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Correlation between tumor induction and the large external transformation sensitive protein on the cell surface

(cold insoluble globulin/transformed phenotype/oncogenicity/adenovirus)

LAN BO CHEN, PHILLIP H. GALLIMORE*, AND JAMES K. MCDOUGALL

Cold Spring Harbor Laboratory, Cold Spring Harbor, New York 11724

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ABSTRACT **The distribution on the cell surface of the large external LETS protein that is transformation sensitive of normal, transformed and tumorigenic cells was examined by immunofluorescent staining. A correlation was established between the expression of fibril-like LETS protein and the oncogenic capabilities of a series of adenovirus-transformed cell lines. In cells expressing a transformed phenotype *in vitro*, LETS protein is only detected in cell-cell contact areas, whereas in "untransformed" cells LETS protein is distributed over the cell surface. Transformed cells capable of inducing invasive tumors, and the cells of established tumor lines, have low or undetectable levels of LETS protein, as measured by this method. The results indicate that LETS protein has a role in cell-cell adhesion and that reduced expression of this protein at the cell surface is related to the oncogenic phenotype. This relationship has been established for experimentally induced and spontaneous tumors.**

The spectrum of host cell response to transformation by oncogenic viruses in culture covers a wide range: from the induction of proteolytic enzymes (1, 2), loss of actin-filament bundle (3), changes in morphology (4), increases in the rate of glucose uptake (5), alteration of surface components (6), increase in saturation density (7, 8), reduction in serum requirement (7, 8), and growth in agar (7, 8), to the appearance of neoantigens, e.g., T-antigen (7, 8). However, some of the host cell responses were suggested to be directly related; for example, loss of actin cable and growth in semi-solid medium (3, 9). By the use of nude mice as a model system, it was suggested that growth in agar is correlated best with tumorigenicity (10, 11).

Recently, we turned our attention to the intriguing observation that there are several adenovirus-transformed cell lines which are nontumorigenic. For instance, cell lines Ad2/F17 and Ad2/F18 which are rat embryo cells transformed by adenovirus type 2 are T-antigen positive and able to grow in low serum and to high saturation density; yet, they are nontumorigenic in both normal syngeneic rats, immunosuppressed newborn syngeneic rats (7, 12, 13), and nude mice. Ad2/F19, on the other hand, is tumorigenic in nude mice, but not in rats, whether immunosuppressed or not. In comparison with the other Ad2 lines studied, Ad2/F19 showed a lower level of tumor induction in nude mice. Ad2/F19 tumors had a longer latent period than the other lines (e.g., T2C4, 7.5 days; REM, 8.5 days; F4, 19 days; F19, 30 days): whereas T2C4, REM, and F4 showed invasion of local mouse tissues, this was not the case with Ad2/F19 which classified histologically as a benign tumor (P. H. Gallimore, manuscript in preparation). Ad2/F4 and Ad2/REM are tumorigenic in immunosuppressed syngeneic rats while some other lines (e.g., T2C4) are tumorigenic in syngeneic rats without immunosuppression (7, 10) (Table 1). This series of cell lines thus provides a spectrum of oncogenicity within a single species.

Recently, a cell surface iodinated protein with nominal molecular weight of about 250,000 was shown to be either undetectable or reduced in various viral-transformed fibroblasts (14–20). This protein has been designated as LETS (large external protein that is transformation sensitive) protein (21) or Z protein (16) or CSP (cell surface protein) (19). More recently it has been shown (22) that LETS protein is closely related to a previously identified plasma protein, cold insoluble globulin (CIG) (23, 24). In this report, we use anti-CIG antibody to study the correlation between tumorigenicity and LETS protein.

MATERIALS AND METHODS

Cells and Media. Cell lines were grown in either Dulbecco's modified Eagle's medium (Gibco) supplemented with 5% dialyzed fetal calf serum or in Joklik's modified Eagle's medium (Gibco) supplemented with 5% dialyzed fetal calf serum. All culture media contained penicillin (50 units/ml) and streptomycin (50 μg/ml). Cultures were incubated at 37° with 10% CO_2 in air. References to the origin of cells are in Table 2.

Immunofluorescence. Cells were grown as monolayers on 12 mm glass coverslips (Bio Glass Co., New York) for 2 days. After fixing in 2% paraformaldehyde in phosphate-buffered saline for 15 min, coverslips were washed in phosphate-buffered saline extensively and placed in a small humidified box. Ten microliters of CIG antibody from rabbit antihuman plasma was diluted 1:80 in P_i/NaCl, layered on the coverslip, and kept at 37° for 20 min. Coverslips were then extensively rinsed in P_i/NaCl and layered with 10 μl of fluorescein isothiocyanate conjugated IgG antibody from goat anti-rabbit serum (Meloy Co., Pennsylvania) diluted 1:20 in buffered saline. After 20 min at 37°, coverslips were washed in phosphate-buffered saline and mounted on microscope slides with Elvanol. Fluorescence was observed by Zeiss Photomicroscope II equipped with epi-illumination. Photographs were taken on Kodak Tri-X film.

Antisera. Rabbit antiserum which contained antibodies to cold insoluble globulin of human plasma was prepared by injecting highly purified CIG into rabbits, and the serum was immunoabsorbed with the supernatant of Cohn fraction I. This antiserum is monospecific as determined by immunoelectrophoresis, and is of high titer. This antiserum was generously provided by A. B. Chen and M. W. Mosesson. An antihuman antiserum prepared in rabbits against plasma Cohn fraction I-4 contained both antifibrinogen and anti-CIG activity and was a gift of J. W. Fenton, II. After extensive immunoabsorption with highly purified bovine fibrinogen and further immunoabsorption with the supernatant of plasma Cohn fraction I, this antiserum was found to be monospecific for CIG. Finally, the antiserum used in this investigation reacts with cellular LETS protein. This was established by K. Burridge (personal communication) by staining whole cell proteins resolved on

Abbreviations: LETS protein, large external protein that is transformation sensitive; CIG, cold insoluble globulin; HEK, human embryo kidney.

* Present address: Department of Cancer Studies, University of Birmingham, Birmingham, England

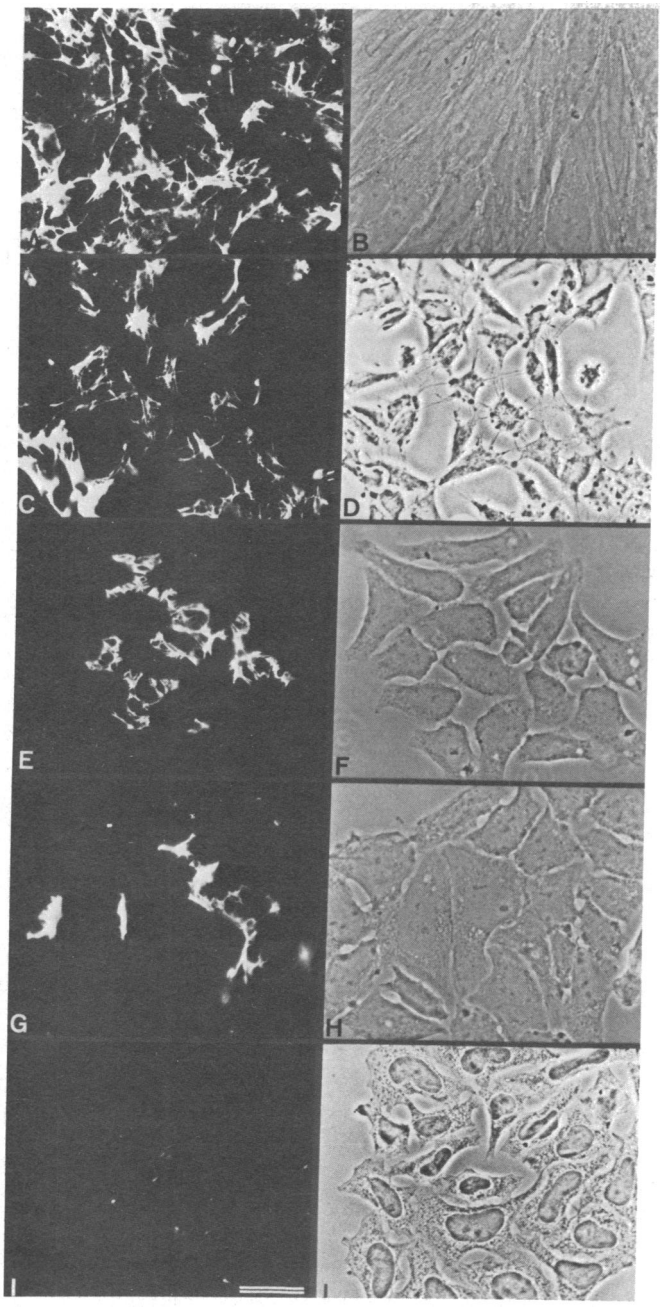

FIG. 1. Indirect immunofluorescent stains of normal and rat embryo fibroblasts transformed by adenovirus-2 with anti-LETS protein antiserum. Photographs A, C, E, G, and I are fluorescent photomicrographs of normal rat cells and cell lines F18, F17, B1 and T8 stained with anti-LETS antiserum, respectively; B, D, F, H, and J are the corresponding phase contrast photomicrographs of the same cell lines. The bar represents 20 μm.

sodium dodecyl sulfate/polyacrylamide gel with anti-CIG antiserum.

RESULTS

Cellular Origin of LETS Protein. With the anti-CIG antiserum, indirect immunofluorescent staining of normal chick embryo fibroblasts is similar to that reported by Wartiovaara *et al.* (25). This observation, together with Burridge's result (personal communication) described above, confirms that CIG in blood is the soluble form of cell surface LETS protein (CSP or Z). We will therefore refer to CIG as LETS protein from this point onwards. Evidence that this surface antigen does not originate from serum has been presented previously (14–17, 22), and we have provided further evidence by the following approach, based on the fact that antiserum raised in rabbits does not react with LETS protein in rabbit serum. If the level of LETS protein detected by immunofluorescence is similar between cells growing in rabbit serum and fetal calf serum, then one can conclude that most of the LETS protein on the cell surface is of cellular origin, not serum. Indeed, we found that in all cell lines tested there is no difference in the level of LETS protein with regard to serum. Moreover, because only paraformaldehyde was used for fixation before incubating with antibody, and live cells gave the same staining pattern, it is most likely that the LETS detected by current techniques is on the cell surface.

Cell Surface Distribution of LETS. When normal rat embryo fibroblasts were examined for the distribution of LETS protein by immunofluorescence, the pattern found was density and time dependent. In a very sparse culture, most of the surface LETS proteins are located at cell-substratum contact area in a fine fibril-like structure. In a monolayer culture grown for 48 hr, surface LETS protein is distributed in a diffuse network over the cell surface of all cells (Fig. 1A). After 6 days, the culture is covered with a massive network of fibril-like structures composed of LETS protein. All the immunofluorescence assays described in this report were made on monolayer cultures after 48 hr of growth.

As shown in Fig. 1, when the distribution of surface LETS protein in a series of adenovirus-transformed rat cells was studied by indirect immunofluorescence, fibril-like antigens, when detected, were always located in the contact area between cells. When cells are not in contact, LETS protein is rarely detected in this series of adenovirus-transformed cells. Fig. 1C and E are typical immunofluorescent stains of surface LETS protein of the nontumorigenic Ad2/F18 and Ad2/F17 cells. Essentially all the cells in cell–cell contact express LETS protein in their respective contact areas, as also shown in Fig. 2A. All the immunofluorescence stains reported here are specific for LETS protein because all the fluorescence disappeared when anti-CIG antibody was first incubated with purified human plasma CIG at 37° for 1 hr.

Correlation of LETS Protein with Tumorigenicity. The percentage of cells positive for LETS protein, among those cells in contact from the different lines of adenovirus-transformed cells, is shown in Table 1. If the tumorigenicity of these lines is compared with the above results, one finds a correlation between increase in tumorigenicity and decrease in the number of cells positive for LETS protein upon cell–cell contact. Ad2/F19 cells produce benign tumors in nude mice and, unlike the tumors induced in nude mice by Ad2/F4, these tumors are neither invasive nor transplantable to syngeneic rats. Thus, there is no correlation between loss of LETS protein and the induction of benign tumors in nude mice by Ad2/F19.

To test the generality of the correlation between tumorigenicity and surface LETS, we randomly chose nine cell lines derived from tumors for surface LETS protein assay. The result is shown in Table 2. Seven out of nine cell lines have completely lost surface LETS protein. The other two cell lines have only about 10% of the cells expressing surface LETS protein upon contact. Fig. 2C shows immunofluorescent stain of surface LETS protein for one of the cell lines, HuTu-80, which was derived from a human stomach carcinoma. Although Fig. 2C shows more than 25% of cells expressing LETS when more than 1000 cells were examined, only about 10% of the cells were LETS positive for surface LETS protein. The existence of a minor population of cells negative for LETS protein may partially explain why, by use of lactoperoxidase catalyzed iodin-

Table 1. Correlation between oncogenicity of type 2 or 12 adenovirus-transformed rat cell lines, human cell lines transformed by type 5 adenovirus, and LETS protein

Cell line[a]	Adenovirus-2 DNA sequences[b] *Hpal* fragments							Tumors induced in:				% of cells for positive fibril-LETS protein[d]
	E	C	F	A	B	D	G	Nude mice[c]	Nude mouse tumor transplants to rat	Immuno-suppressed rat	Immune competent rat	
F17	+	+[e]	0	0	0	0	0	0/20	−	0	0	100
F18	+	+[e]	0	0	0	0	0	ND	−	0	0	100
F19	+	+[e]	0	0	0	0	0	2/10 (30)[f]	0	0	0	100
F4	ND	+	ND	+	+	+	+	10/10 (19)	+	+	0	30
REM	+	+	+	0	+[e]	+	+	10/10 (8.5)	+	+	0	<1
B1	+	+[e]	0	0	0	0	0	ND	ND	+	0	50
B1/T8[g]	+	+[e]	0	0	0	0	0	ND	ND	+	+	<1
T2C4	+	+	+[e]	+[e]	+	+	+	10/10 (7.5)	+	+	+	<1
A5/FG/HEK[h]	(Adenovirus-5)[i]							6/6	+	−	−	<1
A5/FG/HEK nMT/Tpl[j]	(Adenovirus-5)							5/5	+	−	−	<1
A12/0.25/J1	(Adenovirus-12)							5/5	+	ND	+	<1

ND, not determined.
[a] All cell lines were T-antigen positive.
[b] From Gallimore *et al.*, 1974 (26).
[c] Number of animals with tumor/number of animal injected. Numbers in parentheses refer to latent period (days) for initial tumor to appear.
[d] Percent of LETS cells positive for LETS protein is the average value of three experiments. For each experiment more than 1000 cells which are in cell–cell contact were counted.
[e] Only partial fragments detected.
[f] For 8/10 animals, no tumors observed after 165 days. Only 2/10 animals had noninvasive tumors.
[g] Tumor derived from B1 cell line.
[h] From Graham *et al.* 1974 (27). HEK—human embryo kidney.
[i] Contains 1–2 copies of most of the virus DNA molecule/diploid quantity human DNA.
[j] Tumor derived in nude mouse from the A5/FG/HEK cell line.

ation, some LETS protein can still be detected in certain tumorigenic cell lines (39, 40). Furthermore, some tumorigenic cell lines positive for LETS protein, but reduced in quantity when measured by surface iodination, may have an abnormal pattern of distribution of LETS protein on the cell surface. For example, Ad2/T2C4, a tumorigenic line investigated here, actually expresses a small amount of LETS protein; but, by immunofluorescence no fibril-like network of LETS protein has ever been detected. Instead, some diffuse dots of LETS are detected on the surface of rounded-up cells. Perhaps, in addition to the expression of normal quantities of LETS protein, the ability of cells to organize LETS protein on the cell surface properly is also important for the "normality" of a given cell line.

Lack of Correlation with Integrated Viral Sequences. When Table 1 is examined, it is obvious that the amount and portion of the adenovirus genome incorporated into the chromosomes of these adenovirus-transformed cells bears no correlation with tumorigenicity in syngeneic rats. As pointed out previously (7), 14% of the left-hand end of the genome of adenovirus-2 may include a gene for maintenance of the transformation phenotype *in vitro* and for viral T-antigen, but it is not the sole factor in determining tumorigenicity. If, as we propose, the loss of surface LETS protein is an oncogenic phenotype, the presence of this portion of the adenovirus-2 genome is not, *per se* sufficient to induce this characteristic.

DISCUSSION

The cell surface undoubtedly plays an important role in determining oncogenicity. Neoplasm, in a way, may be considered as a disease in which normal regulation and cell-cell communication mediated by the cell surface are lost. In addition, the immune response also plays an important role in determining oncogenicity. Because the interaction of lymphocyte or immunoglobulin with cells is also mediated through the cell surface, alterations at the cell surface level are probably of prime importance among an array of cellular responses induced by oncogenic agents such as viruses, chemical carcinogens or radiation. What are the changes on the cell surface involved in oncogenicity? Among various surface changes involved in the immune response, the appearance of tumor specific transplantation antigens (TSTA) on the cell surface after viral transformation is most noteworthy. That the immune mechanism plays a role in oncogenicity is clearly illustrated in the series of rat cells transformed by adenovirus-2 which we investigated here. For example, Ad2/B1 is tumorigenic in immunosuppressed rats but not in normal rats, which suggests that on Ad2/B1 cells there are surface alterations which are immunologically recognized. This level of surface alteration probably does not involve surface LETS protein.

The second level of surface alterations may be those involved in cell-cell recognition, adhesion, and communication. At present, it is impossible to distinguish between these three types of interactions. In fact, cell–cell adhesion could be the basis of recognition and cell–cell recognition could in turn be the basis for communication. In all, we know very little about these important biological processes. However, in terms of surface alteration after transformation, the observations reported here together with previous findings on LETS protein provide an opportunity to probe the general question of cell–cell interaction at the molecular level. Indeed this protein has recently been shown to be involved in cell–cell adhesion (41), and another surface protein which may be involved in cell–cell adhesion has recently been reported (42). The finding in this report that surface LETS protein is always located in the cell–cell contact area whenever detected in adenovirus-transformed cells further strengthens the possibility that this protein is involved in cell–

Table 2. Correlation of LETS protein with oncogenicity

Cell lines	Growth in 1% serum	High saturation density	Reduction in actin cable[a]	T-antigen	% of cells in contact and positive for fibril-LETS protein	Induction of tumor or derived from tumor	Ref.
Normal							
Chick embryo fibroblast (CEF)	+	+	−	−	100	−	11
Rat embryo fibroblast (REF)	−	−	−	−	100	−	11
Mouse embryo fibroblast	−	−	−	−	100	−	10
Hamster embryo fibroblast	−	−	−	−	100	−	28
CRL-1139, human cystic fibrosis fibroblast	−	−	−	−	100	ND	*b*
WI-38, human fibroblast	−	−	−	−	100	−	29
3T3, mouse fibroblast (Swiss)	−	−	−	−	100	−	11
Adenovirus-transformed							
F17, REF transformed by adenovirus-2	+	+	+	+	80	−	30
F18, REF transformed by adenovirus-2	+	+	+	+	100	−	30
F19, REF transformed by adenovirus-2	+	+	+	+	100	+[c]	30
F4, REF transformed by adenovirus-2	+	+	+	+	30	+	30
B1, REF transformed by adenovirus-2	+	+	+	+	50	+	30
REM, rat embryo myoblast transformed by adenovirus-2	+	+	+	+	<1	+	30
B1/T8, tumor cell derived from B1	+	+	+	+	<1	+	30
T2C4, REF transformed by adenovirus-2	+	+	+	+	<1	+	30
A5/FG/HEK, HEK transformed by adenovirus-5	+	+	+	+	<1	+[c]	27
A5/FG/HEK/nMT1/TP1, transplantable tumor derived from A5/FG/HEK	+	+	+	+	<1	+[c]	—
A12/J1, REF transformed by adenovirus-12	+	+	+	+	<1	+	—
Miscellaneous tumor lines							
RR1022, derived from rat tumor induced by Rous sarcoma virus	+	+	+	ND	<1	+	31
RPM1 1846, derived from melanotic melanoma of Syrian hamster	+	+	+	ND	<1	+	32
MMT 060562, derived from mouse mammary tumor	+	+	+	ND	<1	+	33
LLC-WRC 256, derived from Walker rat carcinoma	+	+	+	ND	<1	+	34
HuTu-80, derived from human carcinoma (stomach)	+	+	ND	ND	10	+	35
NB41A3, derived from mouse neuroblastoma C-1300	+	+	+	ND	10	+	36
N18TG-2, derived from mouse neuroblastoma	+	+	+	ND	<1	+	37
NCTC clone 2472, mouse fibroblast	+	+	+	ND	<1	+	38
NTCT clone 2555, mouse fibroblast	+	+	+	ND	<1	+	38

ND, not determined.
[a] Unpublished result (W. E. Gordon, L. B. Chen, K. Burridge, and J. K. McDougall).
[b] American Type Culture Collection.
[c] Tumors weakly tumorigenic in nude mice only.

cell adhesion. When the number of cells positive for LETS protein upon contact was scored in these lines, a correlation between decrease in the cells positive for LETS protein and increase in tumorigenicity is observed. When other cells with known tumorigenicity were also grown in monolayer and assayed for LETS protein on the cell surface upon contact, the correlation was equally valid. As shown in Table 2, all the adenovirus-transformed rat cells and other tumor cell lines are reduced in the amount of actin cables.

We wish to emphasize that the induction of tumors in animals by viral-transformed cells prepared in culture must result from alterations in a series of cellular properties. Although the cell surface will play a crucial role, it is by no means the sole prime factor involved in oncogenicity. Moreover, even in the domain of cell surface alteration surface LETS protein is unlikely to be the only change relevant to oncogenicity. It is expected that other factors, for example, angiogenesis stimulating factor (to assure a nutrient supply), hydrolytic enzymes (for tissue invasiveness), alterations in the pattern of cell-hormone interactions (for autonomous cell growth), and the whole immune system will all play an important role in the determination of oncogenic potential of transformed cells. In view of such complexity, one may anticipate exceptions where cells which have acquired all the oncogenic phenotypes except for the loss of LETS protein, yet may still induce tumors in animals.

Finally, we do not know why there should be a correlation between the loss of LETS protein and tumor induction. The simplest explanation may be that LETS protein is directly involved in growth control and that the loss of LETS protein is responsible for unrestricted growth during tumor formation. Our previous finding (43) argues against this possibility. In view of the distribution of LETS protein in adenovirus-transformed

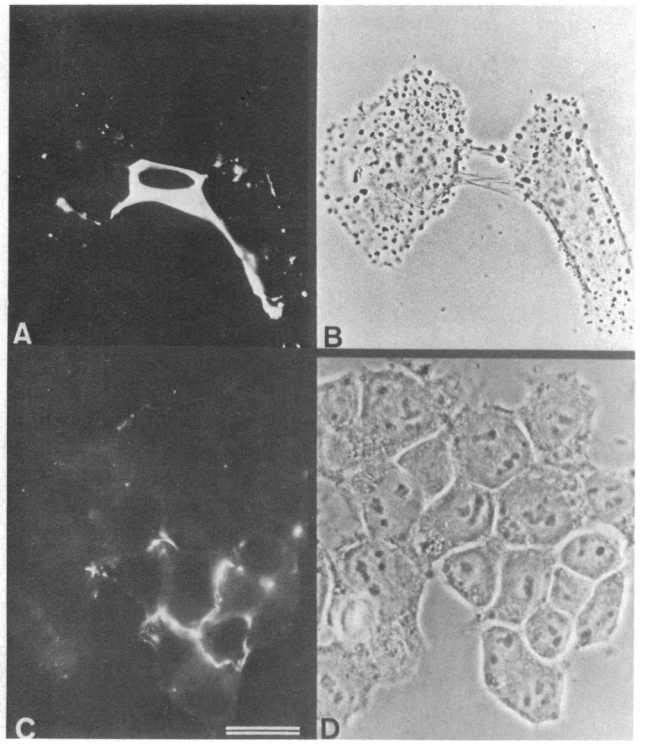

FIG. 2. Indirect immunofluorescent stain of Ad2/F19 and HuTu-80, a cell line derived from human stomach carcinoma, with anti-LETS protein antiserum. A and C are fluorescent photomicrographs of Ad2/F19 and HuTu-80 stained with anti-LETS protein antiserum, and B and D are the corresponding phase contrast photomicrographs. The bar represents 20 μm.

cells reported here, together with the findings of Yamada *et al.* (41), it is possible that LETS protein may be the "glue" involved in the extracellular matrix system. Classical embryology tells us that the extracellular matrix plays an important role in differentiation and organogenesis. Perhaps the maintenance of a normal pattern of cell growth *in vivo* depends on a proper intercellular matrix system. The integrity of such a matrix may be impaired by the loss of one (LETS protein) or two (LETS protein and, for example, collagen) of its elements. An important observation is that of all the adenovirus transformed cell lines described in this report, only Ad2/F17, F18, and F19 produce a three-dimensional matrix of LETS protein on the cell surface after a longer (6-day) period in culture, similar to that seen with normal cells. Results from further experiments indicate that loss of capacity to form such an exoskeleton may be of prime importance in oncogenic behavior.

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