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# Translational Suppression of Syndecan-1 Expression in Ha-*ras* Transformed Mouse Mammary Epithelial Cells

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A cell surface proteoglycan, syndecan-1, has been shown to participate in the maintenance of the epithelial cell morphology. A point mutated activated c-Ha-*ras* gene under the control of the glucocorticoid inducible MMTV-LTR promoter was transfected into the mouse mammary epithelial cell line, NOG-8. The NOG-8 *ras* cells were used to study changes in syndecan-1 expression during epithelial transformation. NOG-8 *ras* cells, when induced to express Ha-*ras*, transformed and formed foci in monolayer cultures and colonies in suspension cultures. Expression of syndecan-1 at the cell surface was markedly reduced in cells showing the transformed phenotype. The accumulation of newly synthesized core protein of syndecan-1 was suppressed in these cells, whereas mRNA levels remained unchanged. This novel finding indicates that syndecan-1 expression is translationally suppressed in the Ha-*ras*–transformed epithelial cells. Hence, syndecan-1 loss during epithelial transformation could take place without altering syndecan gene transcription and, on the other hand, could be one of the critical events involved in malignant transformation.

## INTRODUCTION

Interactions between malignant cells and surrounding extracellular matrix (ECM) are critical for regulation of tumor growth, invasion, and metastasis. ECM receptors, which play a critical role in these interactions, may therefore show significant changes in their expression during malignant transformation. One ECM receptor is syndecan-1 (Jalkanen *et al.*, 1991; Bernfield *et al.*, 1992). It is a heparan sulfate and chondroitin sulfate containing integral membrane proteoglycan (Rapraeger and Bernfield, 1985). Syndecan-1 is the first characterized member in the family of cell surface proteoglycans (Saunders *et al.*, 1989; Mali *et al.*, 1990). This family of syndecans contains at least four distinct proteoglycans, which have similar transmembrane and cytoplasmic domains but different extracellular domains (Bernfield *et al.*, 1992). The prototype of this family, syndecan-1 from epithelial cells, binds selectively to several ECM proteins such as fibrillar collagens (Koda *et al.*, 1985) and fibronectin (Saunders and Bernfield, 1988) but not to the basement membrane components, like laminin or type IV collagen. Syndecan-1 also binds a basic fibroblast growth factor (bFGF) (Kiefer *et al.*, 1990; Elenius *et al.*, 1992). This interaction may be essential for the binding of bFGF to its signal transducing receptor (Rapraeger *et al.*, 1991; Yayon *et al.*, 1991). Recently it has also been demonstrated that syndecan-1 can simultaneously interact with matrix components and bFGF (Salmivirta *et al.*, 1992), suggesting that syndecan-1 is a novel suitable molecule for regulating growth factor responses at cell-matrix interfaces.

Syndecan-1 colocalizes with actin filaments of mammary epithelial cells (Rapraeger *et al.*, 1986). It may thus participate in the maintenance of epithelial phenotype by anchoring cytoskeleton to ECM. This has been supported by studies with S115 epithelial tumor cells, which have shown transformed phenotype and suppressed syndecan-1 expression in the presence of testosterone. Withdrawal of the hormone and concomitant phenotypic shift to an epithelial phenotype has been shown to restore syndecan-1 expression (Leppä *et al.*, 1991). Furthermore, an epithelial phenotype and actin filaments were also restored, if syndecan-1 expression was

reobtained after transfection of syndecan-1 cDNA under a testosterone inducible promoter (Leppä *et al.*, 1992).

Molecular mass of syndecan-1 varies according to cell type and is because of differences in the number and size of glycosaminoglycan (GAG) side chains (Sandersson and Bernfield, 1988; Salmivirta *et al.*, 1991; Bernfield *et al.*, 1992). Transforming growth factor-$\beta$ (TGF$\beta$)–treated epithelial cells have been shown to express increased amounts of chondroitin sulfate chains attached to syndecan-1 (Rasmussen and Rapraeger, 1988). These variations may reflect changes in syndecan-1 function, because syndecan-1 from developing tooth reveals selective and specific binding to tenascin (Salmivirta *et al.*, 1991). These biochemical changes together with its developmentally exclusive expression sites suggest a key role for syndecan-1 in normal differentiation processes (Thesleff *et al.*, 1988; Vainio *et al.*, 1989; Solursh *et al.*, 1990; Trautman *et al.*, 1991).

In earlier immunohistochemical studies syndecan-1 expression has been shown to be suppressed in skin tumors induced by UV-irradiation of hairless mice (Inki *et al.*, 1991), in carcinomas produced from transformed keratinocytes in nude mice (Inki *et al.*, 1992b), or in hormone-induced mammary epithelial tumor cells (Leppä *et al.*, 1991, 1992). In this study we have examined the expression of syndecan-1 in a mouse mammary epithelial cell line (NOG-8) transformed by a point mutated c-Ha-*ras* oncogene. The oncogene expression was under the control of the glucocorticoid inducible mouse mammary tumor virus-long terminal repeat (MMTV-LTR) promoter. After dexamethasone induction the NOG-8 *ras* cells became transformed. We have observed that the amount of cell surface syndecan-1 and the accumulation of newly synthesized core protein is reduced in cells exhibiting the transformed phenotype. However, syndecan-1 mRNA levels remained unaltered, suggesting translational suppression of syndecan-1 expression in these cells. This suppression was not temporally related to the Ha-*ras* expression but rather to the morphological changes of the epithelial cells.

## MATERIALS AND METHODS

### Cell Cultures

NOG-8 cells are a clone of the normal mouse mammary epithelial cell line (NMuMG) (Ciardiello *et al.*, 1988). NOG-8 cells have been transfected with a plasmid containing a glucocorticoid inducible MMTV-LTR promoter linked to a point mutated Ha-*ras* gene (NOG-8 *ras*). In these cells Ha-*ras* gene expression can be induced by dexamethasone (Ciardiello *et al.*, 1988). Cells were grown in RPMI-1640 cell culture medium (GIBCO, Paisley, UK) supplemented with 5% dextrancharcoal (DCC)-treated fetal calf serum (FCS), glutamine (1 mM), penicillin (100 IU/ml), and streptomycin (100 $\mu$g/ml). DCC treatment was used to eliminate steroids that might influence on the expression of the transfected gene. Dexamethasone (Sigma, St. Louis, MO) was supplemented to 1 $\mu$M when indicated.

For suspension cultures bacteriological dishes (10 cm in diameter, Nunc, Roskilde, Denmark) were covered with 1% agar (Sigma) in RPMI-1640 or phosphate-buffered saline (PBS) to prevent possible cell-substrate adhesion. Cells were trypsinized to a single cell suspension to get colonies derived from one cell. Cells were seeded at a density of $1 \times 10^2$ to $1 \times 10^5$/ml. Cell number was determined after trypsinization by using a Coulter Counter (Coulter Electronics, Harpenden, UK).

### cDNA Probes and a Monoclonal Antibody for Syndecan-1

PM-4 is a cDNA clone of mouse syndecan-1 (Saunders *et al.*, 1989), which detects two bands of syndecan-1 mRNA of 2.6 kilobase (kb) and 3.4 kb in normal mouse mammary cells. BS-9 (provided kindly by Dr. Kari Alitalo, University of Helsinki, Finland) is a v-Ha-*ras* cDNA clone, which detects Ha-*ras* mRNA of the 1.5 kb (Ellis *et al.*, 1980). BS-9 does not distinguish endogenous c-Ha-*ras* and transfected Ha-*ras* mRNAs in NOG-8 and in NOG-8 *ras* cells (Figure 1, +/− lanes at day 0). Monoclonal antibody (mAb) 281-2 recognizes the core protein of syndecan-1 (Jalkanen *et al.*, 1985).

### Immunofluorescence Analysis

To analyze syndecan-1 distribution, NOG-8 *ras* cells cultured on glass coverslips were washed two times with ice cold Dulbecco's phosphate buffered saline, pH 7.4, (DPBS) and fixed in methanol for 5 min. Cells were then washed with DPBS and incubated with mAb 281-2 (50 $\mu$g/ml) in DPBS containing 1% bovine serum albumin (BSA) or with 1% BSA-DPBS alone at room temperature (RT). After 30 min the coverslips were rinsed extensively in DPBS and then stained with fluorescein isothiocyanate conjugated anti-rat IgG (dilution 1:40) (Dako, Glostrup, Denmark) in 1% BSA-DPBS for 30 min at RT. The coverslips were washed in DPBS, rinsed in distilled water, and mounted with Glysergel (Dako). Immunofluorescence of the cultures was analyzed using confocal microscopy (CCM, EMBL, Heidelberg).

### Northern Blot Analysis

To prepare RNA, cells were washed twice with ice cold PBS, solubilized in 4 M GIT buffer (4 M guanidine isothiosyanate in 5 mM sodium citrate, pH 7.0, 0.1 M $\beta$-mercaptoethanol and 0.5% N-laurylsarcosine) and isolated by CsCl density centrifugation (Chirgwin *et al.*, 1979). For Northern blot analysis, RNA samples were separated by electrophoresis on 1% formaldehyde-agarose gel and transferred onto the GeneScreen Plus (New England Nuclear, Boston, MA) hybridization membrane. Filters were prehybridized in 1 M NaCl, 1% sodium dodecyl sulfate (SDS), 10% dextran sulfate, 5× Denhardt's solution, 100 $\mu$g/ml salmon-sperm DNA, and 50% formamide at 42°C. For hybridization, PM-4 or BS-9 probes were labeled with [$^{32}$P]-dCTP (Amersham, Arlington Heights, IL) using a multiprime system (Amersham) and then added to the hybridization solution overnight at 42°C. The filters were washed at 65°C for PM-4 and at 60°C for BS-9 in 2× SSC, 1% SDS and autoradiographed on Kodak X-Omat (Rochester, NY) X-ray film.

### Slot-Blot Radioimmunoassay for Syndecan-1

For quantitation of cell surface syndecan-1 expression, cells grown on a culture dish were washed three times with ice cold PBS and then incubated in 0.5 mM K-EDTA in PBS for 10 min at 4°C. Trypsin (Sigma, T-8128) was added to a final concentration of 20 $\mu$g/ml and incubated for 10 min at 4°C. Trypsin releases the ectodomain of syndecan-1 into the medium (Rapraeger and Bernfield, 1985). Then cells were scraped with a rubber policeman, or colonies were suspended, and trypsin inhibitor (Sigma, T-9128) was added to a final concentration of 100 $\mu$g/ml. Cells were centrifuged (10 min, 100 × *g*), supernatants were collected, and cells were counted by a Coulter Counter. Cells in suspension were harvested by allowing them to sedimentate for 5 min. After removal of supernatant colonies were washed three times with ice-cold PBS and treated with trypsin similar to the Petri dish cultures. For quantitation of syndecan-1 ectodomain

in the cell culture medium cells were washed three times with PBS and fresh media were changed. After 23 h media were collected and analyzed after centrifugation (10 min, 100 × *g*).

The amount of supernatant or cell culture medium corresponding $2 \times 10^5$ cells was loaded onto a cationic nylon membrane (Zeta Probe, Bio-Rad, Richmond, CA) in a slot-blot apparatus. The membrane was preincubated in the incubation buffer (10% FCS or 5% nonfat dry milk power, 1 mM natrium azide in PBS) for 1 h at RT. Radioactively labeled (chloramine-T method) mAb 281-2 was added to the incubation buffer to a final concentration of 10 000 cpm/ml and incubated overnight at 4°C. The filter was washed five to ten times with 0.2% Tween-20 in PBS for 10 min at RT and autoradiographed. The autoradiograph was analyzed by GelScan XL ultroscan densitometer (LKB, Bromma, Sweden) and GelScan XL 2400 software (LKB) and standardized with isolated ectodomain (Jalkanen *et al.*, 1987).

### *Immunoprecipitation and SDS-Polyacrylamide Gel Electrophoresis*

To determine the relative molecular mass for the ectodomain of syndecan-1, cells were labeled with 100 μCi/ml [$^{35}$S]-sulfate (New England Nuclear, Boston, MA) for 24 h. Samples were collected as described above for radioimmunoassay, and protease-inhibitors (1 mM phenylmethylsulfonyl fluoride [PMSF], 5 mM EDTA, 5 mM N-ethylmaleimide [NEN]) were added. Aliquots of equal radioactivity were taken to immunoprecipitation. Samples were preabsorbed with normal rabbit serum for 3 h at 4°C followed by incubation with Protein-A Sepharose CL-4B (Pharmacia LKB) for 3 h at 4°C in an end-over-end rotator. For immunoprecipitation mAb 281-2 was coupled to CNBr-activated Sepharose 4B (Pharmacia LKB) according to manufacture's instructions. Preabsorbed samples were incubated with mAb 281-2-Sepharose overnight at 4°C in an end-over-end rotator and then washed three times with ice cold RIPA-buffer (150 mM NaCl, 10 mM tris(hydroxymethyl)aminomethane [Tris]-HCl, pH 7.5, 0.2% SDS, 1% deoxycolic acid, 1% NP-40). Antigen-antibody complexes were dissociated by boiling samples in nonreducing SDS electrophoresis sample buffer. Immunoprecipitates were analyzed on a gradient (2–22%) SDS-PAGE (O'Farrel, 1975). After electrophoresis the gels were fixed in 20% trichloracetic acid for 1 h at RT and incubated sequentially in the following buffers at RT: 7.5% methanol/7.5% acetic acid for 5 min, 100% acetic acid for 30 min, 20% 2,5-difenyloxazole in methanol for 30 min, and distilled water for 1 h. After drying (Model 583 Gel Dryer, Bio Rad), the gels were autoradiographed. The [$^{14}$C]-methylated protein standards (Amersham) were myosin (200 kDa), phosphorylase-b (100 and 92.5 kDa), BSA (69 kDa), and ovalbumin (46 kDa).

### *Detection of Newly Synthesized Syndecan-1 Core Protein*

To study the accumulation of newly synthesized syndecan-1 core protein, cells were treated with Brefeldin A (BFA) (Sigma) at 10 μg/ml for times indicated in Figure 8. Stock solution of BFA (10 mg/ml) was solubilized in methanol and stored at −20°C. After BFA treatment cells were harvested by trypsinization and lysed in an immunoprecipitation buffer (150 mM NaCl, 20 mM Tris-HCl, pH 7.5, 0.5% Triton X-100, 1 mM PMSF, 5 mM EDTA, 5 mM NEN). Samples corresponding $7 \times 10^5$ cells were taken for immunoprecipitation as described above. After electrophoresis in 7.5% SDS-PAGE the proteins were transferred onto the Zeta Probe membrane using a electroblotting apparatus (2005 Transphor, LKB). Syndecan-1 core protein was detected with [$^{125}$I]-labeled mAb 281-2 as described above.

## RESULTS

### *A Point Mutated c-Ha-ras–induced Transformation of NOG-8 Mouse Mammary Epithelial Cell Line*

As a model for epithelial cell transformation, we have used a clone of NOG-8 *ras* cells. These cells have been isolated by transfecting the NOG-8 cell line with the point mutated c-Ha-*ras* gene under the control of the glucocorticoid inducible MMTV-LTR promoter. Activated c-Ha-*ras* proto-oncogene was originally isolated from the human EJ bladder carcinoma cell line (Shih and Weinberg, 1982).

The expression of the Ha-*ras* gene was induced in NOG-8 *ras* cells by adding 1 μM dexamethasone to the cell culture medium. In the absence of dexamethasone, NOG-8 *ras* cells expressed the same Ha-*ras* mRNA levels as the control NOG-8 cells (Figure 1, 0 d, Northern blot autoradiograph in the left top corner). This reflects the level of the normal c-Ha-*ras* mRNA expression in NOG-8 cells. After dexamethasone treatment NOG-8 *ras* cells expressed increased amounts of Ha-*ras* mRNA (Figure 1, + lanes). The maximal level of Ha-*ras* mRNA was reached after 5 days of dexamethasone treatment and was several fold higher than endogenous c-Ha-*ras* expression in NOG-8 cells. Dexamethasone treatment itself did not affect endogenous c-Ha-*ras* mRNA expression (Figure 1; − lanes). It has been previously shown that p21$^{ras}$ protein expression increases concomitantly with the Ha-*ras* mRNA expression in these cell lines (Ciardiello *et al.*, 1988).

Ha-*ras* expression enhanced the growth rate of the NOG-8 *ras* cells (Figure 1, ●). NOG-8 *ras* cells reached also higher density than normal NOG-8 cells. This effect was more evident when the serum concentration was lowered from 5% to 1%. Under these conditions, pro-

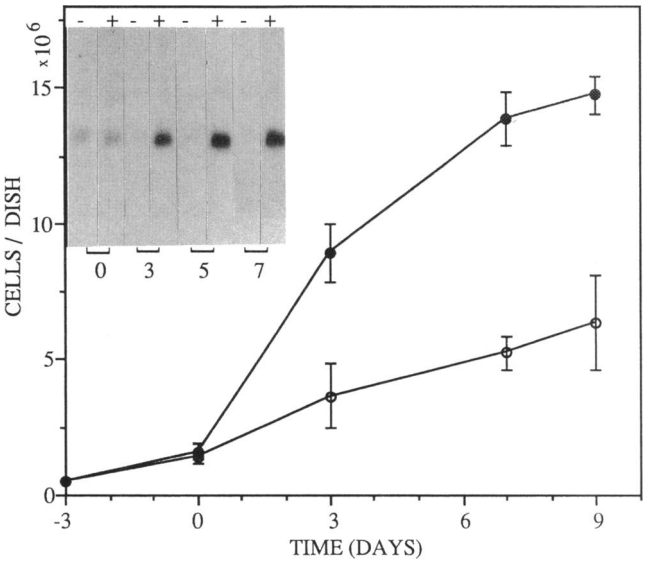


**Figure 1.** The effect of the transfected Ha-*ras* expression on cell growth. NOG-8 cells, ○. NOG-8 *ras* cells, ●. Cells were plated on day −3 and the dexamethasone treatment was initiated on day 0. Northern blot: The mRNA expression of the Ha-*ras* gene in NOG-8 *ras* cells. NOG-8 cells were designated as − lanes and NOG-8 *ras* cells as + lanes. Dexamethasone exposure times are indicated below the Northern blot in days. Cells were plated 2 d before initiation of dexamethasone exposure on day 0.

liferation of NOG-8 cells stopped, but dexamethasone-stimulated NOG-8 *ras* cells continued to divide.

The expression of the transfected Ha-*ras* gene affected cell morphology. There were no morphological differences between NOG-8, unstimulated NOG-8 *ras* (Figure 2A), or chronically Ha-*ras* expressing NOG-8 *ras* (Figure 2B) cells in recently confluent or subconfluent cultures. All cell lines showed a typical epithelial cell morphology with a cuboidal and flattened shape. However, when cell cultures became confluent, Ha-*ras* expressing NOG-8 *ras* cells lost contact inhibition and started to form foci (Figure 2, D and E). NOG-8 (Figure 2C) and unstimulated NOG-8 *ras* cells remained epithelial-like at this stage, and they did not form foci even if cultured several days after they had reached confluence. NOG-8 *ras* cells after Ha-*ras* induction were also capable to form growing colonies in suspension (Figure 2F).

These data show that NOG-8 *ras* cells are fully transformed when they have expressed Ha-*ras* gene for several days. Therefore they can be used as a model to study transformation of epithelial cells. Our major interest was to investigate the regulation of syndecan-1 expression during the process of Ha-*ras* transformation of NOG-8 cells.

### *Ha-ras-induced Transformation Reduces Cell Surface Syndecan-1 Expression*

Previously, it has been suggested that syndecan-1 regulates and stabilizes epithelial cell morphology by connecting cytoskeleton to ECM (Rapraeger *et al.*, 1986; Leppä *et al.*, 1991, 1992). To study the expression of syndecan-1 during Ha-*ras*–induced changes in morphology, NOG-8 *ras* cells were cultured for 10 d until they had reached confluence and started to form foci. The distribution of syndecan-1 was then examined by immunofluorescence and confocal techniques (Figure 3). NOG-8 *ras* cells growing as a monolayer around foci stained intensively with a monoclonal antibody against syndecan-1 core protein (Figure 3A). Cells in the areas

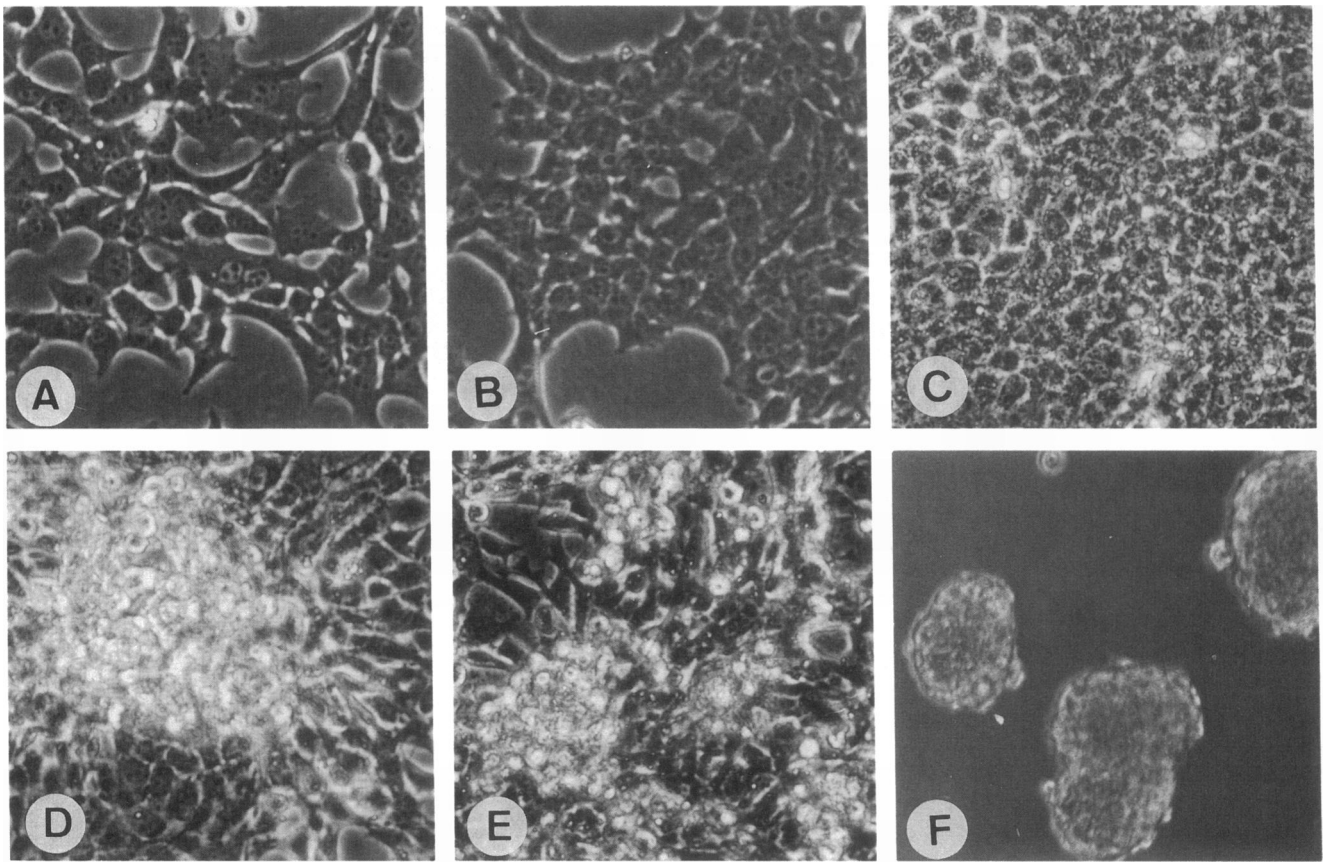


**Figure 2.** The effect of Ha-*ras* expression on epithelial cell morphology. (A) Semiconfluent NOG-8 *ras* cells before induction of Ha-*ras* expression. (B) Semiconfluent NOG-8 *ras* cells chronically expressing Ha-*ras*. (C) Confluent NOG-8 cells. (D) Confluent NOG-8 *ras* cells 5 d after Ha-*ras* induction. (E) Confluent NOG-8 *ras* cells, chronically expressing Ha-*ras*. (F) NOG-8 *ras* cells chronically expressing Ha-*ras* and cultured in suspension for 10 d. A and B are 3 d, C and D are 7 d, and E is 11 d after plating.

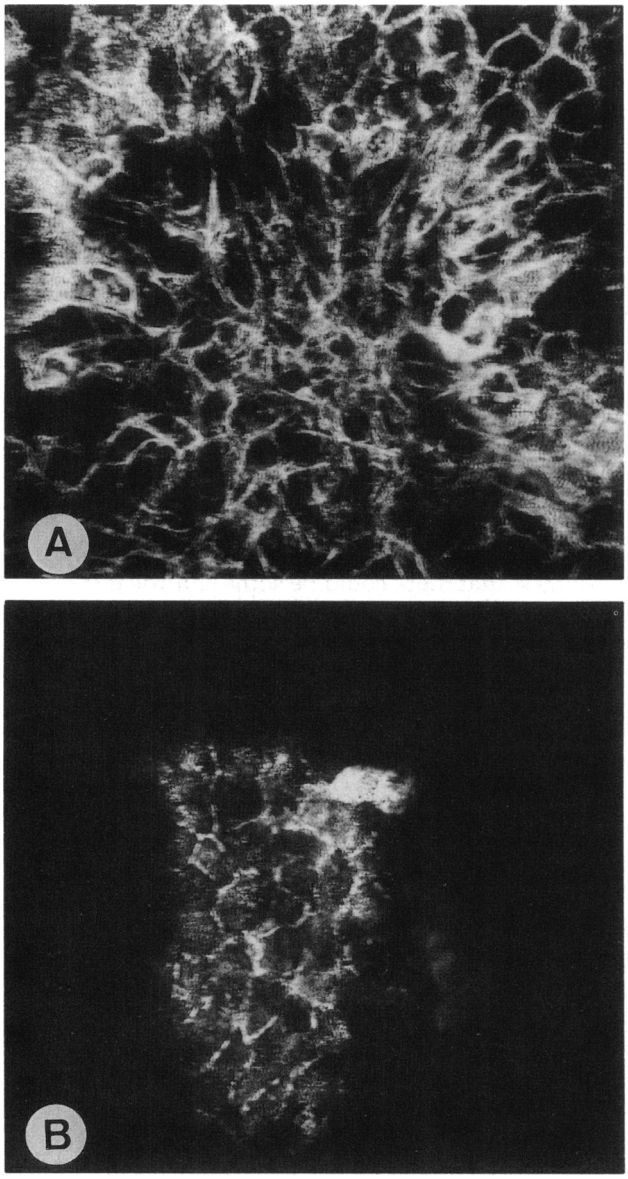


**Figure 3.** Distribution of syndecan-1 in Ha-*ras* expressing NOG-8 cells. Syndecan-1 was localized by immunofluoresence and confocal microscopy. (A) Cross-section through a focus formation and surrounding monolayer. (B) Same focus formation as in panel A but cross-section at a higher horizontal level.

of foci also stained positively for syndecan-1 (Figure 3, A and B). However, when the staining patterns were compared at same horizontal level, it was obvious that the staining was brighter in cell monolayers than in cells forming foci (Figure 3A).

Because immunofluorescence data suggested a decrease in syndecan-1 expression during Ha-*ras*–induced transformation, syndecan-1 expression was quantitated in more detail. For this, the ectodomains of syndecan-1 from NOG-8 and NOG-8 *ras* cells were collected by mild trypsinization and loaded onto a cationic nylon membrane for radioimmune quantitation (Figure 4A). Collective data from several experiments of this kind are shown in Figure 4B. The amount of syndecan-1 in NOG-8 cells declined slightly during the growth of cultures (Figure 4B, closed columns). However, the amount of cell surface syndecan-1 was markedly reduced in Ha-*ras*–expressing cells, when they grew as foci (Figure 4B, open column, 11 days after plating), indicating a correlation between reduced syndecan-1 expression and the disability of epithelial cells to maintain their morphology.

To further investigate syndecan-1 expression in transformed cells, we analyzed the most extreme situation. For this purpose cell surface syndecan-1 was collected from normal and transformed cells 1 d after plating and from transformed cells grown anchorage-independently in suspension as colonies for 10 d (Figure 5A). The amount of cell surface syndecan-1 in chronically Ha-*ras* expressing NOG-8 *ras* cells grown in suspension was only about 10 to 20% of the amount of syndecan-1 that was expressed by cells attached to the cell culture dish (Figure 5A, column d compared to the columns a–c, f). This reduction in syndecan-1 expression was reversible. When colonies were trypsinized and plated as single cells onto a culture dish, the level of cell surface syndecan-1 returned to that of cells grown on a plate (Figure 5A, column f). The expression of cell surface syndecan-1 was about the same in NOG-8, unstimulated NOG-8 *ras*, and chronically Ha-*ras* expressing NOG-8 *ras* subconfluent cell cultures (Figure 5A, columns a, b, c, respectively). These cultures were also indistinguishable by morphological criteria as shown earlier in Figure 2.

One possible mechanism reducing the amount of syndecan-1 at the cell surface is its increased shedding into the cell culture medium (Jalkanen *et al.*, 1987). The

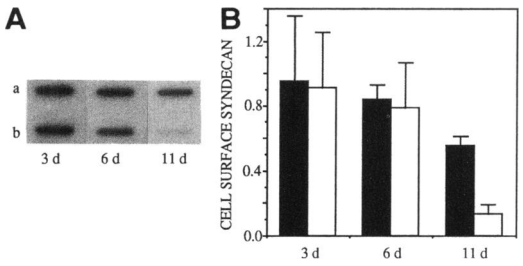


**Figure 4.** Quantitation of cell surface syndecan-1 expression in monolayer cultures. (A) An example of slot-blot autoradiograph. Row a: NOG-8 cells. Row b: NOG-8 *ras* cells. The ectodomain of syndecan-1 were collected by mild trypsinization from $2 \times 10^5$ cells, blotted on a cationic nylon membrane, and detected with [$^{125}$I]-labeled mAb 281-2. (B) Closed columns: NOG-8 cells. Open columns: NOG-8-*ras* cells. Time is indicated as days after plating. The expression of Ha-*ras* was induced on day 3 in NOG-8-*ras* cells (row b or open columns). Three separate samples were collected on each day and mean +/− SD are shown.

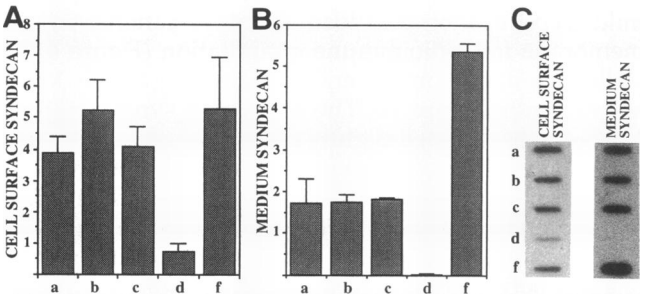


**Figure 5.** (A) Comparison of cell surface syndecan-1 amounts in monolayer and suspension cultures. Column a: NOG-8 cells in monolayer. Column b: NOG-8-*ras* cells before Ha-*ras* induction, in monolayer. Column c: NOG-8-*ras* chronically expressing Ha-*ras*, in monolayer. Column d: NOG-8-*ras* chronically expressing Ha-*ras*, in suspension. Column f: NOG-8-*ras*, chronically expressing Ha-*ras*, cultured first in suspension and then plated on cell culture dish. (B) Comparison of medium syndecan-1 in monolayer and in suspension cultures. Columns are as above. All samples were collected 24 h after plating, except for cells grown in suspension, which were cultured for 10 d. Each column gives the average value from three independent samples and +/− SD. (C) The examples of slot-blot autoradiographs. Symbols as in A and B.

accumulation of the ectodomain of syndecan-1 was quantitated by collecting the cell culture medium during 23 h and blotting it onto a cationic nylon membrane for radioimmunoassay. Anchorage-independently grown Ha-*ras* expressing NOG-8 *ras* cells shed minor amounts of syndecan-1 ectodomain into the cell culture medium (Figure 5B, column d) compared to their adhesive and normal counterparts (Figure 5B, columns a–c). This indicates that the suppression of the expression of cell surface syndecan-1 in these cells is not because of increased shedding of syndecan-1 ectodomain. The replated Ha-*ras* expressing cells grown in suspension showed relatively higher amounts of syndecan-1 in the cell culture medium compared to their cell surface levels (Figure 5, A and B, column f).

The suppression of the cell surface syndecan-1 in transformed cells was not because of intracellular accumulation, because permealized NOG-8 *ras* cells in foci stained weakly for syndecan-1 (Figure 3). Furthermore, it was not possible to release more immunopositive material when trypsinated chronically *ras* expressing NOG-8 *ras* suspension cells were extracted with 1% Triton-0.5 mM KCl buffer (corroborative data).

The relative molecular mass of syndecan-1 ectodomain in chronically Ha-*ras* expressing NOG-8 *ras* cells in suspension was greater than that isolated from normal NOG-8 monolayer cells (Figure 6). The estimated average molecular masses were 400 and 300 kDa, respectively, and similar size differences were also observed when analyzed by Sepharose CL-4B column (data not shown). Thus, Ha-*ras*–transformed cells synthesize low amounts of syndecan-1 with a higher molecular size than syndecan-1 from normal epithelial cells.

### *Syndecan-1 mRNA Level Is Unaltered in Ha-ras–Transformed Cells*

Syndecan-1 gene expression in NOG-8, in unstimulated NOG-8 *ras*, and in chronically Ha-*ras*–expressing NOG-8 *ras* cultures was analyzed from total RNA samples by the Northern technique using syndecan-1 cDNA as a probe (Saunders *et al.*, 1989). The levels of both 2.6 and 3.4 kb mRNAs remained almost the same in all three monolayer cultures (Figure 7A, lanes 1–3). In addition, the cell morphology did not affect syndecan-1 mRNA level, because subconfluent cultures (Figure 7A, lanes 1–3) showed nearly the same amounts of syndecan-1 mRNA as the foci forming cultures (Figure 7A, lanes 11 and 12). Furthermore, Ha-*ras* expressing NOG-8 *ras* cells in suspension (Figure 7C, lane 2) expressed the same syndecan-1 mRNA levels as NOG-8 cells in monolayer (Figure 7C, lane 1). Minor alterations in syndecan-1 mRNA levels in chronically *ras*-expressing NOG-8 *ras* cells on plastic (Figure 7A, lanes 3, 6, 8 and 12) suggest that there could be some control at the level of transcription. However, as shown above cell surface syndecan-1 expression was markedly reduced in transformed NOG-8 *ras* cells (Figures 3–5). Therefore, it is likely that the suppression of syndecan-1 occurs mostly at a posttranscriptional level in transformed NOG-8 *ras* cells.

### *The Expression of Syndecan-1 Is Suppressed Translationally in Ha-ras–expressing Epithelial Cells*

Proteoglycans such as syndecan-1 are transported immediately after translation into the Golgi complex, where the GAG side chains are synthesized. The molecular mass of proteoglycan increases concomitantly with GAG side chain elongation. BFA, a fungal metabolite, blocks the anterograde protein transport out of the endoplasmic reticulum (Klausner *et al.*, 1992) and inhibits GAG-side chain elongation as has been shown for human melanoma-associated proteoglycan (Spiro *et al.*, 1991). BFA treatment was used as a tool to estimate the amount of newly synthesized syndecan-1 core protein. BFA has no effect on overall protein synthesis at

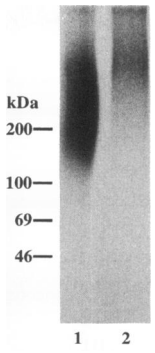


**Figure 6.** Determination of the molecular mass of syndecan-1 ectodomain. Lane 1: Syndecan-1 from NOG-8 monolayer culture. Lane 2: Syndecan-1 from NOG-8 *ras* chronically Ha-*ras* expressing suspension culture. [$^{35}$S]-sulfate labeled syndecan-1 ectodomains were immunoprecipitated and analyzed on a gradient (2–22%) SDS-PAGE.

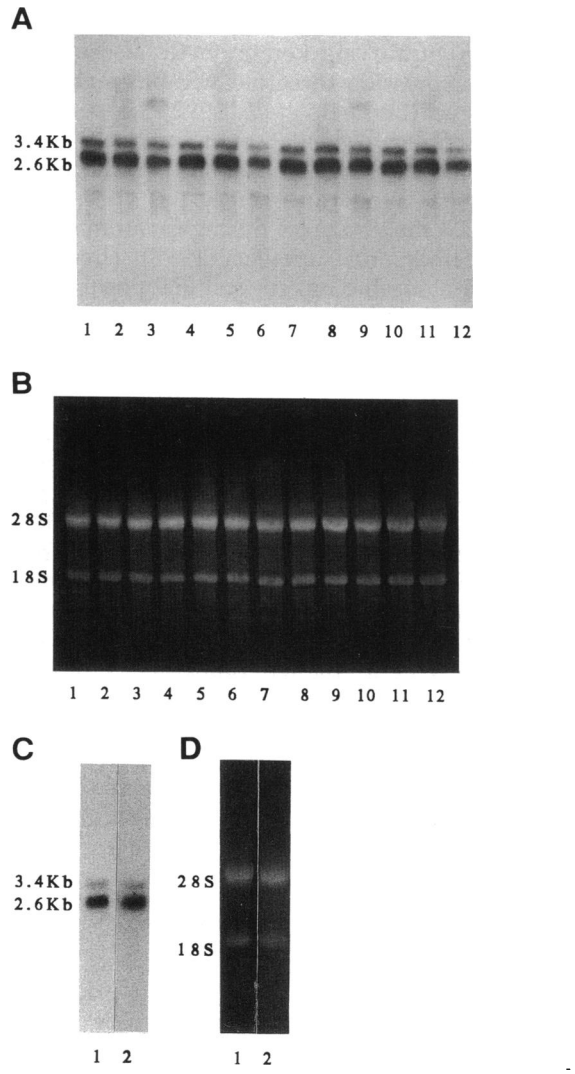


**Figure 7.** Regulation of syndecan-1 mRNA content in NOG-8 cell lines. (A) Syndecan-1 mRNA expression in monolayers. Lanes 1, 4, 7, and 10: NOG-8 cells, 3-, 6-, 8-, and 10-d cultures, respectively. Dexamethasone was added at day 3. Lanes 2, 5, 8, and 11: NOG-8 *ras* cells, cultured as above. Lanes 3, 6, 8, and 12: NOG-8-*ras* cells, chronically expressing Ha-*ras*, cultured as above. (B) The gel (A) stained with ethidium bromide. (C) Syndecan-1 mRNA expression in suspension. Lane 1: NOG-8 cells, cultured 12 d on a cell culture dish. Lane 2: NOG-8-*ras* cells chronically expressing Ha-*ras* cultured 12 d in suspension. (D) The gel (C) stained with ethidium bromide. RNA samples were separated on 1% formaldehyde agarose gels and transferred on Gene Screen Plus (New England Nuclear) hybridization membrane. The filters were hybridized with [$^{32}$P]-dCTP labeled PM-4, a cDNA clone of mouse syndecan-1, which detects two bands of 2.6 kb and 3.4 kb.

concentrations ≤50 μg/ml (Spiro *et al.*, 1991) and allows visualization of the syndecan-1 core protein as a sharp band by Western-blot analysis. To confirm that BFA works well in the different cell cultures, NOG-8 monolayer and Ha-*ras*–expressing NOG-8 *ras* suspension cultures were labeled with [$^{35}$S]-sulfate for 8 h with and without 10 μg/ml BFA. The typical smears of syndecan-1 could be observed on a gradient SDS-PAGE loaded with samples isolated from BFA-untreated cell cultures, but nothing could be detected on the lanes loaded with samples isolated from BFA-treated cell cultures. This indicates that BFA inhibits similarly the GAG synthesis in monolayer and suspension cultures. The cell cultures did not show any morphological changes during 8-h incubation time, suggesting that they tolerate well short BFA treatment.

To study the amount of newly synthesized syndecan-1 core protein, normal NOG-8 monolayer cultures and Ha-*ras*–expressing NOG-8 *ras* suspension cell cultures were treated with 10 μg/ml BFA for 1, 3, and 6 h. Cells were then lysed in the immunoprecipitation buffer, and samples were analyzed on 7.5% SDS-PAGE after immunoprecipitation with mAb 281-2-Sepharose after electroblotting onto a cationic nylon filter and detection with [$^{125}$I]-labeled mAb 281-2. Syndecan-1 core protein, ~80 kDa in SDS-PAGE, accumulated in normal NOG-8 monolayer cell cultures representing the amount of syndecan-1 core protein synthesized during BFA treatment (Figure 8, lanes 2, 4 and 6). However, the accumulation of the syndecan-1 core protein was below the detection limit in Ha-*ras*–expressing NOG-8 *ras* suspension cell cultures (Figure 8, lanes 1, 3, 5). This suggests that the syndecan-1 mRNA translation was suppressed in these cells, because syndecan-1 mRNA levels were the same in the both cell culture conditions (Figure 7C).

## DISCUSSION

We have studied the expression of syndecan-1 in Ha-*ras*–transformed epithelial cells. Our major finding in this study is a reduction of cell surface syndecan-1, when Ha-*ras*–transformed epithelial cells show anchorage-independent phenotype. The accumulation of newly synthesized syndecan-1 core protein during BFA treatment was only observed in normal cells although

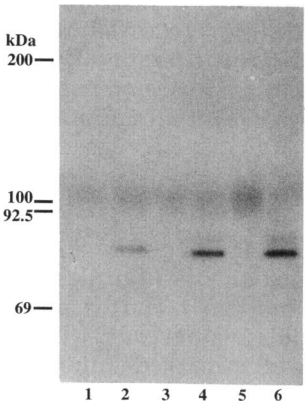


**Figure 8.** Accumulation of newly synthesized syndecan-1 core protein. Lanes 2, 4, and 6: NOG-8 monolayer cultures treated 1, 3, and 6 h with BFA. Lanes 1, 3, and 5: Chronically Ha-*ras*–expressing NOG-8 *ras* suspension cell cultures treated similarly with BFA. Samples were analyzed on 7.5% SDS-PAGE and detected with [$^{125}$I]-labeled mAb 281-2 after electroblotting on a cationic nylon membrane.

both cell types revealed equal syndecan-1 mRNA levels. This indicates that syndecan-1 expression is suppressed translationally in Ha-*ras*–transformed cells.

### *Morphological Changes and Cell-Substrate Adhesion Correlate to Altered Syndecan-1 Expression*

Normal epithelial cells, when EDTA-rounded and cultured in suspension, lose cell surface syndecan-1 by shedding the ectodomain into the culture medium (Jalkanen *et al.*, 1987). This shedding is probably because of a protease cleavage of syndecan-1 core protein, because the ectodomain is separated from the membrane domain. Maturating B-cells express cell surface syndecan-1 in lymph nodes, but when circulating in the blood stream they are syndecan-1 negative, reexpressing syndecan-1 again in tissues as plasma cells (Sanderson *et al.*, 1989). In this work, Ha-*ras*–transformed cells grown as colonies in suspension contained low amounts of cell surface syndecan-1. The reduction was not because of increased shedding of syndecan-1 ectodomain (Figure 5B). According to these results, maintaining of syndecan-1 at the cell surface seems to be related to cell-substrate adhesion regardless of the mechanism that causes anchorage-independent growth. Our findings also support the theory that syndecan-1 is involved in the anchoring of epithelial cells to matrix (Jalkanen *et al.*, 1991).

Syndecan-1 expression has also been shown to associate with epithelial phenotype in epithelial-fusiform conversion of steroid-induced phenotype shift of mouse mammary tumor S115 cells (Leppä *et al.*, 1991, 1992). TGF$\beta$ treatment of NMuMG has been shown to change the composition of syndecan-1 GAG-side chains, and this change, which increases the proportion of chondroitin sulfate, associates with elongation of epithelial cells (Rasmussen and Rapraeger, 1988). Induction of Ha-*ras* expression in subconfluent cells failed to cause changes in cell morphology, and it also failed to alter syndecan-1 expression (Figures 2–5). Syndecan-1 expression, however, declined in confluent cells, which responded to Ha-*ras* induction by forming foci in the monolayer cultures. These measurements also included cells with normal morphology, and thus the results represent average values of two distinct phenotype populations of the monolayer. Indeed, it was shown that cells in foci expressed less syndecan-1 than cells in monolayer around foci (Figure 3).

### *Posttranscriptional Regulation of Syndecan-1 Expression*

During tissue formation and regeneration a close correlation has been demonstrated with immunoreactive syndecan-1 and its mRNA expression (Elenius *et al.*, 1991; Vainio *et al.*, 1991). In a developing kidney, however, a marked stimulation of immunopositive syndecan-1 expression has been observed without any detected changes in syndecan-1 mRNA levels (Vainio *et al.*, 1992). Stratifying keratinocytes also increase syndecan-1 expression threefold without a change in the mRNA level (Bernfield *et al.*, 1992). More evidence for posttranscriptional regulation of syndecan-1 expression comes from the study of Yeaman and Rapraeger who showed that syndecan-1 mRNA is specifically accumulated by macrophages during inflammatory recruitment (Yeaman and Rapraeger, 1993). These results together with our findings suggest that posttranscriptional regulation might be a common way to control syndecan-1 expression. Furthermore, our data suggest that this regulation may occur at a translational level, because the accumulation of newly synthesized syndecan-1 core protein was reduced in spite of high mRNA level in Ha-*ras*–transformed NOG-8 *ras* cells. Translational regulation may provide a mechanism to control syndecan-1 expression during early embryo morphogenesis, because immunopositive syndecan-1 has been detected in the mouse embryos at the 4-cell stage (Sutherland *et al.*, 1991), but the overall nuclear gene transcription is suppressed at this stage and the most of translationally active mRNAs are of maternal origin (Gilbert, 1988). Interestingly, a recently described protein, epimorphin, which is another developmentally regulated cell surface protein that participates in epithelial-mesenchymal interactions during mouse morphogenesis similar to syndecan-1, is also posttranscriptionally regulated (Hiral *et al.*, 1992).

### *Transformation Alters Expression of Syndecan-1*

Tissue matrix system consists of nuclear, cytosol, and ECM, which is a continual matrix network. This system is disrupted in transformed cells leading to an altered phenotype and possible altered signal transduction (Pienta *et al.*, 1989). ECM receptors are one part of the system between ECM and intracellular cytoskeleton. Signal transduction through fibronectin receptor can induce protease expression, which suggests that the events in ECM can directly be reflected to gene expression (Werb *et al.*, 1989). As shown in this study, translational suppression of syndecan-1 expression associates with morphological changes induced by Ha-*ras* transformation.

Immunohistochemical studies have shown that the expression of syndecan-1 correlates with histopathological malignancy of mouse skin tumors and tumors derived from injected malignant keratinocytes in a hairless mouse (Inki *et al.*, 1991, 1992b). Immunopositive staining is weaker in tumors showing anaplastic phenotype than those graduated as adenomas. The reduction of syndecan expression during transformation process is linked to concomitant increase of the molecular mass in some cases as shown in this (Figure 6) and previous studies with malignant keratinocyte cultures (Inki *et al.*, 1992a). Hence, there seems to be a tendency that

the expression of cell surface syndecan-1 is reduced in malignant transformation of several different models. However, the level of regulation may vary depending on the cell type. The suppression of syndecan-1 and concomitant posttranslational modifications may disturb the normal function of syndecan-1 and may maintain the malignant growth of cells.

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