described in Materials and methods. This blot and human multiple tissue western (MTW) blot (Clontech) were probed with N8 antisera (1:5000 dilution) and donkey anti-rabbit Ig (Amersham, 1:2500) as described in Methods. The protein data was compared to RNA data as described in Chen *et al*. (1996). +/–, trace amount; +, relative amount of N8 RNA or protein levels in different tissues; ND = not determined

Properties of N8 protein

N8 protein can form homodimers or multimers in vitro We have previously shown that the N8 protein contains a putative leucine zipper domain with the consensus sequence Lx(6)Ix(6)Lx(6)Lx(6)L starting at amino acid 43 (Chen *et al*., 1996). Since leucine zipper domain containing proteins have been shown to form homo or heterodimers, we examined if N8 can form homo or heterodimers. For this purpose, we used both 32 kDa recombinant N8 protein as well as N8 protein made in reticulocyte lysate. Proteins were cross linked with glutaraldehyde, and products were analysed by SDS–PAGE as described in Materials and methods. Both recombinant and *in vitro* synthesized N8 protein migrated as dimers and trimers only in the presence of glutaraldehyde (Figure 4a and b). These results suggest that the N8 protein can form multimers.

Identification of critical regions of N8 necessary for multimerization To determine if the N8 leucine zipper is important for dimerization, we used N8LZ contain-

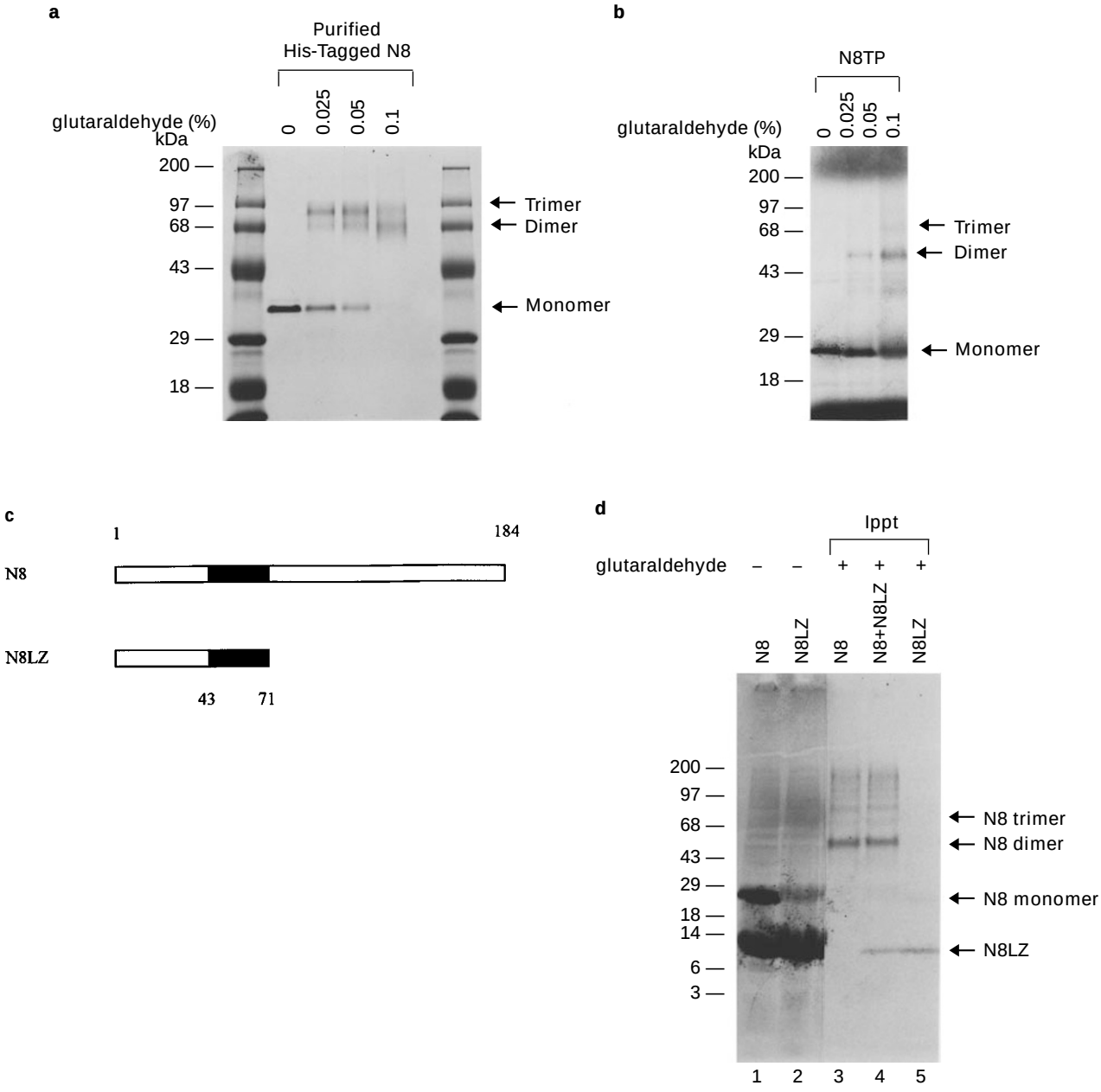

Figure 4 Multimerization of N8 protein *in vitro*. (**a**) Purified recombinant N8 protein was cross-linked with 0.025%, 0.05% and 0.1% glutaraldehyde and proteins were separated on 12% tris-glycine gel and stained with Coomassie Blue. Dimers and timers were shown on the right-hand side with arrows. (**b**) N8 total translation products (TP) was cross-linked with 0.025% and 0.05% glutaraldehyde. Proteins were separated on 12% tris-glycine gel and visualized by fluorography. Dimers and trimers were shown on the right-hand side with arrows. (**c**) Schematic representation of N8 and N8LZ. N8LZ contains the putative leucine zipper domain but lacks 113 C-terminal amino acids. The shaded area indicates leucine zipper domain. (**d**) N8 and N8LZ TPs were cross-linked with 0.05% glutaraldehyde. Lanes 1 and 2, N8 and N8LZ TPs, respectively. Lanes 3 to 5, glutaraldehyde cross-linked TPs were immunoprecipitated with N8 antisera at 1 : 5000 dilution. Immunoprecipitated TPs were separated on 10–20% gradient tricine gel and visualized by fluorography. Monomer, dimer and trimer are indicated on the side. Ippt, immunoprecipitation

ing leucine zipper, but lacking 113 carboxy terminal amino acids (Figure 4c). DNA sequence analyses confirmed that N8LZ terminates at amino acid 71 of the N8 protein and generates a ~10 kDa protein (Figure 4d, lane 5). It is also specifically recognized by N8 antisera, indicating it contains some of the N8 protein epitopes. These results demonstrate that the N8LZ is a C-terminal truncated N8 protein. We used immunoprecipitation to enrich the population of N8 dimers and multimers because N8LZ co-migrated with the globin protein of reticulocyte lysate (Figure 4d, lanes 1 and 2). Dimers and multimers of N8 can easily be detected with full-length N8 protein (Figure 4d, lane 3). However, N8LZ failed to form homodimers. It was not capable of forming dimers, even with full-length N8 protein (Figure 4d, lanes 4 and 5). These results indicate that amino acids C-terminal to the leucine zipper are also necessary for formation of dimers or multimers.

Identification of heteromeric partners for N8 Functions of zipper proteins are often modified by the association with the other partner proteins. To find out if N8 could associate with other proteins, NCI-H209 cells were metabolically labeled with ^{35}S-methionine, and lysed in Triton X-100 buffer and immunoprecipitated with N8 antisera. Results are shown in Figure 5. As expected, N8 antisera specifically immunoprecipitates the 24 kDa N8 protein from H209 cell extracts (Figure 5a) and its immunoprecipitation was blocked by an excess of N8 antigen, and was not recognized by preimmune sera. Although other proteins are immunoprecipitated with N8 antisera, they appear to be non-specific because they are also immunoprecipitated with preimmune sera (Figure 5a) and not competed by an excess of N8 antigen. However, weak association of N8 with other proteins may not be detected with this method. These results suggest that the N8 protein does not appear to interact strongly with other heteromeric partners. Alternatively, N8 antisera could effectively block association with other partners. Further experiments are needed to address these issues.

N8 protein is phosphorylated on serine residues A search for different motifs in the N8 protein revealed that it contains multiple phosphorylation sites. We tested the possibility that the N8 protein could be a substrate for cAMP-dependent protein kinase A or protein kinase C. For this purpose, NCI-H209 cells were labeled with ^{32}P orthophosphate in the presence of either phorbol ester (direct activator of protein kinase C) or Forskolin (activator of cAMP-dependent protein kinase A). ^{32}P-labeled cells were lysed in Triton X-100 solution. N8 protein was immunoprecipitated and analysed by SDS–PAGE and autoradiography (Figure 5b). The phosphorylation of N8 protein was not affected by either treatment (data not shown). To identify the amino acid residues involved in the basal phosphorylation, we carried out phosphoamino acid analysis of N8 protein. Results shown in Figure 5c

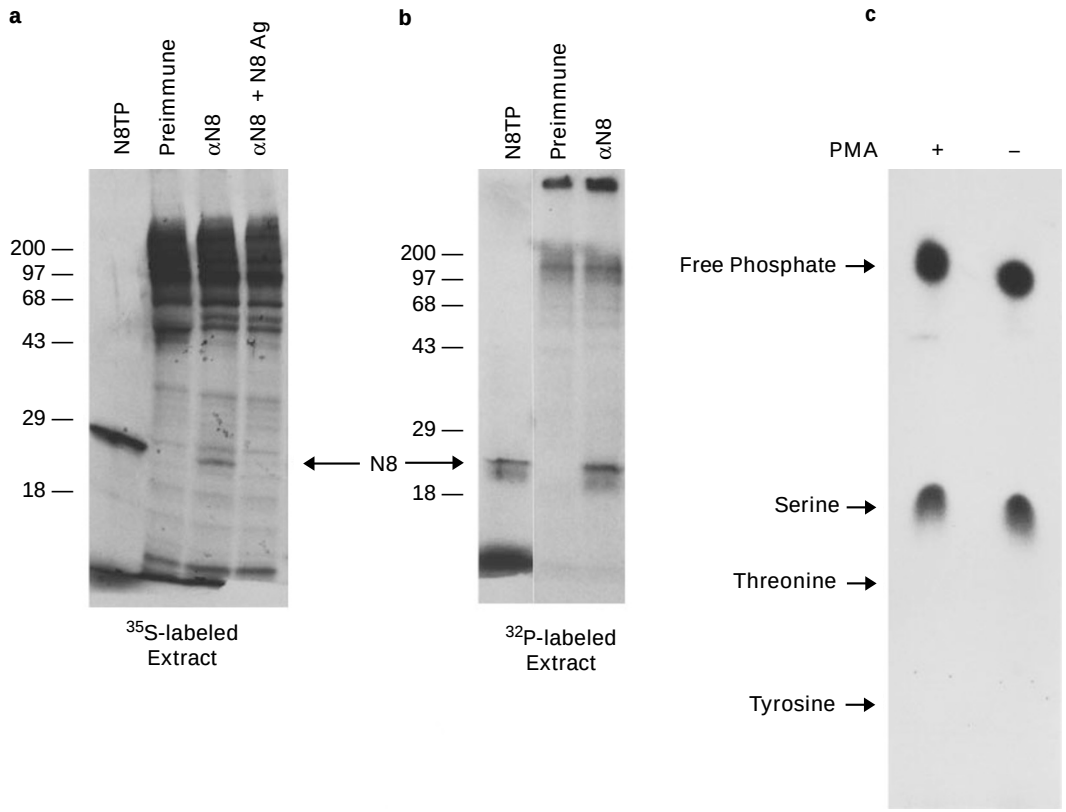

Figure 5 *In vivo* labeling of N8 protein using ^{35}S-methionine and ^{32}P orthophosphate. (**a**) ^{35}S-labeled cell extracts were immunoprecipitated with preimmune sera (lane 2) or N8 antisera (lane 3). Lane 4, N8 antisera was mixed with 2 μg of SDS–PAGE purified N8 protein before immunoprecipitation. (**b**) ^{32}P-labeled cell extracts were immunoprecipitated with preimmune sera (lane 2) or N8 antisera (lane 3). Immunoprecipitated proteins were separated on 12% Tris glycine gel (**a** and **b**). ^{35}S-labeled proteins were visualized by fluorography. (**c**) ^{32}P-labeled cell extracts were immunoprecipitated using N8 antisera, analysed by SDS–PAGE, and protein was transferred to Immobilon-P membrane for phosphoamino acid analysis as described in Materials and methods. The positions of phosphoserine, threonine and tyrosine are indicated on the left side by arrows

indicate N8 is phosphorylated on serine residues. In the presence or absence of phorbol esters, phosphorylation on the serine residues did not change. These results taken together demonstrate that the N8 protein is a serine phosphoprotein and it may not be the direct substrate for protein kinase C or protein kinase A.

N8 is a cytosolic protein Location of a protein often yields some clue to its function. We used both *in situ* immunofluorescence and biochemical fractionation to identify the subcellular localization of N8 protein. NCI-H520 cells expressing high levels of N8 proteins were lysed in mild detergent solution and fractionated into nuclear and post nuclear supernatants. Fractions were analysed for N8 protein by Western blot analysis. N8 protein was more abundant in the post nuclear supernatant fraction than in the nucleus (Figure 6). To further confirm the subcellular localization of N8 protein, we performed immunofluorescence analyses on cells expressing either high (NCI-H520) or very low levels (CCD11Lu) of N8 protein. NCI-H520 cells were fixed in methanol and stained with N8 antisera. As shown in Figure 7a and b, N8 protein was localized predominantly in the cytoplasm. The immunofluorescence was competed by an excess of N8 protein (data not shown) and cells stained with preimmune sera did not show any fluorescence (Figure 7c and d). Furthermore, cells not expressing N8 protein (CCD11Lu) did not exhibit any fluorescence (data not shown). These results taken together demonstrate that the antibody staining is specific and N8 protein is a cytoplasmic protein.

N8 is overexpressed in epithelial cells Mesenchymal to epithelial interconversion is essential in normal embryonic development (Birchmeier and Birchmeier, 1993). The majority of tumor cell lines are carcinomas having some characteristic features of epithelial cells, whereas cell lines derived from normal lung tissues are of fibroblast origin. We have previously shown that N8 mRNA is expressed at very low levels in normal lung tissue derived cell lines (WI38, CCD8Lu, 11Lu and 19Lu) and at high levels in lung tumor-derived cell lines (NCI-H69, H209, H82, H146, H446, H460 and H520) (Chen *et al*., 1996). N8 protein is also expressed at high levels in cells of epithelial origin compared to fibroblasts (Figure 8a). Higher expression of N8 protein is not due to difference in the amount of cell extract loaded because we normalized the amounts of protein loaded on the gel based on coomassie blue staining of proteins (data not shown). Furthermore, vimentin, which is expressed at high levels in cells of mesenchymal origin, is expressed at high levels in CCD8Lu and 11Lu, but not in lung carcinoma cells (Figure 8b). These results indicate that N8 protein is expressed at higher levels in epithelial cells than in mesenchymal cells, thus suggesting that N8 may play an important role in mesenchymal to epithelial conversion.

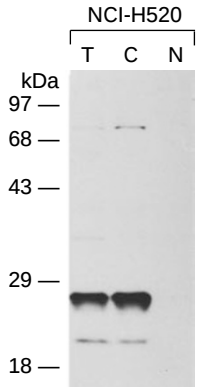

Figure 6 Subcellular localization of N8 protein by Western blotting. Cell extracts of subcellular fractions from the NCI-H520 cell line were prepared as described in Materials and methods. Equal amounts of cell extracts were separated on 12% tris-glycine gel and transferred to nitrocellulose membrane for Western blot analysis. T, total. C, cytoplasmic, N, nuclear. N8 antibody was used at 1 : 5000 dilution and the second antibody, Donkey anti-rabbit Ig conjugated with horseradish peroxidase, at 1 : 2500 dilution

N8 protein is expressed in intetstinal epithelial cells Intestine is a good model system to study the cell proliferation, differentiation, lineage commitment and cell migration. The principal cell type at the surface of the colon mucosa is columnar epithelium, which is developed from undifferentiated cells located near the base of the crypt of the intestine. Undifferentiated cells in the depths of the crypts divide and differentiate into mucin-producing goblet cells and into columnar epithelium as they migrate upward to the surface of the mucosa. Figure 9a shows N8 antisera stain mainly in the epithelial cells lining the crypt and the epithelium on the surface of mucosa. Both undifferentiated and differentiated epithelial cells expressed N8 protein. Very little staining is found in lamina propria (where most cells are fibroblasts), muscularis mucosae and submucosa regions (Figure 9a). No staining is observed using preimmune sera (Figure 9b). These results suggest that the N8 protein may be important in the development of intestinal epithelial cells.

Elevated levels of N8 proteins in fibroblast cells converted to epithelial-like cells To test our hypothesis that the N8 may be involved in mesenchymal to epithelial conversion, we used a model system developed by Steven Frisch. He has shown that fibroblasts spontaneously immortalized by deletion of the p53 gene (Li-Fraumeni fibroblasts; FB) were converted to epithelial-like cells by expression of adenovirus-5E1a gene products (Frisch, 1994). Results presented in Figure 10 demonstrate that the N8 protein level is increased in E1a-expressing epithelial-like cells, whereas N8 protein was not expressed in the parental fibroblast cell line. These results further support our hypothesis that the N8 protein may be associated with the development or maintenance of epithelial characteristics.

Overexpression of human N8 proteins in NIH3T3 cells alters its morphology Based on the results shown in Figures 8, 9 and 10, and Tables 1 and 2, it appears that N8 protein may play an important role in mesenchymal to epithelial conversion or the maintenance of epithelial characteristics. To gain further support for this hypothesis, we expressed human N8 protein under the control of the CMV promoter in NIH3T3 cells and two independent clones were used for further analyses.

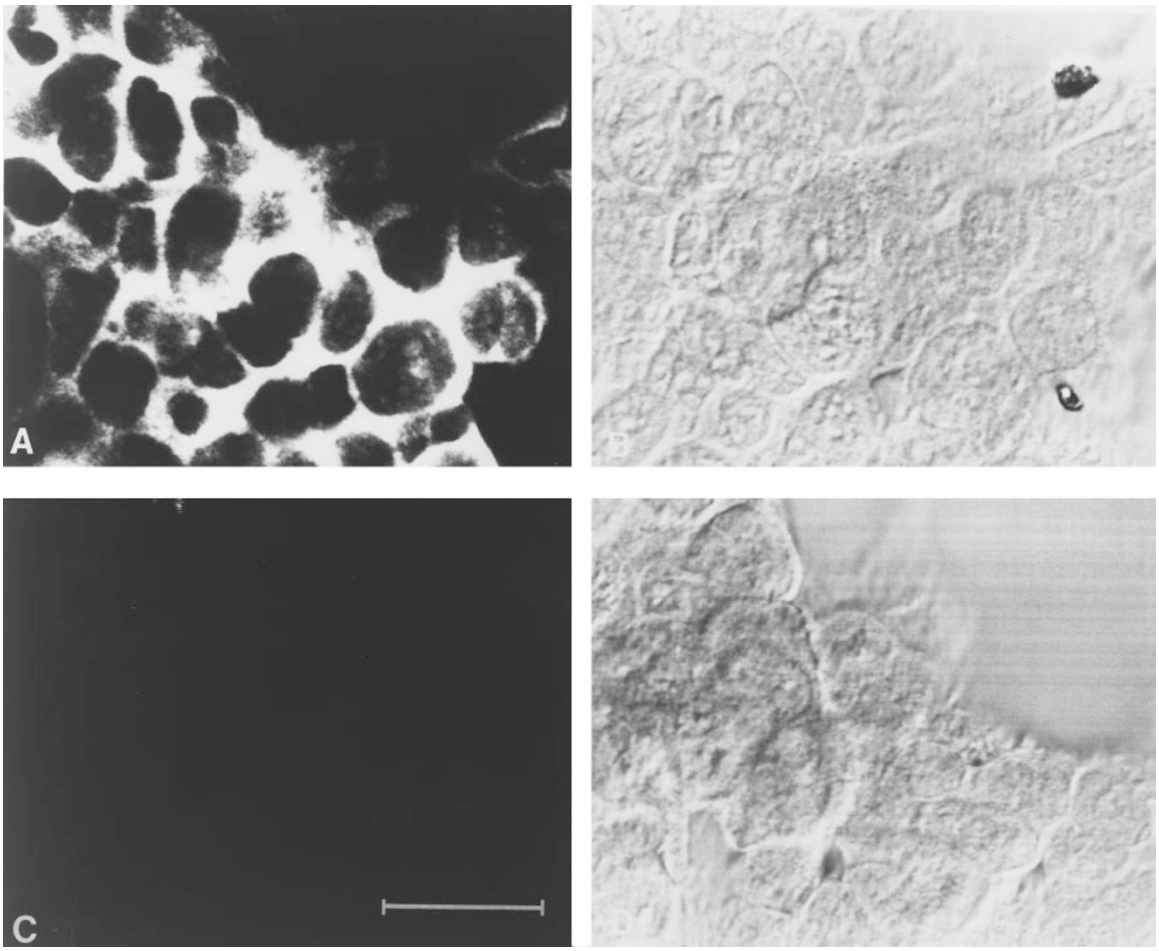

Figure 7 Subcellular localization of N8 protein by immunofluorescence analysis. The cells were fixed with 100% methanol and stained with N8 antisera as described in Materials and methods. (**a**) NCI-H520 cells stained with N8 antisera; (**b**) phase-contrast field of (**a**) without antibody; (**c**) NCI-H520 cells stained with rabbit preimmune sera; (**d**) phase-contrast field of (**c**) without antibody

As shown in Figure 11A, 24 kDa protein is expressed in clones containing N8 cDNA but not in parental NIH3T3 cells containing empty vectors (Figure 11A, lanes 2, 4 and 6). The apparent size of the N8 protein expressed in NIH3T3 cells is the same as the one expressed in H209 cells (Figure 11A, lanes 1, 4 and 6). Since N8 cDNA is under the control of the CMV promoter, its expression should be induced by phorbol ester. Consistent with this hypothesis, the 24 kDa N8 protein is induced by phorbol esters in NIH3T3 transfectants containing N8 cDNA (Figure 11A, lanes 4 and 6 *versus* 5 and 7, respectively). Endogenous murine N8 protein is not induced by phorbol ester treatment (Figure 11A, lanes 2 and 3). Taken together, these results demonstrate that in NIH3T3 transfectants, N8 protein is expressed from human N8 cDNA.

It has been shown that NIH3T3 cells grown under serum-free conditions, forms spheroid-like structures (Halverson *et al*., manuscript in preparation). These structures have been shown to express markers of epithelial cells, such as cell to cell contacts, cytokeratins and syndecans. We used similar conditions, to find out if NIH3T3 cells expressing N8 protein can form spheroids. Although parental NIH3T3 cells containing empty vectors (N4-3) overgrew and piled up, they never formed spheroids (Figure 11B, a). However, clones N8-6 and N8-10, which express N8 protein, were able to form spheroids under low serum (Figure 11B, b and c). Taken together, these results support the hypothesis that the N8 protein may be associated with the mesenchymal to epithelial conversion or the maintenance of epithelial characteristics.

Discussion

In this study, we demonstrate that the N8 antisera recognizes a major 24 kDa protein by Western blot analysis, immunoprecipitation and immunohistochemical studies. N8 protein is expressed at higher levels in lung tumor tissues than normal lung cells and at high levels in several tumor-derived cell lines. The N8 protein can form homodimers or multimers *in vitro*. It is a cytoplasmic protein and is phosphorylated at serine residue. Our results demonstrate that the N8 protein expression is higher in epithelial cells than in mesenchymal cells. When fibroblast cells are converted to epithelial-like cells, the N8 protein level is increased dramatically. Furthermore, ectopic expression of N8 protein in NIH3T3 cells lacking endogenous N8 protein induced the formation of spheroid-like structure which has the characteristic of epithelial cells. These results suggest the N8 protein may be associated with the development or maintenance of epithelial cells.

The N8 protein contains a putative leucine zipper domain and the full-length N8 protein can form multimers *in vitro*. However, the truncated N8 protein (N8LZ) containing the leucine zipper domain and its N-terminal region failed to form multimers (Figure 4d), indicating protein sequence C-terminal to the leucine zipper domain might play a role in the formation of N8 multimers. Studies with other leucine zipper-containing proteins have shown the formation of homo- and heterodimers depends on the leucine zipper domains (Blackwood and Eisenman, 1991; Amati *et al*., 1993; Beckmann and Kadesch, 1991; O'Shea *et al*., 1989a,b; Landschulz *et al*., 1989; Abdel-Hafiz *et al*., 1993). The mechanism of protein-protein interaction of N8 protein may be different from that of other leucine zipper proteins. At this time, it is hard to assess the importance of the protein sequence C-terminal to the N8 leucine zipper domain in the formation of multimers. It would require further experiments using a series of mutant proteins lacking different C-terminal protein sequences to study their effects on dimer formation.

Recently, a mouse homolog of the N8 gene (mD52) was identified (Byrne *et al*., 1996). The deduced mouse N8 protein sequence is 91% similar to the human N8 protein. Consistent with the high conservation of amino acid sequences between human and mouse species, N8 antisera also recognizes 24 kDa murine protein. In addition, a search in the GenBank database revealed that N8 protein has a homology (38% identities) with a protein encoded by the CEF13E6 locus of *C. elegans*. The homology is higher (~48% identities) in the leucine zipper region. However, a search within the saccharomyces cervesiae database revealed no such homology, indicating N8 gene expression may be coincident with the evolution of specialized cell type.

Results presented in this paper strengthen the above argument and we hypothesize that the N8 gene products may be associated with the development or maintenance of epithelial cells for the following reasons: (i) N8 mRNA and protein are detected at high levels in several epithelial-like tumor cell lines; (ii) normal tissues containing large number of epithelial cells (brain, kidney, intestine and pancreas), express high levels of N8 gene products; (iii) E1A-expressing Li-Fraumeni fibroblast cell line which acquired the epithelial cell characteristics, also expresses N8 protein; (iv) N8 protein is expressed at higher levels in the epithelial layer of intestine than in surrounding cells of mesenchymal origin; and (v) NIH3T3 cells expressing human N8 protein forms spheroid structure which also have some epithelial cell characteristics (Halverson *et al*., manuscript in preparation). Taken together, these results demonstrate that the N8 protein may play an important role in the development or maintenance of epithelial cell characteristics.

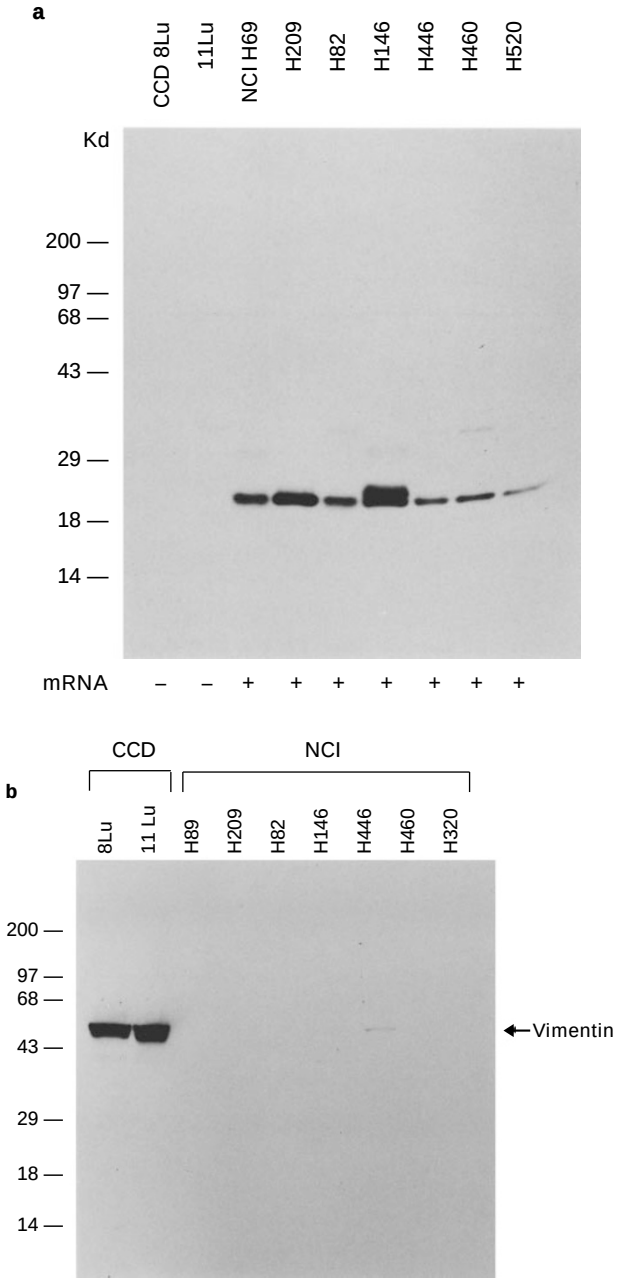

Figure 8 N8 protein is expressed in epithelial but not in mesenchymal cells. (**a**) cell extracts from normal and tumor-derived lung cell lines were separated on 12% tris-glycrine gel and transferred to nitrocellulose membrane for Western blot analysis using N8 antisera and second antibody as described in Figure 6; (**b**) anti-vimentin antibody was used at 1:500 dilution to probe the same blot as in (**a**). Sheep anti-mouse Ig antibody conjugated with horseradish peroxidase was used at 1:2500 dilution as second antibody

Materials and methods

Cell lines and tissues

FNC267 B1, FNC267 B1/Ki, HPV-18, HPV-18/Ki and MLC SV40 were gifts from Dr J Rhim. The rest of the cell lines were obtained from the American Type Culture Collection (ATCC) and grown according to their protocol. Liquid nitrogen frozen human lung tumor tissues and surrounding non-tumor tissues were gifts from Dr A Salesiotis.

Preparation and purification of recombinant N8 protein

N8 open reading frame (ORF) was synthesized by reverse transcription polymerase chain reaction (RT–PCR) using total RNA isolated from normal human lung tissue. RT–PCR was carried out as described by Chen *et al*. (1996) using 5′ (5′-CGCGGATCCCATGGACCGCGGCG AGCAAG-3′) and 3′ (5′-CGCGGATCCCAATCTCACA GGCTCTCCTGTG-3′) primers containing *Bam*HI sites. PCR products were cloned in PCR3 vector by TA cloning

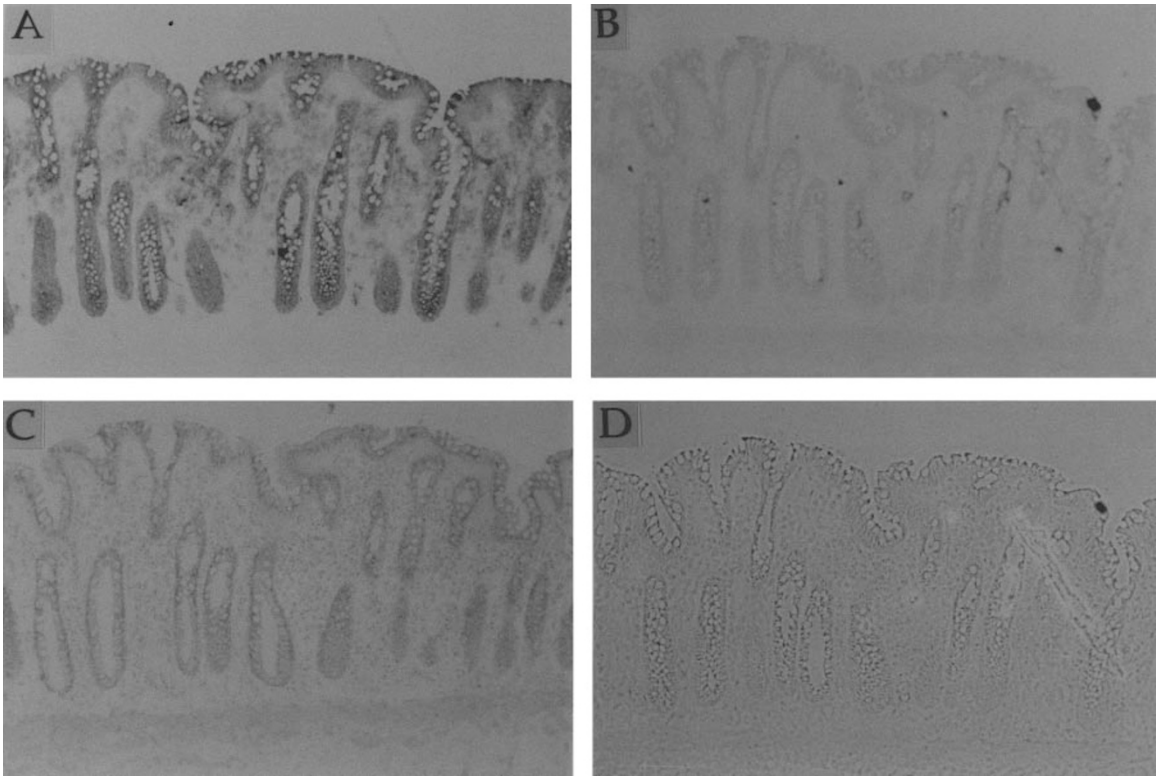

Figure 9 Immunohistochemical study of human colon using N8 antisera and preimmune sera. (**a** and **b**) were stained with N8 antisera and preimmune sera, respectively. (**c**) was stained with H&E (**d**) is the phase-contrast image of unstained adjacent section

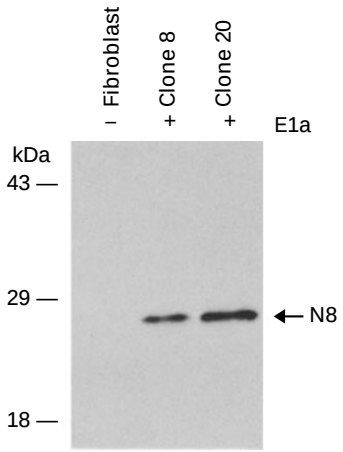

Figure 10 Overexpression of N8 protein in fibroblasts expressing E1a protein. Cell extracts of adenovirus-5E1a protein expressing Li-Fraumeni fibroblast cell lines (clones 8 and 20) were separated on 12% tris-glycine gel and transferred to nitrocellulose membrane and probed with N8 antisera as described in Figure 6

(Invitrogen) and characterized further by DNA sequencing. The *Bam*HI fragment containing N8 ORF was subcloned into *Bam*HI site of a modified PET15b expression vector (Novagen). The modified PET15b expression vector contains His tag at amino terminal end and contains a heart muscle kinase site and FLAG peptide (Kodak) inserted between thrombin and *Bam*HI sites of PEt15b vector. The PET vector containing N8 ORF was transformed into BL21 (DE3) host bacteria and the N8 protein was induced by IPTG addition. Recombinant N8 protein was purified using His-bind resin according to the manufacturer's protocol (Novagen). Since N8 protein eluted with imidazole buffer containing additional proteins, it was further purified by SDS polyacrylamide gel electrophoresis as described before (Bhat and Papas, 1992). N8 protein was stained with ice cold 0.2 M KCl and excised from the gel and eluted from the gel by soaking in 10 ml 0.1% SDS solution at room temperature for overnight. The gel pieces were pelleted by centrifugation at 4K r.p.m., 4°C for 10 min and proteins were re-extracted once more. The supernatants from two extractions were pooled and filtered through 0.45 μm membrane and the filtrate was concentrated using Macrosep centrifugal concentrator (Filtron) with 10K molecular weight (MW) cut off at 5K r.p.m., 4°C overnight. Purified recombinant N8 protein migrates as a 32 kDa protein and its identity was further confirmed by amino acid sequence analyses.

Preparation and purification of N8 antisera

N8 antisera was raised by injecting N8 protein eluate collected from His-bind resin into rabbit at the NCI-FCRDC facility. The 32 kDa recombinant N8 protein was immobilized on an AminoLink column following the manufacturer's protocol (Pierce). Ten ml of rabbit N8 antisera was mixed with an equal volume of storage buffer (1 × TBS (10 mM Tris-HCl, pH 7.5, 0.15 M NaCl), 0.05% NaN_3) and loaded on a column immobilized with 32 kDa recombinant N8 antigen. The column was washed with 17 ml of binding buffer (storage buffer). One ml fraction was collected and their OD at 280 nm were measured to determine the removal of uncoupled antibodies. The bound N8 antibodies were eluted using 10 ml elution buffer (0.1 M glycine, 0.15 M NaCl, 0.02% NaN_3, pH 2–3), and 200 μl of 1 M Tris-HCl, pH 7.5 was added to each 1 ml fraction eluate immediately. The eluates with high OD reading were pooled, diluted with 1 × TBS buffer to 10 ml and concentrated using a Macrosep centrifugal concentrator (Filtron) with 10K MW cut off at 6K r.p.m., 4°C for 2.5 h to a final volume of about 3 ml and stored in aliquots at −70°C.

Western blot analysis

Cell extracts of tumor and normal cell lines were isolated as described by Bhat and Papas (1992). Tissues were homogenized (0.25 g tissue/ml) in nuclear isolation buffer (NIB, 0.3 M sucrose, 10 mM KCl, 10 mM Tris-HCl, pH 8.0, 0.2 mM EDTA, 0.2 M EGTA, 0.5 mM DTT, 0.5 mM PMSF, 1% Aprotinin and 0.05% NP40) using a tissue homogenizer. Homogenates were centrifuged at 1K, 4°C for 5 min and the supernatants were processed as described for the cell line. Cell extracts of Li-Fraumeni (FB) and E1a-expressing FB cell lines (clone 8 and 20) were kindly provided by Dr Steve Frisch (La Jolla Cancer Research

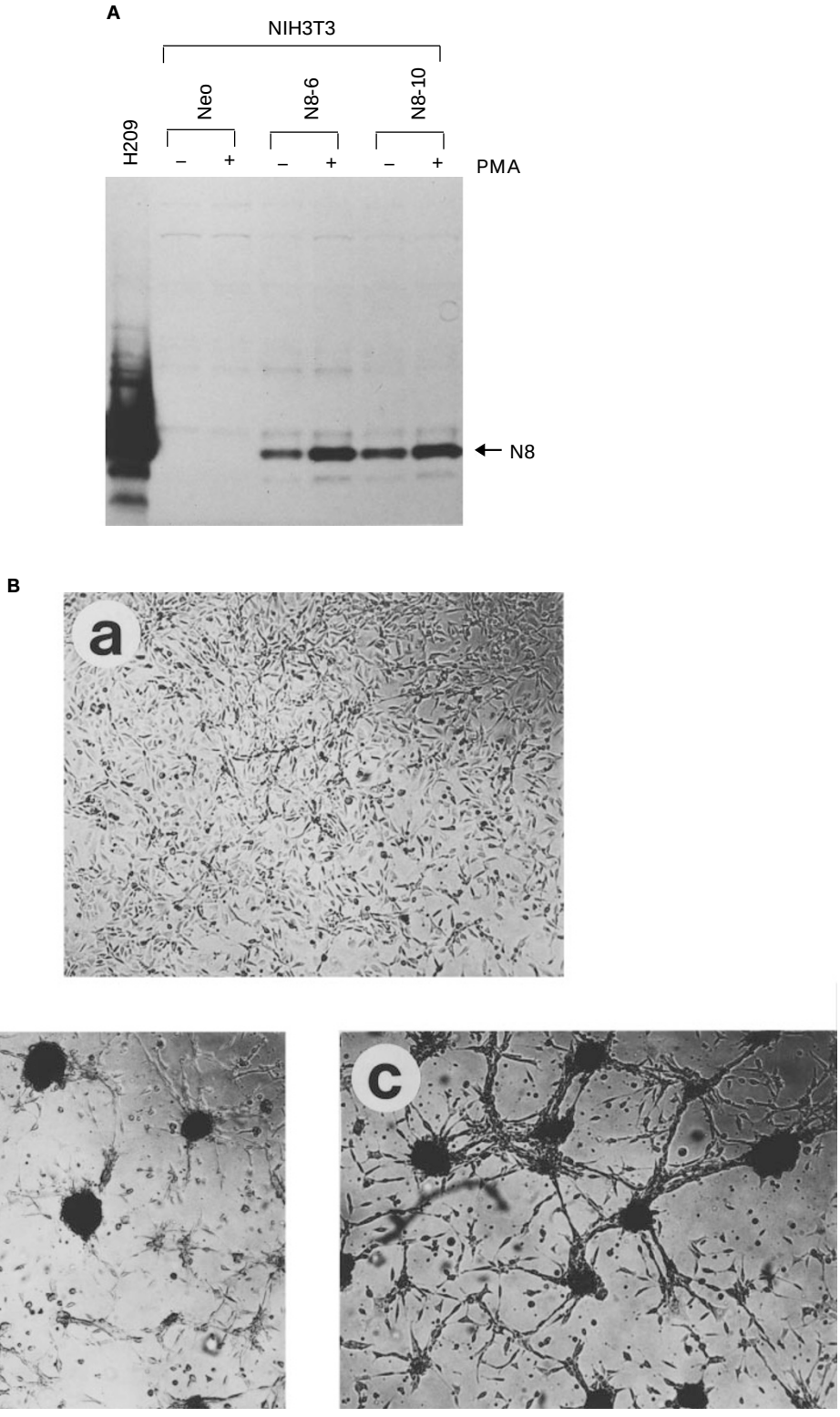

Figure 11 Ectopic expression of N8 in NIH3T3 cells alters its morphology. Stable NIH3T3 transfectants expressing human N8 proteins were selected as described in Materials and methods. (**A**) Western blot analysis of NIH3T3 cells transfected with N8 cDNA. The blot was probed with N8 antisera as described previously. Neo, NIH3T3 cells containing only the PCR3 vector, i.e., N4-3 cell line. N8-6 and N8-10 are two independent NIH3T3 cell lines expressing N8 protein. (**B**) Transfected NIH3T3 cells grown in 0.5% calf serum medium as described in Materials and methods. The picture was taken at 40× magnification using Nikon Diaphot 200. Panel a N4-3 cell line; panel b, N8-6 cell line; panel c, N8-10 cell line

Foundation). Proteins were electrophoretically separated on 12% Tris-glycine gel and blotted onto nitrocellulose membrane (Schleicher & Schuell) using 1 × Western transfer buffer (25 mM Tris Base, 0.19 M glycine, 0.1% SDS, 16.7% methanol) at 75 mA for 2 h. Membranes were submerged in dH_2O for 5 min, then incubated in blocking buffer (10 mM Tris HCl, pH 7.4, 0.1 M NaCl, 0.1% Tween 20 and 1% Nonfat dry milk) at room temperature for 1 h. The membrane was probed with either N8 or actin antisera (clone C4, Boehringer Mannheim) at 1 : 5000 dilution in blocking buffer for 1 h at room temperature. Anti-vimentin antibody was used at 1 : 500 dilution in blocking buffer. The membrane was washed in wash buffer (blocking buffer with 0.5% BSA) for 15 min, four times, and was incubated in blocking buffer containing appropriate second antibody (1 : 2500 dilution) at room temperature for 1 h. The membrane was washed with wash buffer as described above and N8 protein was visualized using ECL detection kit (Amersham). For competition experiment, 2 μg of purified N8 protein was mixed with either N8 or actin antibody in 500 μl blocking buffer at room temperature for 5 min and then diluted with 9.5 ml blocking buffer before use.

In vivo *labeling of cellular N8 protein*

NCI-H209 cells were labeled with either L-^{35}S methionine (1175 Ci/mmole, NEN) or [^{32}P]orthophosphate (8500–9120 Ci/mmole, NEN). For ^{35}S labeling, NCI-H209 cells were washed twice with PBS and RPMI methionine free media. The cells (6×10^6) were resuspended in 3 ml RPMI methionine free medium and starved for 1 h, and labeled with L-^{35}S-methionine (200 μCi/ml) for 3 h. Cells were washed twice in ice cold PBS and lysed in 6 ml TX100 buffer (150 mM NaCl, 50 mM Tris-HCl, pH 8.0, 1% Triton X-100, 1 mM EDTA, 0.1 mM EGTA, 1% aprotinin, 0.5 mM PMSF, 0.1 mM TPCK, 0.1 mM TLCK). Insolubles were removed by centrifugation at 13 000 g for 30 min at 4°C. For ^{32}P labeling, cells were suspended in phosphate free RPMI media and labeled (500 μCi ^{32}P/ml) for 3 h. Cell lysis was done in TX100 buffer containing phosphatase inhibitors (10 mM pyrophosphate, 1 mM sodium vanadate, 10 mM NaF and 1 mM β-glycerophosphate). Either 5×10^6 ^{32}P (4.95 ng) or 100×10^6 ^{35}S TCA (0.766 μg) insoluble CPM was immunoprecipitated with N8 antisera as described before (Bhat and Papas, 1992). For the competition experiment, N8 antisera (0.4 μg) and N8 protein (2 μg) were mixed in 100 μl of TX100 buffer and incubated at room temperature for 5 min before mixing with ^{35}S or ^{32}P labeled cell extracts.

Immunofluorescence assay

Human lung carcinoma cell line NCI-H520 and cell line derived from normal lung tissue CCD11Lu, were cultured on tissue culture chamber slide (Nunc) for 2 days. The cells were washed with PBS and fixed in 100% methanol at −20°C for 20 min and then were preincubated with PBS containing 20% swine serum (Sigma) at 37°C for 30 min. After washing for 5 min three times, the slides were incubated with N8 antisera (1 : 500 dilution) at 37°C for 2 h. The cells were washed again and incubated with swine biotinylated anti-rabbit IgG (1 : 400, DAKO) at 37°C for 30 min, and then stained with streptavidin conjugated with fluorescein isothiocyanate (1 : 100, DAKO). The slides were washed and examined under a Zeiss confocal laser scanning microscope.

Glutaraldehyde binding assay

A 2.0 μg of 32 kDa recombinant N8 protein (2.5 μl) in 1 × TBS was mixed with equal volume of 0.05%, 0.1% or 0.2% glutaraldehyde, incubated at room temperature for 5 min. Cross linking was terminated by suspending samples in 5 μl of protein sample buffer (150 mM Tris-HCl, pH 6.8; 6 mM EDTA, 6% w/v SDS, 20% glycerol, 10% β-mercaptoethanol, 0.01% w/v Bromophenol blue) and boiling for 3 min. Proteins were separated on 12% Tris-glycine gel and visualized by staining with Coomassie Blue (10% glacial acetic acid, 40% methanol, 0.1% Coomassie Brilliant Blue R-250). Cross linking was also done with N8 translation products (TP) as described above except that proteins were visualized by fluorography.

Subcellular fractionation

NCI-H520 cells were trypsinized and centrifuged at 1100 r.p.m. at 4°C for 5 min. The cells were washed twice with PBS and suspended in 300 μl of NIB buffer (NIB) (0.05% NP40, 0.3 M sucrose, 10 mM KCl, 10 mM Tris-HCl, pH 8.0, 0.2 mM EDTA, 0.2 mM EGTA, 1% aprotinin, 0.5 mM DTT and 0.5 mM PMSF) and mixed well. One-hundred microliters of mixture were mixed with an equal volume of protein sample buffer and was considered as total cell extract. The rest of the mixture was centrifuged (1000 g) and the post-nuclear supernatant (SN) was transferred to a fresh tube. One-hundred microliters of the SN was mixed with an equal volume of protein sample buffer. The nuclear pellet was rinsed with PBS twice and resuspended in 200 μl of NIB and mixed with an equal volume of protein sample buffer. Samples were vortexed and boiled for 2 min twice, and proteins were fractionated on a 12% Tris-glycine gel and transferred to nitrocellulose membrane and processed for detection of N8 protein as described above.

Phosphoamino acid analysis

^{32}P-labeled cell lysates were immunoprecipitated with N8 antisera and analysed by SDS–PAGE. Proteins were transferred to Immobilon-P membrane (Millipore) and N8 protein (24 kDa) was detected by autoradiography. The N8 protein band was cut off the membrane and analysed for phosphoamino acids following the protocol described in Current Protocols in Immunology (Coligan *et al*., 1991).

Transfection of NIH3T3 cells

N8 ORF was cloned into the PCR3 expression vector, as described above. The PCR3 vector (Invitrogen) and N8 expression vectors were transfected into NIH3T3 cells using LipofectAMINE (BRL), following the manufacturer's protocol. Four micrograms of DNA and 25 μl of LipofectAMINE each in 300 μl serum-free DMEM medium were mixed and incubated at room temperature for 40 min. The DNA-LipofectAMINE complex was mixed with 2.4 ml serum-free DMEM medium and added to NIH3T3 monolayer cells and individual colonies were selected in a medium containing 600 μg/ml Geneticin. Transfectants expressing human N8 protein were identified by Western blot analysis. N8-6 and N8-10 are the established permanent cell lines containing N8 ORF in the PCR3 vector and expressing human N8 protein. N4-3 is the cell line containing the PCR3 vector, but does not express N8 protein.

Immunohistochemical study

Cell pellets of NCI-H520 and CCD8Lu were fixed in 4% paraformaldehyde. Paraffin block of cell pellets were used to make slides. All immunohistochemical staining were

done in the Pathology/Histotechnology laboratory of NCI-FCRDC using a VECTASTAIN ABC Kit (Vector Laboratories). N8 antisera was used at 1 : 500 dilution.

Construction of truncated N8 protein (N8LZ) and immunoprecipitation

Human N8 cDNA clone HK4a1 (20 pg) (Chen *et al*., 1996) was used as a template and N8LZ cDNA fragment was generated by PCR using 5′ primer (5′-CGCGGATCC-CATGGACCGCGGCGAGCAAG-3′), and 3′ primer (5′-CGCGGATCCCTCAAAGTTTCCGCTTGATC-3′), as described previously (Chen *et al*., 1996). A 'TGA' termination codon was introduced into the 3′ primer right after the leucine zipper domain. PCR was carried out under the following conditions: 94°C, 5 min and then 94°C 1 min, 65°C 1 min for 30 cycles and an extensive of 72°C for 5 min. The PCR product was subcloned into PCR3 vector by TA cloning (Invitrogen). The sequence of the clone, N8LZ, was confirmed by DNA sequencing N8 and N8LZ TPs were obtained using the TNT reticulocyte lysate system (Promega) according to the manufacturer's protocol. Either N8 (3 μl) or N8LZ (6 μl) TP was mixed with an equal amount of 0.1% glutaraldehyde, incubated at room temperature for 10 min and added to 450 μl L100 buffer (100 mM KCl, 10 mM Tris-HCl, pH 7.5, 10% glycerol, 0.5% NP-40). The mixture was centrifuged for 10 min at room temperature and the supernatant was transferred to fresh tube for immunoprecipitation using N8 antisera at 1 : 5000 dilution. The mixture was rocked at room temperature for 1 h, then 50 μl of protein A Sepharose was added and rocked at room temperature for 30 min. Protein A Sepharose was spun down and washed with L100 buffer three times and resuspended in 30 μl Tricine sample buffer. The sample was boiled for 3 min and run on a 10–20% tricine gel and N8 proteins were visualized by fluorography. For competition experiments using N8 TPs, either 200 ng or 2 μg of N8 protein was mixed with N8 antisera in 100 μl L100 buffer at room temperature for 5 min before immunoprecipitation.

Spheroid assay

Cell lines N4-3, N8-6 and N8-10 were maintained in Dulbecco's medium (Quality Biological, Inc., Gaithersburg, MD) containing 8% calf serum, then shifted to QBSF-51 medium (Quality Biological, Inc., Gaithersburg, MD) containing 2% calf serum. Fourteen days later all cell lines were shifted to QBSF-51 plus 1% calf serum. After 2 more weeks cells were shifted to QBSF-51 plus 0.5% calf serum. Within 10 days cell line N8-6 had formed small diameter spheroids that had detached from the growth surface. Floating spheroids were collected and seeded in new flasks with fresh medium and formed spheroids. Cell line N8-10 had formed spheroids within 18 days. Both cell lines were repeatedly passaged as floating spheroids, and maintained as such thereafter. Although cell line N4-3 (control) overgrew and piled up, it never formed spheroids, even after many generations.

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