Suppression of Tumorigenicity by Plakoglobin: An Augmenting Effect of N-Cadherin

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Abstract. Plakoglobin is a major component of the submembranal plaque of adherens junctions and desmosomes in mammalian cells. It is closely related to the Drosophila segment polarity gene armadillo which has a role in the transduction of transmembrane signals that regulate cell fate. Like its close homologue β-catenin, plakoglobin can associate with the product of the tumor suppressor gene APC that is linked to human colon cancer. We have studied the effect of plakoglobin overexpression, and the cooperation between plakoglobin and N-cadherin, on the morphology and tumorigenic ability of cells either lacking, or expressing cadherin and α- and β-catenin.

Overexpression of plakoglobin in SV40-transformed 3T3 (SVT2) cells suppressed the tumorigenicity of the cells in syngeneic mice. Transfection with N-cadherin conferred an epithelial phenotype on the cell culture, but had no significant effect on the tumorigenicity of the cells. Cotransfection of plakoglobin and N-cadherin into SVT2 cells, however, was considerably more effective in tumor suppression than plakoglobin overexpression alone. Finally, transfection of plakoglobin into a human renal carcinoma cell line that expresses neither cadherins nor plakoglobin, or α- and β-catenin, resulted in a dose-dependent suppression of tumor formation by these cells in nude mice. Plakoglobin, in these cells, did not exhibit junctional localization and was diffusely distributed in the cytoplasm, with a significant amount of the protein also localized in the nucleus. The results suggest that plakoglobin can efficiently suppress the tumorigenicity of cells in the presence of, or independently of the cadherin-catenin complex.

Recent studies have established that the modulation of cell adhesion, using genetic or immunochemical approaches, could exert a major effect on the invasive or metastatic potential of tumor cells. Overexpression of various junctional molecules, including the adhesion receptor molecules integrin and cadherin, and the plaque proteins vinculin and α-actinin, was shown to increase the adhesiveness of the transfected cells and effectively suppress their tumorigenicity (Behrens et al., 1989; Rodríguez Fernández et al., 1992a,b; Glück et al., 1993; Vleminckx et al., 1991; Navarro et al., 1991; Frixen et al., 1991; Giancotti and Rousslahti, 1990; Tsukita et al., 1993). This suggests that a highly adhesive phenotype may not be compatible with malignant growth. Similarly, antibody-mediated inhibition of cell adhesion and antisense suppression of junctional protein expression led to a decrease in cell adhesion, and, consequently, to an increase in tumorigenic capacity (Behrens et al., 1989; Vleminckx et al., 1991; Rodríguez Fernández et al., 1993; Glück and Ben-Ze’ev, 1994).

In the present study, we have investigated the ability of plakoglobin, a major constituent of the submembrane plaque of cell–cell adherens junctions (Cowin et al., 1986) to affect tumorigenicity, when overexpressed either alone, or in conjunction with cadherins. We have chosen to study plakoglobin for the following reasons: (a) Previous studies on the potential role of junctional plaque components in tumor suppression were conducted with proteins associated with both cell–cell and cell–matrix adhesions. It was therefore of interest to determine whether molecules which are uniquely associated with cell–cell junctions (such as plakoglobin), could have a similar effect. (b) Recent studies have suggested that malignant transformation, in certain tumor cell lines, correlates with reduced plakoglobin expression (Navarro et al., 1993; Sommers et al., 1994). (c) In addition, plakoglobin is closely related to the Drosophila segment polarity gene armadillo (Peifer and Weischaus, 1990; Peifer et al., 1992) which was demonstrated to function in the transduction of transmembrane signals that regulate cell fate (Bejsovec and Wieschaus, 1993). (d) Plakoglobin, like its close homologue β-catenin (Su et al., 1993; Rubinfeld et al., 1993), can associate with the product of the tumor suppressor gene APC (Shibata et al., 1994; Hülsken et al., 1994) that is linked to human co-
Materials and Methods

Cell Culture and Transfection

Balb/c 3T3 and SVT2 cells, which are Balb/c 3T3 cells transformed by SV40, MDCK, and A431 cells were all grown in Dulbecco’s modified Eagle medium plus 10% calf serum (GIBCO BRL, Gaithersburg, MD). Renal carcinoma cell lines of the KTCTL series were obtained from Dr. M. Zöller (The German Cancer Research Center, Heidelberg, FRG). These lines were established from human renal tumors whose clinical and pathological characteristics were defined. The cells were grown in RPMI medium containing 10% calf serum.

Cells were transfected with a full-length human plakoglobin cDNA (Franke et al., 1989) or the human N-cadherin (Salomon et al., 1992). The cDNAs were cloned into the EcoRI site of the polylinker of the pJ4fl expression vector (Rodríguez Fernández et al., 1993) that consists of the Mo-MuLV LTR promoter-enhancer sequence, the SV40 small t-antigen intron, and the SV40 large T polyadenylation signal in the pBR322 plasmid. The neomycin resistance (neo) gene, which was cotransfected with the pJ4fl construct containing the full-length human plakoglobin or N-cadherin, was subcloned into the pSVL expression vector. Transfection was carried out by the calcium phosphate precipitation method and colonies resistant to 500 µg/ml hygromycin (Calbiochem, San Diego, CA) and 500 µg/ml neomycin (Calbiochem, San Diego, CA) were isolated.

The ability of the different transfected clones to grow in suspension in soft agar was determined as described (Rodríguez Fernández et al., 1993). The distribution of cells in the different phases of the cell cycle was determined by flow cytometry and the percentage of cells in the various stages of the cell cycle was chosen since it does not express detectable levels of this protein (Fig. 1 A), while expressing α- and β-catenin (Figs. 1 C and 2 A). In contrast, 3T3 cells express significant levels of plakoglobin (Fig. 1, A and B). The tumors were excised and examined histologically after HE staining. In addition, frozen sections of the excised tumors were immunostained with antibodies to plakoglobin, N-cadherin, and vinculin.

Results

Suppression of Tumorigenicity in SVT2 Cells Transfected with Plakoglobin

To study the effect of plakoglobin on tumorigenicity, a highly malignant SV40-transformed 3T3 cell line, SVT2, was chosen since it does not express detectable levels of this protein (Fig. 1 A), while expressing α- and β-catenin and N-cadherin (Figs. 1 C and 2 A). In contrast, 3T3 cells express significant levels of plakoglobin (Fig. 1, A and B). SVT2 cells were cotransfected with a full-length plakoglobin cDNA and the neo gene and colonies resistant to 500 µg/ml hygromycin (Calbiochem, San Diego, CA) and 500 µg/ml neomycin (Calbiochem, San Diego, CA) were isolated. Western blots of equal amounts of total cell protein from such clones were incubated with anti-plakoglobin antibody and several SVT2 clones expressing plakoglobin at different levels were identified (Fig. 1 A). Southern blot hybridization indicated that multiple copies...
of the transgene were integrated into the genome of the positive clones (data not shown), and Northern blot hybridization with the plakoglobin cDNA showed a correlation between the level of the plakoglobin protein and RNA in the different transfected SVT2 clones (Fig. 1 B).

The forced expression of plakoglobin in SVT2 cells did not lead to gross changes in cell shape (Fig. 1, D and F), or actin and vinculin organization (data not shown). Immunofluorescent staining revealed, however, that the transfected plakoglobin was localized at cell–cell junctions (Fig. 1 G), indicating that the other junctional protein(s), necessary for the interaction of plakoglobin with the membrane were available within the cells.

To study the effect of plakoglobin overexpression on the tumorigenic phenotype, the ability of SVT2 clones expressing different levels of plakoglobin to grow in soft agar and the formation of tumors in syngeneic mice were examined (Table I). While no significant differences were obtained in the cloning efficiency in agar of the various plakoglobin-expressing SVT2 clones (~23% colony formation), there was a dramatic inhibition of tumorigenicity in cells expressing high levels of plakoglobin (Table I). This inhibition corresponded to the level of plakoglobin expressed: i.e., the clone expressing the highest plakoglobin level was the least tumorigenic, while the clone expressing only low levels of plakoglobin was as tumorigenic as the parental SVT2 (Table I).

### Augmenting Action of N-Cadherin and Plakoglobin in the Inhibition of Tumorigenicity

To investigate the possible cooperation between cadherin and plakoglobin in suppressing the tumorigenicity of SVT2 cells, N-cadherin was overexpressed in these cells, either alone, or together with plakoglobin (Fig. 2). Unlike 3T3 cells, SVT2 cells express low levels of N-cadherin (Fig. 2 A, compare lanes 1 and 4). After transfection with N-cadherin cDNA, colonies expressing increased levels (up to 14-fold higher than control SVT2, see Table II) of N-cadherin were obtained (Fig. 2 A, CAD1 and CAD2). These clones gained an epithelial morphology (Fig. 3 A), with N-cadherin, α-, and β-catenin present at cell–cell borders (Figs. 2 B, 3, B and C). Immunoblot analysis with antibodies to β-catenin revealed that the level of this protein increased in the N-cadherin overexpressing clones (Fig. 2 A). The tumorigenic ability of SVT2 clones overexpressing N-cadherin was, however, not significantly affected when compared to parental SVT2 cells, or to hygro control cells (Table II). This is in sharp contrast to the plakoglobin-transfected cells which did not show an increase in neither α- or β-catenin, nor in cadherin expression (Figs. 1 A and 2 A), but efficiently suppressed the tumorigenic ability of cells (Table I). In addition, growth in agar indicated that the cadherin-transfected cells remained anchorage independent, like the parental, nontransfected cells (Fig. 4).

To study the interrelationships between plakoglobin (which does not induce shape changes, but is tumor suppressive) and N-cadherin (which causes epithelialization, but does not reduce tumorigenicity), we have double-transfected SVT2 cells with cDNAs encoding the two proteins (Fig. 2 A, CAD/PG1-5). The morphology of the double overexpressing clones was variable (Fig. 3 D), largely reflecting differences in the level of cadherin expressed by the cells (Table II). Immunofluorescence analysis of these clones invariably showed that plakoglobin and N-cadherin, as well as α- and β-catenin were enriched at cell–cell junctions (Fig. 2, D and E, 3, E and F). In addition, in cells doubly transfected with plakoglobin and N-cadherin, the level of Triton X-100 insoluble plakoglobin was higher than in cells transfected with plakoglobin alone (Fig. 2 A, 54% vs 42%, in CAD/PG3 vs PG3). These cells also displayed a drastically reduced tumorigenicity as compared to either control SVT2, or even to the clones expressing plakoglobin alone (Table II). Examination of colony formation by the different clones in soft agar revealed that while the number of colonies in agar was similar to that of SVT2 cells, or of cells overexpressing the individual proteins, the size of the colonies in the double transfectants was significantly smaller (Fig. 4 D), suggesting a decrease in growth rate. The results summarized in Fig. 5 demonstrate that cells transfected with either plakoglobin or cadherin have a slower growth rate than control-untransfected cells and that double-transfected clones show slowest proliferation. Possible changes in cell cycle in the different clones was directly investigated by flow cytometry (Table III). The less tumorigenic clones contained a smaller number of cells in the S phase and an increased number of cells in the G1 and G2 phase, with the double

| Table I: Tumorigenicity of Plakoglobin-transfected SVT2 Cells |
|-----------------|----------------|----------------|
| **Cell line**   | **Tumor incidence** | **PG level** |
| SVT2            | 5/5             | 0             |
| N1              | 5/5             | 0             |
| PG1             | 4/5             | 3.5           |
| PG2             | 3/5             | 12.8          |
| PG3             | 3/5             | 14.2          |
| PG4             | 1/5             | 110.7         |

Groups of five Balb/C syngeneic mice were injected with 3 × 10⁶ cells/mouse and the development of tumors was followed for 3 mo. Animals with tumors died within 4–5 wk, while the tumor-free animals did not develop tumors even after 3 mo when the experiment was terminated.

N1, neo SVT2 control.

*The levels of plakoglobin were determined by densitometer scanning of the gel shown in Fig. 1 A and taking the value of MDCK cells as 100%.

| Table II: Tumorigenicity of Plakoglobin- and N-Cadherin-transfected SVT2 Cells |
|-----------------|----------------|----------------|
| **Cell line**   | **Tumor incidence** | **PG** | **N-CAD** |
| SVT2            | 5/5             | 0     | 0         |
| H1              | 5/5             | 0     | 0         |
| CAD1            | 5/5             | 0     | 0         |
| CAD2            | 5/5             | 0     | 0         |
| PG3             | 3/5             | 0     | 0         |
| CAD/PG1         | 1/5             | 0     | 0         |
| CAD/PD2         | 1/5             | 0     | 0         |
| CAD/PDG3        | 1/5             | 0     | 0         |
| CAD/PDG4        | 1/5             | 0     | 0         |

Groups of five syngeneic Balb/C mice were injected s.c. with 3 × 10⁶ cells of the different clones and the development of tumors after 3 wk is shown. After 3 mo the animals without tumors remained tumor-free, while those with tumors died ~4–5 wk after injection. H1, hygro SVT2 control; CAD, N-cadherin-transfected SVT2; PG, plakoglobin-transfected SVT2; CAD/PG, double-transfected SVT2 with N-cadherin and plakoglobin.

*The numbers in parentheses are arbitrary units representing the levels of expression of the transgenes after densitometer scanning of the gels shown in Fig. 2 A.
transfected cells displaying the greatest effect. Taken together, these data suggest that plakoglobin can suppress the tumorigenicity of cells when overexpressed either alone, or in conjunction with N-cadherin, and that the growth of the doubly transfected cells is reduced.

Suppression of Tumorigenicity in Renal Carcinoma Cells by Plakoglobin Transfection in the Absence of Cadherins and α- and β-Catenin

The augmenting effect of N-cadherin on the suppression of tumorigenicity in plakoglobin-transfected SVT2 cells raised the possibility that the effect of plakoglobin might be attributable to increased assembly of adherens junctions in the transfected cells. We, therefore, looked for tumor cell lines that lack all the major constituents of cell-cell adherens junctions including plakoglobin, α- and β-catenins as well as cadherin, as candidates for transfection with plakoglobin. Screening of a variety of cell lines derived from various human tumors revealed several renal carcinoma lines which lack all these proteins, as well as the desmosomal cadherins and desmoplakins (Fig. 6, A and C). The clone selected for the present work (KTCTL 60) produced tumors with high efficiency in nude mice. The cells were cotransfected with the human plakoglobin and the neo' cDNAs and G418-resistant colonies were analyzed for ex-
Figure 2. Western blot and immunofluorescence analysis of SVT2 clones transfected with N-cadherin and plakoglobin. Cells were transfected with either N-cadherin alone (CAD1, 2), with plakoglobin alone (PG3), or with N-cadherin after transfection with plakoglobin (CAD/PG1-5), and individual hygro colonies were isolated. (A) Equal amounts of total cell protein were analyzed by Western blotting with anti-N-cadherin (CAD), plakoglobin (PG), or β-catenin (β-CAT) antibodies. The levels of N-cadherin in 3T3 (lane 1); CAD1 (lane 2); CAD/PG1 (lane 3); SVT2 (lane 4) and the neo H1 clone (lane 5) were compared. The levels of Triton X-100 soluble (s) and insoluble (i) plakoglobin in several clones is shown. (B and C) The N-cadherin-transfected clone CAD1, and the N-cadherin plus plakoglobin-transfected clone CAD/PG1 (D and E) were immunostained with anti-N-cadherin (B and D) or plakoglobin (C and E) antibodies. H1, hygro-transfected control. The bars represent 10 μM.

Expression of the transgene (Fig. 6B). G418-resistant plakoglobin negative clones, and several positive clones, including one that expressed high levels of the protein (No. 14, similar to plakoglobin levels in MDCK cells, Fig. 6B), were identified. Eight of these clones were injected into nude mice and the rate of tumor development (tumor diameter at different times after injection) determined (Fig. 7). While all the clones, except one (No. 14), eventually formed tumors in nude mice, the rate of tumor formation was inversely correlated to the level of plakoglobin ex-
pressed. Neo clones which did not express detectable levels of plakoglobin (clones 4 and 9, Fig. 6 B), formed large, anaplastic, and undifferentiated tumors within 10 d, while the tumors formed by clones expressing low levels of plakoglobin (clones 1, 3, 11, 12, and 13, Fig. 6 B) exhibited similar morphology (data not shown), yet developed significantly more slowly (Fig. 7). Clone 14 with a highest plakoglobin level, comparable to that of MDCK epithelial
Figure 4. The growth in soft agar of SVT2 cells transfected with either plakoglobin, N-cadherin, or both N-cadherin and plakoglobin. Cells were seeded (250 and 1,000 cells per 35-mm dish) in soft agar and the colonies were photographed 2 wk later. (A) SVT2 control; (B) N-cadherin-transfected cells (clone CAD2); (C) plakoglobin-transfected cells (clone PG4); and (D) cells transfected with both N-cadherin and plakoglobin (clone CAD/PG1). The diameter of the colonies was smaller by 25% in N-cadherin transfectants, by 30% in plakoglobin-transfected cells, and by 62% in the double transflectants. The decrease in volume, assuming a spherical shape for the colonies was 2.4-, 2.8-, and 17.7-fold, respectively. The bars represent 100 μM.

Table III. The Effects of Plakoglobin and N-Cadherin Overexpression on Cell Cycle Distribution

| Cell cycle phase | H1* | CAD2† | PG3‡ | CAD/PG1†* |
|------------------|-----|-------|------|-----------|
|                  | %   |       |      |           |
| G1               | 63 ± 2.6 | 71 ± 2.2 | 75 ± 2.0 | 81 ± 1.8 |
| S                | 32 ± 2.1 | 23 ± 2.0 | 14 ± 1.0 | 9 ± 1.5  |
| G2               | 5 ± 1.3  | 6 ± 1.1  | 11 ± 2.1 | 10 ± 0.8 |

The values represent percent of transfected SVT2 cells in the different stages of the cell cycle determined by FACS analysis.
*Control, hygromycin-resistant cells (see Fig. 2 A).
†N-cadherin-transfected cells (CAD2, see Fig. 2 A).
‡Plakoglobin-transfected cells (PG3, see Figs. 1 A and 2 A).
*N-cadherin- and plakoglobin-transfected cells (see Fig. 2 A and Table II).
Discussion

Previous studies have demonstrated that increasing the expression of several adherens junction plaque proteins, such as vinculin and α-actinin, may considerably augment cell-ECM adhesion and consequently suppress the tumorigenicity of malignant cells (Rodríguez Fernández et al., 1992b; Glück et al., 1993). It was further shown that targeted decrease in the expression of these proteins in non-tumorigenic 3T3 cells by antisense transfection can conferring “anchorage independence” or even tumor formation in nude mice (Rodríguez Fernández et al., 1993; Glück et al., 1994). These and additional studies of a similar nature (Cunningham et al., 1992; Prasad et al., 1993), suggested that increased cell adhesiveness, triggered by high levels of junctional plaque or microfilament proteins, may suppress the malignant properties of tumor cells and restore adhesion-mediated growth control. These studies however, did not indicate whether junctional molecules such as vinculin and α-actinin exert their tumor suppressing effect by influencing cell–matrix or cell–cell contacts, nor was it excluded that soluble, rather than junctional association of such molecules, are responsible for affecting the tumorigenicity of cells.

Plakoglobin was chosen for the present study since this protein is exclusively associated with cell–cell adhesions (both adherens and desmosomes, Cowin et al., 1986; Cowin, 1994) and was implicated in signal transduction processes in Drosophila (Peifer et al., 1991) and Xenopus (Karnovsky and Klymkowsky, 1995). Moreover, the availability of tumor cells which lack both cadherins and catenins, enabled us to determine whether junction formation is necessary for tumor suppression.

The results presented here demonstrate that expression of plakoglobin in various tumor cells, which lack this protein, results in an effective suppression of their tumorigenic ability in vivo. This was observed in SV40-transformed 3T3 cells (SVT2) which express low levels of cadherin, and α- and β-catenin, as well as in a human renal carcinoma cell line which expresses neither cadherins nor catenins or plakoglobin. Interestingly, transfection with plakoglobin was not accompanied by significant changes in overall cell morphology or in the distribution of actin and vinculin in these cells. This was in contrast with the conspicuous effects on cell morphology exerted by transfection with vinculin (Rodríguez Fernández et al., 1992b; Geiger et al., 1992). In addition, plakoglobin-transfected SVT2 cells, unlike vinculin- and α-actinin–transfected SVT2 (Rodríguez Fernández et al., 1992b; Glück et al., 1993), remained anchorage independent and produced large colonies in soft agar.

Transfection with N-cadherin, while leading to epithelialization of the cell culture, did not affect the tumorigenicity of SVT2 cells, raising the possibility that the suppressive effect of plakoglobin on tumor formation may not require a marked stimulation of cell–cell adhesion. This notion was strongly supported by the results obtained with transfected renal carcinoma cells which displayed neither adherens junctions nor desmosomes, yet their tumorigenicity was effectively suppressed by plakoglobin overexpression. Our results also support previous findings indicating that cadherin overexpression is not always sufficient for changing invasiveness and for suppressing tumor formation (Navarro et al., 1993; Sommers et al., 1994). It is noteworthy, however, that expression of N-cadherin, in addition to plakoglobin, significantly enhances the tumor suppressive effect. This finding further supports the possibility that assembly of the junctional complex which contains cadherin, the catenins, and plakoglobin might be important for signaling pathways that regulate cell growth. This hypothesis is consistent with the results showing a decrease in the number of cells in S phase and the smaller size of the colonies formed in soft agar by cells double transfected with plakoglobin and N-cadherin.

By what mechanism is plakoglobin exerting its suppressive effect in the absence of cadherins and catenins? One possibility could be that plakoglobin might associate with other regulatory molecules, including the EGF receptor (Hoschuetzky et al., 1994) or the human tumor suppressor protein APC (Shibata et al., 1994; Hülskens et al., 1994), in a cadherin- or β-catenin–independent fashion (Hülskens et al., 1994; Knudsen and Wheelock, 1992; Hinck et al., 1994a). Thus, nonjunctional cytoplasmic or nuclear plakoglobin could participate in signal transduction processes that eventually regulate cell growth. Recent genetic analysis in Drosophila supports this notion, by demonstrating
distribution of plakoglobin between the Triton X-100-soluble (s) and insoluble (i) fractions was determined in the parental clone (60) and in the plakoglobin-transfected clones 14, 13, and 9. (C) The expression of cadherin (CAD) (using a Pan-cadherin antibody), α-catenin (α-CAT), β-catenin (β-CAT), desmoplakin II (DP), desmocollin 2 (DC), and desmoglein 2 (DG) by control KTCTL 60 cells and cells transfected with plakoglobin was examined by Western blotting. Only about half of the amount of protein was loaded from clone 14 in C as compared to the other lanes. Cell morphology (D and F) and immunofluorescence with anti-plakoglobin antibody (E and G) of untransfected KTCTL 60 (D and E) and plakoglobin-transfected cells (clone 14, F and G). M, MDCK cell extract. Bars: (D and F) 50 μM; (E and G) 10 μM.
that the plakoglobin- and β-catenin-related molecule armadillo (Peifer and Weischaus, 1990; Peifer et al., 1991) is a segment polarity gene in Drosophila, and is part of the signaling pathway driven by the secreted glycoprotein Wg (Bejsovec and Wieschaus, 1993), the homologue in vertebrates of the head axis determining protein Wnt-1 (Sokol et al., 1993). Wnt-1 was also shown to elicit adhesion-related responses in plakoglobin expression and organization in mammalian cells (Bradley et al., 1993; Hinck et al., 1994b). Furthermore, both β-catenin (Funayama et al., 1995) and plakoglobin mRNA overexpression (Karsnovsky and Klymkowsky, 1995) result in embryonic axis induction in Xeno-

pus, with part of the molecules distributed in the cell nucleus. It remains to be determined whether the tumor suppressor activity of plakoglobin is exerted by an interaction with APC, or other signaling molecules, and to elucidate the mechanisms by which these molecules are involved in bringing about the exit of cells from S-phase.

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