Overexpression of Bamacan/SMC3 Causes Transformation*

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Bamacan can occur in certain cell types as either a secreted proteoglycan assembled into basement membranes or as an intracellular protein known as structural maintenance of chromosome 3 (SMC3). To assess the role of this protein in tumorigenesis, we investigated whether induced overexpression of bamacan/SMC3 could transform normal fibroblasts. We generated a full-length cDNA encoding the entire mouse bamacan/SMC3 and demonstrated appropriate transcription and translation into a 146-kDa protein. All the NIH and Balb/c 3T3 murine fibroblasts overexpressing this bamacan/SMC3 transgene generated foci of transformation and acquired anchorage-independent growth. The increased levels of bamacan/SMC3 expression achieved in the transfected fibroblasts were the same as those detected in a series of spontaneously transformed murine and human colon carcinoma cells. Moreover, a 3-4-fold overexpression of bamacan/SMC3 was detected in ~70% of human colon carcinoma specimens from matched pairs (n = 19, p < 0.0002) and in a cohort of intestinal tumors from Apc-deficient Min/+ mice. These results support the concept that deregulated expression of bamacan/SMC3 is involved in cell transformation.

Originally isolated from the embryonic parietal yolk sac as a high density chondroitin sulfate proteoglycan (1, 2), bamacan was subsequently identified to be a component of the renal mesangial matrix (3), the basement membrane of other tissues (4), and tumor matrix (5). The murine protein is encoded by 31 exons distributed along ~45 kilobase pairs of genomic DNA (6) and is highly conserved across species (7–9). Secondary structure analysis of the protein reveals three globular domains intercalated by two α-helix coiled-coils in an antiparallel arrangement folded at a flexible hinge (10). The terminal ends harbor five glycanation sites to which the attachment of glycosaminoglycan side GAG chains is possible, and a P-loop and a motif called “cohesin”, involved in chromosome cohesion and prevents premature sister chromatid separation (12). The second, termed “cohesin”, is involved in chromosome cohesion and prevents premature sister chromatid separation (12). The second, termed “cohesin”, is involved in chromosome cohesion and prevents premature sister chromatid separation (12).

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The abbreviations used are: SMC3, structural maintenance of chromosome protein 3; PAGE, polyacrylamide gel electrophoresis; RT-PCR, reverse transcriptase polymerase chain reaction; bp, base pair(s); PAGE, polyacrylamide gel electrophoresis; EST, expressed sequence tag.

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addition of the vector encoding luciferase gave the predicted band of ~60 kDa (Fig. 1C, lane 4). Thus, the full-length cDNA encoding bamacan protein is appropriately transcribed and translated into a protein of size (146 kDa) identical to the native bamacan/SMC3.

Overexpression of Bamacan/SMC3 Causes Transformation of Normal Murine Cells—Because bamacan mRNA levels are elevated in a number of tumorigenic cell lines (6), we investigated whether overexpression of this protein could directly produce cell transformation. For this purpose, normal NIH and Balb/c 3T3 fibroblasts, which are both contact inhibited, were transfected with the pcDNA3.1 bamacan/SMC3 expression vector. Of the 22 clones obtained, 3 displayed ~4-fold bamacan overexpression (Fig. 2A). Eighteen of these clones were tested for the ability to generate colonies in soft agar. In these experiments, clones 9 and 22, i.e. those overexpressing bamacan mRNA, displayed anchorage-independent growth and generated $31 \pm 4$ (3) and $23 \pm 4$ (4) colonies $>100 \mu m$ diameter after 3 weeks of growth (Fig. 2B). No colonies were observed in the remaining 16 G418-resistant clones expressing normal bamacan mRNA and in wild type cells. Growth kinetics experiments revealed that the overexpressing clones had lost contact inhibition, confirming they had acquired a transformed phenotype. Initial growth rate did not differ significantly between overexpressing and normally expressing bamacan clones. However, upon reaching confluence, normally expressing clones significantly reduced their duplication rate to ~0.2 doubling/day, whereas clones 9 and 22 maintained a vigorous duplication activity (~0.6–1 doubling/day) (Fig. 2C). All the G418-resistant clones were tested in a transformation focus assay. Whereas wild type and G418-resistant clones expressing normal bamacan/SMC3 mRNA levels did not generate foci of transformation, clones 9 and 22 generated numerous foci of transformation (Fig. 2D). Moreover, cells expressing normal bamacan level (clone 6) formed a uniform monolayer, whereas cells overexpressing bamacan exhibited an haphazard arrangement and lacked contact inhibition (Fig. 2E). In agreement with the Northern blotting data, the cellular levels of bamacan/SMC3 protein in the overexpressing clones were ~3-fold higher than control (Fig. 2F). In contrast, no bamacan levels could be detected in the media conditioned by either wild type or transfected cells (not shown).

To corroborate the transformation potential of bamacan, we performed additional experiments in which bamacan/SMC3 gene was delivered into NIH 3T3 cells using pLXSN retroviral vector. Among 12 clones isolated, one displayed increased bamacan/SMC3 mRNA (clone 1, Fig. 3A) and protein (Fig. 3B) levels. As in the cells transfected with the pcDNA3.1 vector, the retroviral clones overexpressing bamacan formed numerous foci of transformation (Fig. 3C).

Collectively, these findings support the conclusion that induction of intracellular bamacan/SMC3 expression is sufficient to cause transformation of normal, contact-inhibited fibroblasts.

Bamacan Expression Is Abnormally Elevated in Colon Carcinoma Cell Lines and Colon Carcinoma Tissues—To assess whether the degree of bamacan/SMC3 expression that caused transformation of 3T3 fibroblast was similar to that of established neoplastic cells, a series of mouse and human neoplastic cell lines was examined. A non-tumorigenic colon epithelial cell line originated from p53 +/− mice was used as control. These cells do not grow in soft agar and do not generate tumors in either SCID or immunocompetent syngeneic mice (15). Compared with the expression in this primary colon cell line, a 5-fold increase in bamacan/SMC3 expression was observed in CMT-93 mouse colorectal carcinoma cells (Fig. 4A). Similar
Fig. 2. Overexpression of bamacan/SMC3 causes transformation of Balb-c 3T3 fibroblasts. A, Northern blotting of total RNA extracted from clone 6 (a G418-resistant but lacking bamacan/SMC3 expression) and two overexpressing clones. For loading comparison, the ethidium bromide-stained ribosomal RNA (rRNA) is shown in the lower panel. B, growth in soft agar as determined after 21 days of growth. C, growth curve of three individual clones as indicated. D, focus assay of clones as in A. The wild type was identical to clone 6 (not shown). E, morphological changes and lack of contact inhibition in cells overexpressing bamacan/SMC3. F, Western immunoblotting of total cell extracts (~20 μg of protein each) of clone 6 (lane 1) and clone 9 (lane 2) using a rabbit antibody raised against Pro1137-Ser1157 region of murine bamacan. Representative of one of 12 clones expressing normal bamacan/SMC3.

sues (n = 19). About 70% of the colon carcinomas displayed a significant increase in bamacan/SMC3 steady state mRNA levels (Fig. 4C). In the colon carcinomas, bamacan mRNA levels were 3.7 ± 0.4 (mean ± S.E.) higher than matched control tissues (p < 0.0002 by paired Student’s t test) (Fig. 4D).

Bamacan Is Overexpressed in Intestinal Tumors of APC-deficient Min/+ Mice—There is convincing evidence that most human colon carcinomas exhibit loss of heterozygosity at the Apc locus coding for the APC tumor suppressor gene (16). In mice, a non-sense mutation (Min allele) of the Apc gene causes the development of multiple intestinal neoplasia (Min/+). As in humans, the adenomas in Min/+ mice show loss of the wild type Apc allele. Therefore, we sought to establish whether bamacan is also overexpressed in adenomas from Min/+ mice. Careful examination of five animals of eight months of age revealed that all had advanced intestinal neoplasia covering ~60% of the small intestine. In agreement with the data presented above, there was a ~4-fold elevation of bamacan/SMC3 expression in the tumors (Figs. 4E and F). This elevation was specific inasmuch as no difference in bamacan/SMC3 mRNA levels was detected in the proximal or distal colon, or in the testis, when compared with the levels expressed in the same tissues of sex- and age-matched Apc-deficient C57BL/6J mice. The increased expression of bamacan mRNA in the intestinal tumors was confirmed by Western immunoblotting, which showed a ~4-fold increase of bamacan protein in the neoplastic tissues (Fig. 4G). These findings support the concept that an Apc-related pathway may mediate bamacan up-regulation in colon tumors.

Fig. 3. Overexpression of bamacan/SMC3 causes transformation of NIH 3T3 fibroblasts. A, Northern blotting of five independent G418-resistant clones transfected with pLXSN retroviral bamacan/SMC3 expression vector. For loading comparison, the ethidium bromide-stained ribosomal RNA (rRNA) is shown in the lower panel. B, Western immunoblotting of bamacan/SMC3 levels (20 μg of cellular protein) in clone 1, a bamacan overexpressing clone, and clone 2 as representative of one of 12 clones expressing normal bamacan/SMC3 levels. C, focus assay for clones 1 and 2. Results were obtained when normal colon tissue and the matching neoplastic tissue from a patient with colon carcinoma were investigated (Fig. 4B). Five independent human colon carcinoma cell lines displayed the same degree of bamacan overexpression as the colon carcinoma sample (Fig. 4B), suggesting that the phenomenon does not simply reflect the high rate of mitosis of the cells held in culture. We then compared bamacan/SMC3 expression in matched neoplastic and normal colon tissues of sex- and age-matched Apc-deficient Min/+ mice. In agreement with the data presented above, there was a ~4-fold elevation of bamacan/SMC3 expression in the tumors (Figs. 4E and F). This elevation was specific inasmuch as no difference in bamacan/SMC3 mRNA levels was detected in the proximal or distal colon, or in the testis, when compared with the levels expressed in the same tissues of sex- and age-matched Apc-deficient C57BL/6J mice. The increased expression of bamacan mRNA in the intestinal tumors was confirmed by Western immunoblotting, which showed a ~4-fold increase of bamacan protein in the neoplastic tissues (Fig. 4G). These findings support the concept that an Apc-related pathway may mediate bamacan up-regulation in colon tumors.

**DISCUSSION**

The results of this study strengthen the notion that bamacan/SMC3 may play an important role in tumorigenesis (6). The degree of gene activation that leads to cell transformation in normal mouse cells is comparable with that observed in a series of colon carcinomas cell lines and in the majority of human colon carcinoma specimens. The average 4-fold increase supports the idea that bamacan/SMC3 biosynthesis is tightly regulated in normal cells and that an imbalance in the amounts of this protein can directly affect the transformation program.

Several functional roles have been assigned to eukaryotic bamacan/SMC3 for which it is possible to envision mechanisms linking its overexpression to cell growth dis-regulation. The involvement of this protein in chromatid cohesion during metaphase points to a first mechanism (18). The protein is part of
The adenomatous tissue is in lane 2 and colon (N) and colon carcinoma (T) specimens. The intestine of 5-month-old Min/+ mice were dissected under a microscope and the adenomatous areas carefully separated from normal tissue. Other collected Min/+ mice were dissected under a microscope and the adenomatous areas carefully separated from normal tissue. Other collected tissues are as indicated. F, abundance of bamacan mRNA in the different specimens calculated as relative to the matching normal small intestine. In E and F, lanes 1, 3, 5, and 7 represent tissues from a C57BL/6J Apc+/+ mouse, whereas lanes 2, 4, 6, and 8 represent tissues from a Min/+ mouse. The adenomatous tissue is in lane 2. Samples in lanes 9–12 are from two Min/+ mice and represent normal (lanes 9 and 11) and adenomatous tissue (lanes 10 and 12). G, Western immunoblotting of two Min/+ mice (~20 μg of protein each) using anti-bamacan antisera. Lanes 1 and 3 represent normal small intestine, whereas lanes 2 and 4 are the matching neoplastic tissues.

In conclusion, our results support the concept that overexpression of bamacan/SMC3 gene may disrupt the formation of the multimeric protein complexes within the nucleus which could directly contribute to the genesis of a transformed phenotype.

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the cohesin multimeric protein complex that prevents the premature separation of sister chromatids (12). This process occurs in an orderly fashion during anaphase under the control of the cell cycle machinery and, if improperly executed, could result in the segregation of both sisters to the same cell thereby generating an aneuploid karyotype (19). Bamacan/SMC3 is also a necessary component, together with SMC1, of a multimeric complex named RC1 that has DNA recombination/renaturation, DNA ligase, and DNA polymerase activities (13). The enzymes act in concert to repair a gapped or deleted DNA. These different functions of bamacan/SMC3 are mediated by its ability to bind palindromic DNA sequences through its C terminus and by allowing the formation of protein-DNA structures that are accessible to the action of DNA-modifying enzymes (20, 21).

The functional significance of proteoglycan core protein in the nucleus and at the same time extracellularly eludes a simple interpretation. Proteoglycans can affect cell growth and differentiation by acting through different signaling pathways (22). Increased levels of proteoglycans with altered composition have been reported in human tumors, primarily those of epithelial origin, such as breast, colon, and lung. Bamacan proteoglycan has been shown to be a normal constituent of the basement membranes in several tissues (3) and is, thus, plausible that its secretion is a physiological event. Whether abnormal accumulation of extracellular bamacan occurs during tumor formation and the exact extracellular function of this protein needs to be established in future studies.

In conclusion, our results support the concept that overexpression of bamacan/SMC3 alone is sufficient to initiate cell transformation in normal fibroblasts. Thus, abnormal expression of bamacan/SMC3 gene may disrupt the formation of