Proc. Natl. Acad. Sci. USA
Vol. 74, No. 1, pp. 243–247, January 1977
Cell Biology

Role of cell surface carbohydrates and proteins in cell behavior: Studies on the biochemical reversion of an *N*-acetylglucosamine-deficient fibroblast mutant

(cell surface glycoproteins/membrane mutant/growth control/phenotypic transformation/3T3 Balb cells)

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Communicated by P. Roy Vagelos, October 15, 1976

ABSTRACT **AD6, a mutant derived from 3T3 Balb/c cells, is characterized by low adhesion to substratum, round shape, increase in surface microvilli, increase in agglutinability by concanavalin A, and loss of directional motility. These properties are often observed in transformed cells. However, the mutant has normal growth properties and anchorage-dependence of growth, and it does not form tumors. In AD6, the biosynthesis of complex carbohydrates and glycoproteins is impaired because of a block in the acetylation of GlcN-6-P. This defect is responsible for all the surface alterations because feeding of GlcNAc to AD6 cells corrects the defects in the synthesis of complex carbohydrates and the exposure of glycoproteins at the outer surface of the plasma membrane. Parallel to this biochemical reversion, there is full restoration of the altered biological properties. In contrast, GlcNAc has no effect on the morphologic features of two lines of transformed cells.**

Our results suggest that the carbohydrate portion of cell surface proteins has an important role in adhesion and related aspects of cell behavior. The fact that a defined alteration of the cell surface induces many properties often encountered in transformed cells, without affecting control of cell division, strongly suggests that these alterations in properties are not sufficient to account for the loss of growth regulation.

The surface of an animal cell undergoes marked biochemical changes after malignant transformation (1–4). Such alterations have been thought to be responsible for some of the typical properties of transformed cells, including (*i*) rounder shape, (*ii*) decrease in adhesion to substratum, (*iii*) decrease in contact inhibition of movement, and (*iv*) increase in agglutinability by plant lectins. However, whether these four alterations are primarily required for the establishment of the "transformed state" or are secondary events is unknown.

To investigate this question we (5) isolated a low-adherent mutant (AD6) from a nontransformed fibroblast, Balb 3T3, and analyzed its biological behavior and the biochemical alterations in its cell surface. We found a decrease in cell surface carbohydrates in clone AD6 as a result of a block in the acetylation of GlcN-6-P (refs. 6 and 7) (Fig. 1). This early defect in the biosynthesis of amino sugars leads to incomplete glycosylation of glycoproteins. One consequence of the defect is a decrease in the exposure of glycoproteins at the outer surface of the cell (5). In addition, the mutant cells are rounder, less adherent to substratum, and more agglutinable by plant lectins than are the wild-type cells. However, like cells of the parental line, their growth remains anchorage-dependent (5, 8) and they do not give rise to tumors.

We report here that feeding GlcNAc to this mutant (the next intermediate after the block) restores the synthesis of the carbohydrate portion of the glycoproteins to normal, and the cell surface glycoproteins become normally exposed. Such a biochemical reversion is accompanied by complete restoration of the altered biological properties: flatter shape, increase in cell-to-substratum adhesion, decrease in the number of microvilli, decrease in agglutinability by concanavalin A, and recovery of directional motility.

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MATERIALS AND METHODS

Cell Culture. Balb/c 3T3 mouse fibroblasts, simian virus 40 and Kirsten sarcoma virus transformed derivatives, and mutant clone AD6 were grown in Dulbecco-Vogt's modified Eagle's medium supplemented with 10% calf serum, penicillin (50 units/ml), streptomycin (50 μg/ml), and, when specified, 10 mM GlcNAc. Cells were passaged with trypsin, 250 μg/ml in Ca^{2+} and Mg^{2+}-free phosphate-buffered saline.

Radioiodination of the Cell Surface. Cells were planted at 5×10^4 to 5×10^5 cells per 100-mm plate and grown for 4 days. The medium was changed every 2 days. Lactoperoxidase iodination (9) was performed as described previously (5) except that $Na^{131}I$ was used instead of $Na^{125}I$. Whole cells were solubilized in sodium dodecyl sulfate, and proteins were separated in 5% polyacrylamide slab gels as described (5, 10).

Adhesion. Kinetics of detachment of the cells from the substratum with trypsin was studied as previously described (5).

Motility. Migration of the cells in Falcon dishes was monitored with a phase contrast inverted microscope coupled to a Bolex H16M camera. The cells were maintained in a Plexiglas box at 37° in a 95% air/5% CO_2 atmosphere. Photographs were taken at 1-min intervals, starting 2 days after planting (sparse cells) and 1 day after medium change. Cell migration was analyzed by projecting the film on white paper and marking the location of the nucleus of each of the single cells every 10 min. Cells in contact and mitotic cells were not analyzed.

Tumorigenicity. AD6, wild type 3T3, and simian virus 40 3T3 Balb/c transformed cells were grown to confluency, trypsinized, and resuspended in complete medium; 1×10^6 cells were injected subcutaneously in the interscapular region of Balb/c mice. Animals were observed at weekly intervals and scored for tumors for a period of more than 4 months.

Agglutinability by Concanavalin A. Quantitative agglutination with concanavalin A was performed as previously reported (11). With this assay, most transformed cells have agglutination indices of >100, whereas most normal cell lines show indices of <20.

Scanning Electron Microscopy. Cells were grown on UV sterilized carbon-Formvar support films (glow-discharged) mounted on 50-mesh nickel grids. The grids were mounted in culture dishes on double-sided Scotch tape. The cells were fixed in 2% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4, at 37° for 30 min followed by 2% OsO_4 in cacodylate buffer at 23° for 30 min. They were dehydrated in ethanol, critical-point-dried

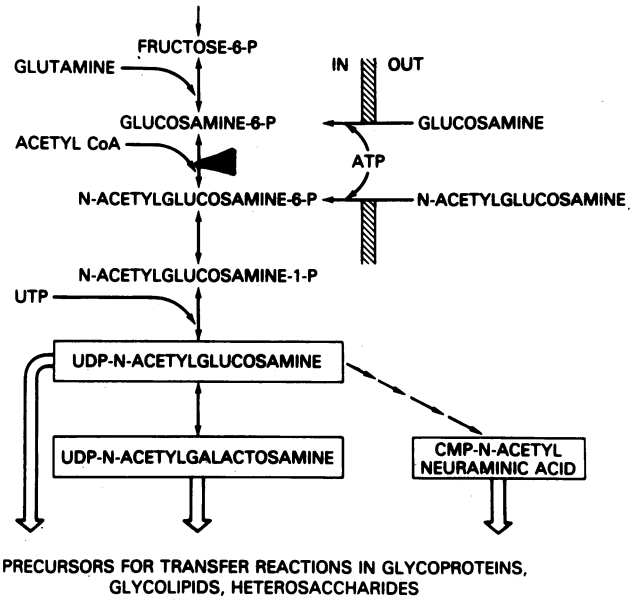

FIG. 1. Metabolic pathway of amino sugars according to Warren (20). ◀ indicates the position of the block in AD6 cells.

with CO_2 as the transition fluid, and shadowed with carbon followed by gold-palladium on a tilting rotary fixture. The grids were examined with a Hitachi HU-12A electron microscope equipped with an HSE-2 scanning attachment. Polaroid photographs were taken with type 52 P/N film at 75 kV and a pointed filament electron source.

RESULTS

Reversion of Cell Surface Glycoproteins and Carbohydrates with GlcNAc. The alterations in the cell surface proteins of ADG are apparently due to a block in the acetylation of GlcN-6-P (6, 7). Such a biochemical defect would result in the synthesis of partially glycosylated or perhaps even "unglycosylated" glycoproteins (7). Because it has been suggested that glycosylation is necessary for the proper insertion of proteins into the plasma membrane, a defect in glycosylation should result in proteins not being accessible to lactoperoxidase-catalyzed iodination (5). To test whether the acetylation block is directly responsible for the diminished surface iodination we attempted to correct the defect by feeding GlcNAc to the AD6 cells. Fig. 2A shows that feeding GlcNAc restored the iodination pattern of AD6 to that of the wild-type cells. This chemical restoration was reversible: 3 days after removal of GlcNAc from the medium of AD6, the cell surface iodination defect reappeared.

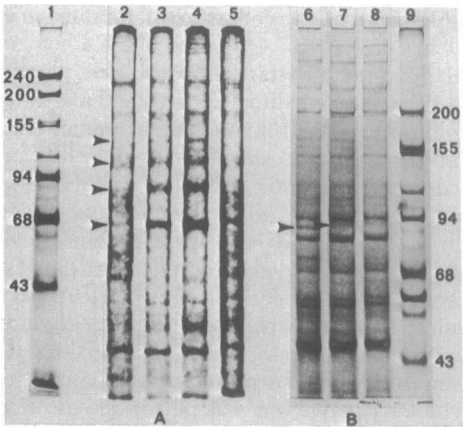

FIG. 2. Reversion of cell surface proteins. (A) Autoradiograms of 5% sodium dodecyl sulfate/polyacrylamide slab gel electrophoresis of whole cells labeled with $Na^{131}I$. 1 = protein standards; 2 = AD6 cells; 3 = AD6 cells grown with GlcNAc, 10 mM; 4 = wild-type cells; 5 = AD6 3 days after removal of GlcNAc from medium. Arrows indicate protein bands poorly iodinated in AD6. (B) 5% sodium dodecyl sulfate/polyacrylamide slab gel electrophoresis of whole cell extracts stained with Coomassie blue. 6 = AD6 cells; 7 = AD6 grown with GlcNAc, 10 mM; 8 = wild-type cells; 9 = protein standards. Arrows indicate mobility of 92,000 molecular weight protein (lane 7) and its precursor (lane 6) (see *text*).

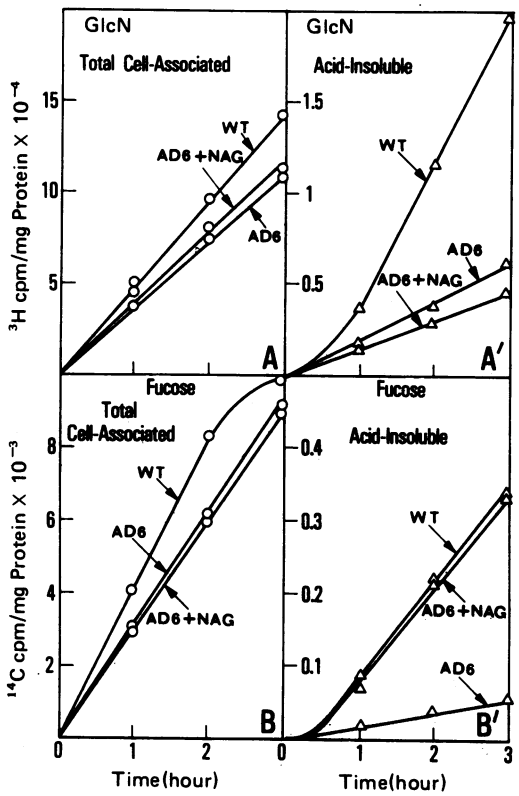

FIG. 3. Rates of uptake of D-glucosamine and L-fucose in total cell and acid-insoluble fractions of wild-type, AD6 mutant, and biochemically reverted AD6 mutant cells. Cells were planted at 10^5 cells per 60-mm dish and used for the experiment 3 days later and 1 day after medium change. The cell monolayers were washed twice with 2 ml of growth medium containing 10% dialyzed calf serum and 1 mM glucose, and the reaction was started by addition of either D-[^{3}H]-glucosamine (5 μCi/ml, 50 μM; upper panels) or L-[^{14}C]fucose (2.5 μCi/ml, 48 μM; lower panels) in 2 ml of the above defined growth medium. Incubation was at 37° in a CO_2 incubator. The reactions were stopped by removing the medium and washing the cell monolayer four times with 2 ml of growth medium. The cells were then scraped off the dish in isotonic saline and radioactivity was measured to determine total sugar uptake (left panels) and uptake into the acid-insoluble fractions (right panels). An aliquot of each reaction point was used for protein determination. WT = 3T3 Balb/c cells; AD6 = mutant cells; AD6 + NAG = AD6 mutant cells grown for 6 days in presence of 10 mM GlcNAc.

One of the main alterations in AD6 was reflected in the failure to iodinate a protein of apparent molecular weight 92,000. This protein is synthesized by AD6 but has a higher mobility in gel electrophoresis. GlcNAc feeding corrected the mobility of this glycoprotein. The identification of the 90,000 molecular weight protein in AD6 extracts as a nonglycosylated precursor of the 92,000 molecular weight glycoprotein of the cell surface has been achieved by immunological and chemical studies (J. Pouysségur, K. Yamada, unpublished data).

A different and more quantitative demonstration that cell

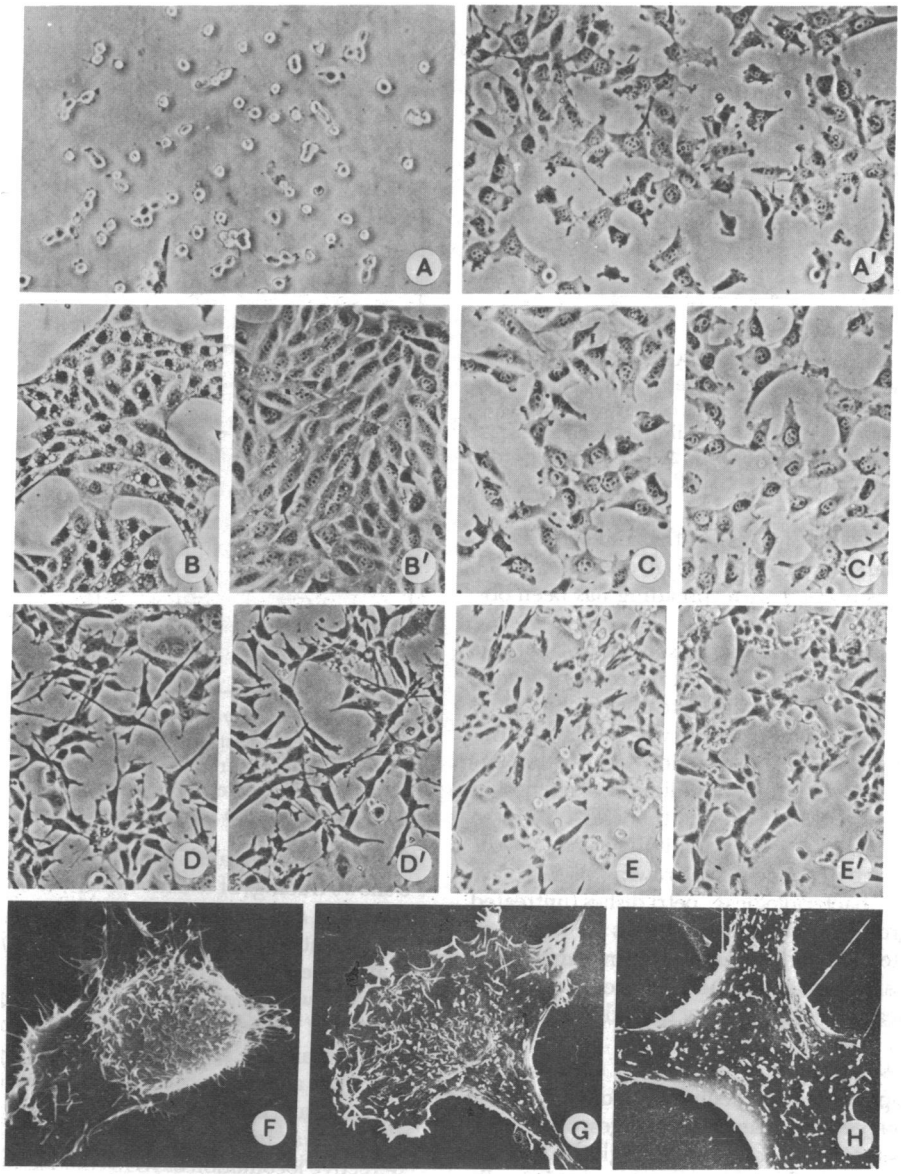

FIG. 4. Effect of GlcNAc on the morphologic features of AD6, wild-type, and virally transformed 3T3 Balb/c cells. Pictures were taken 3 days after planting and 1 day after medium change. GlcNAc was added at 10 mM in the culture medium at the time of planting. *Phase contrast*, (×94): A, Sparse AD6 cells; B, confluent AD6 cells; C, sparse 3T3 Balb/c; D and E, 3T3 Balb/c transformed by Kirsten sarcoma virus and simian virus 40, respectively. These all were grown in regular medium. The corresponding letters, A′, B′, C′, D′, and E′, refer to the same cells grown in presence of GlcNAc. *Scanning electron micrographs:* Note the villous surface features of AD6 cells (F) and smoother appearance of AD6 after GlcNAc treatment (G) or of a parental Balb/c 3T3 cells (H). (F = ×1728; G = ×118; H = ×1152.)

surface glycoprotein synthesis reverts to normal after GlcNAc feeding was obtained by measuring the incorporation of fucose into macromolecules. This terminal sugar is incorporated into glycoproteins at a low rate in AD6 (15% of the rate in wild type), even though fucose enters the cell at a normal rate (Fig. 3B and B′). However, when AD6 was grown with 10 mM GlcNAc, the normal rate of incorporation of fucose into glycoprotein was recovered. In spite of the correction of glycoprotein synthesis resulting from growth in GlcNAc, GlcN incorporation into macromolecules still was low (Fig. 3A′). This result suggests two conclusions: (*i*) the metabolic block is still present in AD6 after biochemical reversion; and (*ii*) this block is the cause of the defect in glycoprotein synthesis, not a consequence of it.

Morphology. A change in the shape of AD6 cells was evident after 2 days of growth with 10 mM GlcNAc and was maximal after 3–4 days. A lower concentration (1 mM) was less effective. The major change was that the round, compact shape of sparse AD6 cells (Fig. 4A) was replaced by a flat shape (Fig. 4A′). Further, the cytoplasmic vacuoles that were evident in dense cultures of AD6 (Fig. 4B) did not form in growth medium containing GlcNAc (Fig. 4B′). GlcNAc had no effect on the growth or morphologic features of three nonmutant cell lines tested: the parent 3T3 Balb/c cells (Fig. 4C, C′) and derivatives of the parent transformed by a RNA tumor virus (Fig. 4D and D′) or a DNA tumor virus.(Fig. 4E and E′). GalNAc (10 mM), which can give rise to GlcNAc, also restored the flat shape; ManNAc was less effective. The nonacetylated analog, GlcN, is toxic at 10 mM; at a lower nontoxic concentration, 1 mM, it did not change the shape of AD6 but instead induced rounding of the wild-type cells (data not shown).

When examined by scanning electron microscopy, the surface of AD6 was covered with many microvilli and ruffles (Fig. 4F), similar to many transformed cells (12). Growth in the presence of GlcNAc induced spreading and decreased the vil-

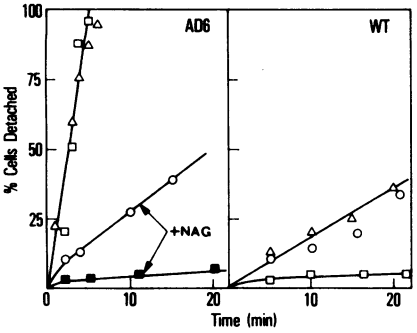

FIG. 5. Kinetics of detachment of 3T3 and AD6 cells with trypsin (250 μg/ml) at 37° (see ref. 5) 2 days after the cells were planted at 5 × 10^4 cells per 60-mm dishes. △, No addition; ○, cells grown for 2 days with GlcNAc at 10 mM; □, cells treated with dibutyryl adenosine 3′:5′-cyclic monophosphate (cAMP), 1 mM, for 24 hr; ■, cells grown with both 10 mM GlcNAc and dibutyryl cAMP.

lous formation of AD6 cells (Fig. 4G) so that the surface resembled that of the parent cell (Fig. 4H). A decrease in the number of microvilli associated with spreading has been observed by others (13, 14).

Adhesion. We evaluated adhesion to substratum by two methods. In the first we measured the kinetics of detachment with trypsin. Fig. 5 shows that the high rate of detachment of AD6 cells was decreased to that of wild-type cells when the mutant was grown with GlcNAc. Adhesion was further increased, and to the same degree, when dibutyryl adenosine 3′:5′-cyclic monophosphate was added to the wild-type or to the GlcNAc-treated mutant. Another qualitative assay for adhesion uses the anchorage-dependence of growth (8). AD6 cells do not adhere at all in "bacteriological" petri dishes (untreated polystyrene) and therefore do not grow, whereas the wild-type cells adhere enough to the poorly adhesive substratum to grow normally (5). AD6 cells cultivated with GlcNAc were able to grow on bacteriological dishes, suggesting that adhesion to that substratum had been restored.

Agglutinability. Agglutinability of cells by various plant lectins is another property that has been found to be altered in transformed cells, suggesting chemical changes of the cell surface (15). In general, transformed cells agglutinate more rapidly than do their untransformed counterparts. AD6 cells, like transformed cells, are highly agglutinable (Table 1). However, when AD6 was grown with GlcNAc, its susceptibility to agglutination by concanavalin A fell to a value close to that of wild-type cells. This cell line constitutes another example in which the presence of microvilli is correlated with increased agglutinability with concanavalin A (16).

Motility. Locomotion of the cells *in vitro* proceeds via a cytoplasmic contraction that takes place between the area of adhesion at the leading edge and less adhesive points at the posterior of the cell (17, 18). The obvious role of adhesion in motility led us to investigate this property in the adhesion-

Table 1. Cell agglutinability by concanavalin A

Cell line	Number of experiments	Agglutination index
3T3 Balb/c	1	0–4
3T3 Balb/c (+GlcNAc)	1	0–5
AD6	3	107–117
AD6 (+GlcNAc)	2	22–27

Agglutination assay was performed with concanavalin A at 100 μg/ml; indices were calculated as in ref. 11.

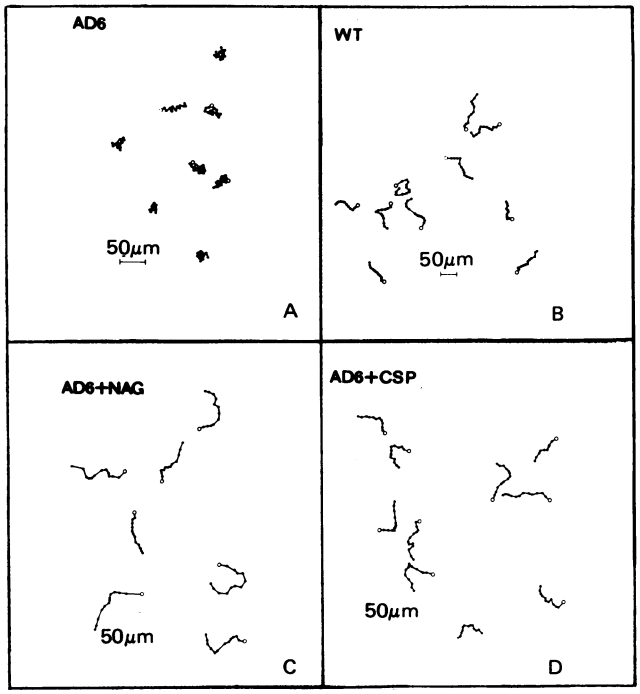

FIG. 6. Motility of wild-type and mutant cells in Falcon plastic dishes. (A) AD6 mutant; (B) wild type; (C) AD6 grown with 10mM GlcNAc; (D) AD6 treated for 48 hr with cell surface protein at 50 μg/ml (21). Each tracing represents the locomotion of a cell, recorded every 10 min, over a period of 120 min.

deficient mutant and in its biochemically reverted form. Fig. 6 shows tracings of the movement of sparse cells for a 2-hr period. The distance moved by AD6 was much less than that of the parent cell. The membrane of AD6 was more active than that of the wild-type cell; processes that were not strongly associated with the substratum were rapidly emitted and retracted at various parts of the cell periphery. Associated with, and perhaps the result of, this intense peripheral ruffling activity was a nonoriented pattern of cell locomotion. When AD6 was grown in the presence of GlcNAc, its directional locomotion pattern was fully restored to that of the wild-type cells. The defective locomotion of AD6 could also be corrected by adding the major cell surface glycoprotein of chick embryo fibroblasts to the medium (Fig. 6D). This glycoprotein is known to increase the adhesion of transformed cells (4). Conversely, growing the wild-type cells on a poorly adhesive substratum (bacteriological dishes) abolished the oriented locomotion of these cells (data not shown).

Tumorigenicity. We have already reported that AD6 has normal growth properties *in vitro*. Like the wild-type parent and other untransformed cells, AD6 is anchorage-dependent for growth (8). Furthermore, it does not grow in agar (5) or in bacteriological dishes to which it cannot adhere. When 1 × 10^6 AD6 cells were injected into eight Balb/c mice, no tumors were produced although an equivalent number of simian virus 40-transformed 3T3 cells produced tumor in all eight mice treated (Table 2). It has been previously emphasized that there is a strong correlation between tumorigenicity and the loss of anchorage-dependent growth (19).

DISCUSSION

Our hypothesis that the defect in glycoprotein biosynthesis in AD6 is probably due to a block in the acetylation of GlcN-6-P is strengthened by the demonstration that feeding the mutant cells the next intermediate after the putative block restores

Table 2. Tumor production in Balb/c mice

Cell type injected	Tumor incidence*
3T3 Balb/c	0/8
AD6	0/8
SV40-3T3 Balb/c	8/8

Animals were inoculated with 10^6 cells.
* Number of animals with tumor per number of animals inoculated.

glycoprotein synthesis to normal and corrects the altered biological properties of AD6. As expected from the position of the block (Fig. 1), GlcN was ineffective in the biochemical reversion whereas GalNAc, which can be isomerized to GlcNAc, was effective (20). Therefore, AD6 possesses all the characteristics of a mutant defective in the biosynthesis of GlcNAc.

Our finding that restoration of glycoproteins at the cell surface is accompanied by correction of the defect in cell-to-substratum adhesion suggests that one or more of these molecules play a direct role in cellular adhesion. Other recent reports favor this conclusion. The major cell surface glycoprotein of chick embryo cells increases adhesion when added to various transformed cell lines (21). Patients with one of two different types of bleeding disorders due to a defect in an early stage of platelet adhesion or aggregation have platelets that are altered in two specific cell surface glycoproteins (22, 23). The specific aggregation of two different types of ameba has been related to a difference in glycoprotein composition (24).

Glycosaminoglycans also appear to be made in decreased amounts in AD6, and they increase after GlcNAc is fed (data not shown). This is to be expected from the site of the block in the carbohydrate synthetic pathway. To our knowledge there is no evidence to suggest that these polysaccharides are adhesive molecules. Furthermore, enhanced hyaluronic acid synthesis in Rous sarcoma virus-transformed cells is often accompanied by decreased cell-to-substratum adhesion, and a Chinese hamster ovary cell variant characterized by an increased cell-to-substratum adhesion no longer synthesizes hyaluronic acid (25).

In addition to the decreased adhesion, AD6 has a rounder shape, an increase in microvilli, an increase in the agglutinability by concanavalin A, an altered motility, and a decrease in contact inhibition of movement. This last change is suggested by an increase in the number of nuclear overlaps in cultures of AD6 (data not shown). All these alterations seem to be due to a unique mutation because they all revert to normal after correction of the biochemical block with GlcNAc.

The biological changes observed in AD6 are similar to those encountered in malignant cells. However, unlike transformed cells, AD6 has normal growth properties *in vitro* and does not give rise to tumors *in vivo*. The addition of the transformation-sensitive surface glycoprotein to transformed cells restores adhesion, morphology, and the nuclear overlap ratio to normal but does not correct the loss of growth control (21). On the basis of these two types of studies, we conclude that a round shape, low adhesiveness, increase in microvillous formation, increase in agglutinability by plant lectins, and increased number of nuclear overlaps are dissociable from the mechanisms that control cell growth.

We thank Dr. Kenneth Yamada for providing us with cell surface protein.

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