Temperature-Dependent Surface Changes in Cells Infected or Transformed by a Thermosensitive Mutant of Polyoma Virus

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ABSTRACT Infection of BALB/3T3 cells by polyoma virus causes an alteration in the cell surface, characterized by enhanced agglutination of the cells by wheat germ agglutinin or concanavalin A. Infection by the thermosensitive mutant of polyoma, ts-3, causes the cell surface alteration at the permissive temperature, but not at the nonpermissive temperature. The cell surface alteration requires cellular DNA synthesis, but not viral DNA synthesis. BHK cells transformed by ts-3 show the surface alteration when grown at the permissive temperature, but not when grown at the nonpermissive temperature. It is concluded that the surface alteration in transformed cells is under the control of a viral gene.

Cell surface alterations that accompany transformation can be detected by enhanced agglutination of the transformed cells by wheat germ agglutinin (agglutinin) or concanavalin A (Con A) (1-4). Agglutination of normal cells, or of variants of SV40-transformed cells that do not grow to high cell densities in culture, occurs only at much higher concentrations of agglutinin than the concentrations required for agglutination of transformed cells (5, 6). A site in the cell surface containing carbohydrate is responsible for the binding of wheat germ agglutinin (1). This site is exposed in transformed cells, and can be exposed in normal cells by mild protease treatment (7). Release of 3T3 cells from inhibition of growth in crowded cultures occurs concomitantly with exposure of the agglutinin site by protease (8).

Infection of 3T3 cells by polyoma virus (Py) causes a surface alteration that is similar to the alteration in transformed cells resulting in enhanced agglutination of the infected cells by agglutinin (9). The surface alteration does not take place in the presence of inhibitors of DNA synthesis (9). A host-range mutant of Py that is unable to cause cell transformation does not cause the surface alteration after infection of nonpermissive cells (9).

Conditional lethal mutants of Py have been isolated to determine what properties of transformed cells may be controlled by viral genes (10). A thermosensitive mutant of Py, ts-3, has been shown to be defective in the induction of cellular DNA synthesis and movement in BALB/3T3 cells infected at the nonpermissive temperature, and to render one property of transformed cells, inhibition of cellular DNA synthesis by topographical factors (topoinhibition) tempera-

ture-dependent, implying that topoinhibition of transformed cells can be controlled by a viral gene (11).

In order to investigate the relationship between surface alterations and growth properties of transformed cells, we have studied the agglutination of cells infected or transformed by ts-3 by agglutinin. Enhanced agglutination of BALB/3T3 cells does not occur after infection by ts-3 at the nonpermissive temperature. BHK cells transformed by ts-3, which show temperature-dependent topoinhibition, also show temperature-dependent surface alterations; enhanced agglutination by agglutinin or Con A occurs with cells grown at permissive temperature, but not with cells grown at nonpermissive temperature. Therefore, surface alterations of transformed cells, as well as the growth property, topoinhibition, can be under the control of a viral gene.

MATERIALS AND METHODS

Cell cultures were maintained in reinforced Eagle's medium supplemented with 10% calf serum. BALB/3T3 cells were kindly provided by Dr. Stuart Aaronson. BHK cells were an early passage provided by Dr. Michael Stoker in 1962, and were kept frozen until shortly before they were used for transformation.

Virus was the wild type polyoma large plaque (LP) and ts-3, a thermosensitive mutant derived from LP by Dr. Marguerite Vogt. The properties of ts-3 will be described in detail elsewhere.

Infection was carried out using subconfluent BALB/3T3 cultures in 9-cm plastic tissue culture dishes (NUNC, Denmark). The cultures were washed once with Tris-buffered saline and infected with 0.5 ml of a virus suspension of $0.5-2 \times 10^8$ plaque-forming units/ml. After adsorption, the layers were covered with medium supplemented with 10% calf serum. Mock infection was carried out with buffer containing serum. In some experiments, hydroxyurea (HU) was added to the medium at a concentration of 3×10^{-3} M. This concentration of HU reduced incorporation of thymidine to less than 1% of that in untreated cultures. The effect of HU on thymidine incorporation was rapidly reversed upon removal of HU after treatments as long as 40 hr. The HU was recrystallized prior to use.

Transformation of BHK cells by LP or by ts-3 was carried out by methods described previously (11). Two clones of BHK cells transformed independently by ts-3 were used. Ts-3 C17C had been cultured at 32°C for 3 months before being used in

Abbreviations: Con A, concanavalin A; HU, hydroxyurea; WSR, wound serum requirement; Py, polyoma virus; LP, large plaque (strain of Py); agglutinin, wheat germ agglutinin.

FIG. 1. Time course of appearance of surface alteration. ○---○, mock-infected; ■---■ infected with wild type polyoma virus. WGA, wheat germ agglutinin.

these experiments. Ts-3 Cl 1 and WT Cl 1-A (BHK transformed by LP) were frozen shortly after being isolated from agar, and were grown at 39°C for 10 days prior to the experiments.

Wheat germ agglutination assay

Infected and transformed cells were assayed for their ability to be agglutinated by agglutinin. Subconfluent cultures were used in all experiments. Cultures were rinsed several times with saline at 37°C to remove serum. The cells were then rinsed once or twice quickly with Tris-buffered saline, pH 7.4, containing 5×10^{-4} M EDTA, which was prewarmed to 45°C so that its temperature did not fall below 35°C when layered over the cultures. When the cells loosened from the dish, they were suspended in the EDTA-saline solution and dispersed by gentle pipetting. The cells were washed twice with saline by very gentle centrifugation at about 500 rpm in the swinging bucket rotor of an International Model HN table centrifuge. Care was taken to avoid damage to the cells that might result in the release of proteolytic enzymes. The agglutination tests were performed by placing 0.1 ml of a cell suspension, at $1-2 \times 10^6$ cells/ml, in the well of a hemagglutination tray. 0.01 ml of a pure agglutinin solution in saline was placed at the edge of the well, and cells and agglutinin were mixed in the

well. After one minute, a small drop of the suspension was placed in the well of a concavity slide, and the slide was inverted on a rocking platform with a temperature control block at 23°C to form a hanging-drop suspension. After 5 min, the suspension was examined on the concavity slide with an inverted microscope, and the proportions of single cells and clumped cells were counted in several fields. Cells not treated with agglutinin were tested at the same time. Scores of 0, +, ++, +++, and ++++ correspond to 0, 50, 75, 90, and over 97% of cells present in clumps. The concentration of agglutinin required for half-maximal agglutination (++) was determined by interpolation of scores obtained using 3-4 different concentrations of agglutinin.

RESULTS

Agglutination of cells after infection

BALB/3T3 cells were infected with wild type Py as described in *Methods*, and assayed at various times after infection for agglutination by agglutinin. The results of this experiment are shown in Fig. 1. Enhanced agglutinability of the infected cells begins to appear about 18 hr after infection, and reaches a maximum 36 hr after infection.

BALB/3T3 cells were infected with ts-3 and incubated at 39 and 32°C. Agglutination of the cells was tested at various times after infection. The results of this experiment are shown in Fig. 2. Enhanced agglutination does not occur with cells infected by ts-3 at 39°C, but does occur with cells infected at 32°C. Enhanced agglutinability appears more slowly in cells infected by wild type virus at 32°C than at 39°C, presumably because the infection proceeds more slowly at 32°C.

The lack of enhanced agglutination with cells infected by ts-3 at 39°C is not the result of failure of the virus to penetrate BALB/3T3 cells, because ts-3 behaves as thermosensitive after infection with viral DNA (W. Eckhart, unpublished observations). Lack of enhanced agglutination is not simply the result of lack of cytopathic effect at 39°C because other mutants which fail to grow at 39°C, and which do not cause a cytopathic effect at 39°C, nevertheless produced enhanced agglutinability (R. Dulbecco, manuscript in preparation).

FIG. 2. Surface alteration after infection with ts mutants. ●---●, ts-3 at 39°C; ■---■, wild type at 39°C; ○---○, ts-3 at 32°C; □---□, wild type at 32°C; ▲---▲, ts 616 at 39°C.

TABLE 1. Agglutination of transformed cells

Cells	Temperature, °C	Concentration of agglutinin required for half-maximal agglutination μg/ml	
		Wheat germ	Concavalin A
WT Cl 1-A	32	45	100
ts-3 Cl-1	32	53	
ts-3 Cl-7C	32	40	80
WT Cl 1-A	39	37	
ts-3 Cl-1	39	160	
	39 + trypsin	60	
ts-3 Cl-7C	39	180	240
	39 + trypsin	36	

Cells were grown at the temperatures indicated for several days and then tested for agglutination by wheat germ agglutinin or concanavalin A. Trypsin treatment was with 0.0003% trypsin, 5 min incubation at 37°C.

(Left) FIG. 3. Requirement for DNA synthesis for surface alterations. Cells were infected at 39 and 32°C in the presence of 3×10^{-3} M hydroxyurea. Samples were tested for agglutination before and at various times after removal of HU 24 or 36 hr after infection. Same symbols used as in Fig. 2 except for Δ - Δ , mock-infected and treated with HU at 39°C for 24 hr.

(Right) FIG. 4. Appearance and disappearance of surface alteration after shift in temperature of ts-3-transformed BHK cells.

Δ - Δ , clone 7-C } Shifted from 39 to 32°C
 $\bullet$ - $\bullet$, clone 1 }
 Δ - Δ , clone 1 }
 $\circ$ - $\circ$, clone 7-C } Shifted from 32 to 39°C

Requirement for DNA synthesis for surface alteration after infection

BALB/3T3 cells were infected with wild type Py and incubated in the presence of HU after infection as described in *Methods*. HU was removed from the cultures at 24 hr after infection at 39°C, and at 36 hr after infection at 32°C. Agglutination of the cells by agglutinin was tested at various times after the removal of HU. The results of this experiment are shown in Fig. 3. Enhanced agglutination does not occur with cells incubated in the presence of HU. After removal of HU, enhanced agglutinability appears rapidly, and is complete by 10 hr after removal of HU. HU acts to inhibit DNA synthesis by blocking the action of ribonucleoside diphosphate reductase, preventing the formation of deoxynucleotide precursors of DNA (12). Therefore, the lack of enhanced agglutination of cells after infection in the presence of HU argues strongly that DNA synthesis is required in order for the surface alteration to take place.

In order to test whether viral DNA synthesis is required for the surface alteration to occur, we infected BALB/3T3 cells with another thermosensitive mutant, ts 616, which is defective in viral DNA synthesis at 39°C. The cell surface alteration does occur after infection by ts 616 at 39°C (Fig. 2), implying that the requirement for DNA synthesis in order for the surface alteration to occur is a requirement for cellular DNA synthesis, rather than for viral DNA synthesis.

Agglutination of transformed cells

Two clones of ts 3-transformed BHK cells, and one clone of wild type transformed BHK cells were tested for agglutination by agglutinin and Con A after being grown at 39 and 32°C. The results are shown in Table 1. Wild type transformed cells show enhanced agglutinability when grown at 39°C or at 32°C. Ts-3-transformed cells show enhanced agglutinability when grown at 32°C, but not when grown at 39°C. The surface properties of ts-3-transformed cells grown at 39°C are similar to those of normal cells, in that enhanced agglutinability can be produced by mild protease treatment (Table 1).

Appearance and disappearance of surface alterations in ts-3-transformed cells

In order to determine the length of time necessary for the surface alteration to appear and disappear in ts-3-transformed cells grown at different temperatures, we shifted ts-3-BHK cells grown at 39 to 32°C, and cells grown at 32 to 39°C. Agglutination was tested at various times after the shift in temperature. The results of this experiment are shown in Fig. 4. Masking of the surface alteration begins to be detectable between 4 and 8 hr after a shift of the cells from low to high temperature, and is essentially complete by 24 hr after the shift. Appearance of the surface alteration after shift from high to low temperature is not detectable by 12 hr after the shift, but is substantial by 24 hr, and is essentially complete by 36 hr after the shift.

DISCUSSION

It has been reported that infection of 3T3 cells by Py leads to enhanced agglutinability of the infected cells by agglutinin and that this surface alteration does not occur in the presence of inhibitors of DNA synthesis (9). We have repeated these observations, using BALB/3T3 cells.

The ts-3-mutant is defective in causing a surface alteration after infection at the nonpermissive temperature. In ts-3-transformed BHK cells, expression of the surface alteration is temperature dependent. Therefore, surface changes in infected and transformed cells can be under the control of a viral gene.

In a previous communication, two of us reported that the ts-3 mutant is partially defective in induction of cellular DNA synthesis and movement after infection of BALB/3T3 cells at the nonpermissive temperature and that BHK cells transformed by ts-3 show temperature-dependent expression of one attribute of transformed cells, inhibition of DNA synthesis by topographical factors (topoinhibition) (11). Other attributes of transformed cells, growth in agar and serum requirement for initiation of DNA synthesis in a wound (WSR), are not temperature dependent in ts-3-BHK.

The attributes of infected or transformed cells that can be controlled by the ts-3 gene may have a common origin in the surface changes in infected or transformed cells. Alteration of the cell surface by the ts-3 gene product could result in release of restraints on cellular DNA synthesis and growth such as has been observed after protease treatment of 3T3 cells and chick cells (8, 13). Topoinhibition could be mediated by surface alterations that are temperature dependent in ts-3-transformed cells.

The relation between surface changes in transformed cells, and the properties of growth in agar and WSR, which are not temperature-dependent in ts-3-BHK, is not clear. If the ts-3 gene product has a pleiotropic effect, and if different properties of transformed cells require different amounts of the gene product for expression, it is possible that the reduced amount of functional gene product present in ts-3-transformed cells at the nonpermissive temperature is sufficient to allow expression of growth in agar and WSR, but not sufficient to allow expression of enhanced agglutinability or loss of topoinhibition. Alternatively, growth in agar and decreased WSR could be unrelated to the surface changes detected by agglutination.

Characterization of ts-3 and other viral mutants that affect the properties of transformed cells should clarify some of the relationships between cell surface alterations and cell growth properties.

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