

THE CELL SURFACE AND MALIGNANT TRANSFORMATION

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A malignant cell has lost many restrictions to which normal cells are subjected. For example, cell division is no longer tightly regulated by cell-cell contact or specific serum factors, serum-mediated hormonal feedback loops are broken, and cell recognition of neighboring cells no longer leads to maintenance of cell position.

As such differences (Table 1) between normal and malignant cells are described in ever greater detail, mechanisms to explain how the cell maintains any or all of them have remained sorely lacking.

Very recently, different laboratories working on widely different biological systems have reported a series of experiments (1-7) which, taken together, begin to suggest possible mechanisms for the maintenance of the transformed state. Most of these new lines of research have directly involved experiments on the surfaces of normal and malignant cells.

In this review we have attempted to make explicit some implied connections among the results of these laboratories. Our hope is that our admittedly premature synthesis might be predictive, and so stimulate some new critical experiments.

STRUCTURE OF THE CELL SURFACE

In normal and transformed cells alike the cell surface acts both as a transmitter and as a transducer of signals from other cells and from serum or culture medium. The signals received by the surface may be virus-induced gene products [e.g. the proteolytic activity of Reich and co-workers (6)] or hormones [e.g. insulin (8)]; and output signals may be issued to the interior of the cell via membrane-bound enzyme systems [e.g. adenylate cyclase (9)], or the active transport systems (10), or possibly by changing the attachment of membrane proteins and glycoproteins to a postulated internal structure involving microtubules (11).

A widely accepted current view of the molecular structure of the surface consistent with those functions is illustrated in Figure 1 (12, 13). The membrane is a lipid

bilayer with hydrophobic hydrocarbon tails 14–24 carbons long in the interior, and either (a) polar head groups linked by phosphate or (b) polysaccharide structures of variable complexity at the outer surface. The membrane thickness has been determined by small angle X-ray diffraction to be 46 Å (14).

Membranes from different tissues contain varying amounts of the rigid molecule, cholesterol, (15) and it has been argued that parts of the membrane may be in a gel or crystalline state (16). Recent studies of the membrane-bound enzyme adenylate cyclase (17) show a discontinuity in activation energy for the enzyme at 32° C, and while this temperature is nonphysiological, the unexplained effect suggests that we should expect complex interactions between the enzymatically active proteins of the membrane and the lipid matrix.

It is most promising to look to the protein and glycoprotein component, the embedded globular particles of Figure 1, for the control and signal properties of the membrane. Experiments with intact erythrocytes and erythrocyte ghosts (18–21) establish, for the red blood cells at least, that while most membrane proteins and glycoproteins are exposed only at the inner surface, a major protein and a major

Table 1 Properties of cells transformed by DNA or RNA tumor viruses

Growth

- High or indefinite saturation density
- Reduced serum requirement
- Growth in agar or methocel suspension
- Tumor formation on injection into susceptible animals
- Reduced contact inhibition of movement
- Disoriented growth pattern
- Growth on monolayers of normal cells

Surface

- Increased agglutinability by plant lectins
- Changes in composition of glycoproteins and glycolipids
- Virus-induced transplantation antigen
- Increased rate of transport of nutrients

Enzymatic

- Production of cell factor component of fibrinolysin T
- Increased anaerobic glycolysis

Evidence of Virus

- Virus-specific antigens present
- Virus DNA or RNA sequences detected
- Virus rescued by heterokaryon formation

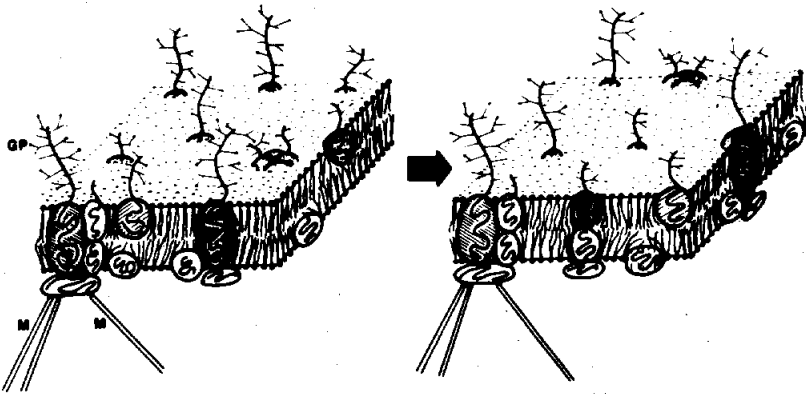


Figure 1 The fluid mosaic model of the cell membrane (12) adapted from Singer & Nicolson (13) with hypothetical cytoplasmic restraints. Some membrane components (glycoproteins, glycolipids, and lipids) are rapidly diffusing in the membrane plane under physiological conditions (left sketch → right sketch), while others such as the glycoprotein complex (GP), illustrated at the left, have their mobility impeded by membrane-associated interior cellular structures (M). Used with permission from *Control of Proliferation in Animal Cells*, Cold Spring Harbor Laboratory, 1973.

glycoprotein penetrate the lipid bilayer and are exposed at both the inner and outer surfaces of the cell. In Bretscher's experiments (19, 20), for example, the labeling reagent [^{35}S] formyl-methionyl (sulfone) methyl-phosphate was used to add formyl-methionyl sulfone groups to any exposed amino (and possibly hydroxyl) groups on the membrane particles. Exposing intact erythrocytes only labels groups on the outside surface of the membrane, while exposing lysed ghost cell membranes permits labeling of both outside and inside groups. By this technique, Bretscher showed that a single major 100,000-dalton protein was exposed on both surfaces of the red cell membrane. To prove that some parts of the protein are outside and others inside, Bretscher (20) isolated the protein and prepared a "fingerprint" of its tryptic digest. Only a few spots of the fingerprint appeared when the protein was labeled in intact cells but a more complete fingerprint, which included the earlier spots, appeared when ghosts were labeled.

The freeze fracture technique (22) cleaves the membrane at a midplane between the lipid layers. Electron microscopy of the newly exposed previously interior surfaces of the membrane reveals particles (23, 24); since the sizes of the particles are as predicted from membrane protein molecular weights (24), the number of particles (24) agrees with the number predicted from chemical analysis (19), and the particles observed by electron microscopy are digested by proteases (25, 26), we can conclude that the observed particles are the membrane proteins and glycoproteins. Usually the particles are found randomly distributed laterally in the membrane, but e.g. in *Tetrahymena*, Satir et al (27) find that a rosette of particles appears at the

point where a mucocyst begins to merge with the cell membrane. It will perhaps not be surprising to find that over the next few years such phenomena as nutrient and ion transport, agglutination, and cell division are successfully accounted for in terms of the composition and distribution of membrane particles and their connections to the interior of the cell.

Whatever their function, membrane glycoproteins appear to be free to move about rapidly in the lipid bilayer, giving the membrane as a whole the somewhat paradoxical properties of transmembrane impermeability and lateral fluidity. Frye & Edidin (28) and Edidin & Weiss (29) have obtained direct evidence for lateral mobility of membrane glycoproteins and proteins in mouse and human fibroblasts. Cells of a mouse cell line and a human strain were fused with the aid of inactivated sendai virus. Mouse H2 antigens (glycoproteins) and human species antigens (believed to be proteins) were localized at light microscopic resolution on the surface of the fused cell with fluorescent antibodies. Immediately after fusion the two groups of antigens were segregated. The number of cells with segregated antigens then decreased exponentially with a half-life dependent on temperature, but not on generation of ATP or on protein or amino sugar synthesis in the cell. These data suggest that the mixing of antigens results from simple diffusion. In view of the drastic effect of sendai virus on the membrane in promoting fusion one may worry about applying this result to normal cells, but under the conditions of the experiment it is clear that membrane proteins and glycoproteins are free to move laterally.

Lateral fluidity was related to the general problem of transformation when Edidin and co-workers showed that the mobilities of the particular surface antigens they detect were far higher in transformed than normal cells. They measured a half-life of only 20 min for intermixing in the case of fusion of a mouse L cell with an SV40-transformed human fibroblast, but about a 2-hr half-life for fusions of 3T3 with secondary human fibroblasts, and of a revertant line (30) with the untransformed diploid strain of human fibroblasts WI38. The question of mobility of membrane particles will come up again below when we discuss agglutinability. This property shows every sign of developing into a major clue to the nature of transformation.

As yet, no compelling functional role in transformation has been devised for the glycolipids and we will somewhat reluctantly pass them by, making reference to chapter 3 of Tooze (15).

TWO MISSING LINKS

Normal cells *in vivo* or *in culture* may, during the G_1 phase of the cell cycle, elect or be induced to enter a differentiated resting state usually called G_0 . Cells in G_0 can be induced to reenter the cell cycle by a variety of agents, many acting at the cell surface.

Therefore, we consider missing link I in our understanding of control of proliferation to be the sequence of biochemical events occurring between stimulation of the surface and initiation of DNA replication. We devote the following two sections to presumed components of this signal system. Of course, some inducers of departure

from G_0 such as replacement of a missing amino acid for a starved culture or supply of Ca^{2+} to the G_0 chick fibroblast system of Balk et al (31) are not likely to act directly on the membrane. Accordingly, our first missing link is really a missing network of which one link includes the surface and a culminating link initiates synthesis of a critical precursor to DNA replication. In view of the variety of input effectors, the link at which the network yields to analysis may be the culminating and not the surface link.

Missing link II is the sequence of biochemical events leading from the integration of the genome of the oncogenic virus into the host chromosome to modification of "the surface," or (as a realistic generalization) to modification of the proliferation control network which we have just named missing link I. We devote the last three sections of this review to missing link II. In the section on viral transformation we discuss the changes in growth, surface, and other cell parameters associated with transformation and consider briefly two useful virus-cell systems. In the last sections we discuss two cellular effects that are perhaps as close to the transforming virus as we have yet come. One effect is the freeing of some surface receptors that are immobilized in untransformed cells; the second is the induction of a proteolytic activity which probably acts at the cell surface and may have the effect of freeing the receptors.

CYCLIC NUCLEOTIDES

Cyclic AMP (3',5'-adenosine monophosphate) is manufactured from ATP by an enzyme of the plasma membrane (32), adenylate cyclase, and reclaimed to 5'-AMP by phosphodiesterase. A decrease in diesterase activity or an increase in cyclase activity or both will lead to an increase in cyclic AMP. Epinephrine and glucagon in the liver and muscles act by stimulating adenylate cyclase, but in the wide assortment of physiological processes mediated by cAMP (32), both enzymes are important.

Cyclic GMP (3',5'-guanosine monophosphate) was discovered by Goldberg and co-workers (1) in the late 60s in many animal tissues at a concentration one to two orders of magnitude less than that of cAMP. The first correlation of cGMP with physiological function was found (33) in cardiac contractility in the rat, stimulated by isoproterenol and depressed by acetylcholine, and accompanied by a decrease and increase, respectively, in the level of cGMP. In many cases cGMP concentration is altered in a sense opposite to that of cAMP. Many other hormone-induced changes in cell function have since been found to be correlated with cGMP level (34).

The correlation of cAMP level with cell proliferation has been extensively investigated by Pastan (35), Sheppard (36), and many others. There is general agreement that the concentration of cAMP in cells showing reduced density-dependent inhibition of growth is about one half the concentration in related normal cells. Also, it is generally agreed that agents that induce proliferation in resting cultures, e.g. fresh serum, insulin, and trypsin, all reduce cAMP concentration, while serum depletion raises it. Concerning the important question of change in cAMP concentration as a cell population passes from the log phase to the stationary phase of growth,

Sheppard (37) found no change for 3T3 mouse fibroblasts, while Pastan and co-workers (35, 38-40) found a marked increase not only for 3T3 cells but also for normal rat kidney (NRK) cells and human fibroblasts. In NRK cells they found a more than 7-fold increase in cAMP concentration due to a large increase in adenylate cyclase activity overcompensating a minor increase in phosphodiesterase activity. In interpreting these experiments (35) it is important to be sure that serum deprivation did not inadvertently occur as cultures reached confluence (37).

In any event all such experiments are consistent with the hypothesis that a modulation of the activity of membrane-bound adenylate cyclase may control proliferation by causing a net elevation in the level of cAMP.

Temin (8) has shown that insulin stimulates cell division. An accompanying decrease in cAMP concentration was found by Sheppard (37), and very recently, Goldberg and co-workers (34) found in 3T3 cultures an accompanying increase in cGMP concentration—about 10-fold within 3 min at an insulin concentration of 10 mU/ml. Additional provocative but as yet isolated observations include Estensen et al's (41) finding that within 60 sec the tumor promoter phorbol myristate acetate, which induces cell division in 3T3 fibroblasts, produces a 10-fold increase in cellular cGMP concentration; also cGMP concentrations between about 10^{-8} and 10^{-11} M stimulate [3 H] uridine incorporation into RNA by isolated lymphocyte nuclei (36).

It is clear that the molecular biology of the control of adenylate and guanylate cyclase must be pursued intensively, and a search made for enzymes influenced by the cyclic nucleotides and connected with the cell's commitment to DNA synthesis. Whether one of the two purine cyclic nucleotides is master and the other slave, or whether their two cyclases have a common modulator, or whether the interaction yielding a frequently contrary change in cGMP and cAMP levels is more complex is so far unknown. Since the distinctive association of both cyclic nucleotide concentrations with the state of cell proliferation persists for the case of viral transformation, it seems certain that much more of this story will be written over the next few years.

GROWTH STIMULATING FACTORS

If the saturation density of 3T3 cells in culture is plotted against the serum concentration in the nutrient medium, the resulting smooth curve continues without break in slope through and well beyond the point of confluence (42). Holley and Kiernan (42, 43), Temin and co-workers (44-46), and others have concluded from such data that nutrient or control factors in serum or medium are the determining elements for proliferation and that contact-dependent inhibition of division is not per se a separate phenomenon. These workers are looking in depth for growth stimulating factors and the functional role of such factors. One recent product of the search is Multiplication Stimulating Activity (MSA) purified originally from calf serum by Pierson & Temin (44).

The relation of MSA to other such factors is only now becoming clear. Over the past 15 years, studies of growth stimulating factors in human serum have led to the partial purification of several activities originally named simply for their function. One is "sulfation factor" (47), which stimulates the uptake of [35 S]O₄ by cartilage;

another is "nonsuppressible insulin-like activity" [see (48) and references therein], which stimulates incorporation of thymidine in DNA, incorporation of amino acids into rat diaphragm, and a number of other insulin-like activities. This activity is different from that of insulin because it is not suppressed by antiserum to insulin. Apparently, all these activities in human serum may be ascribed to a single small protein (mol wt ~ 7000) named somatomedin (48). Hintz et al (48) have shown that somatomedin competes with insulin for binding to receptors which in turn have been shown by Cuatrecasas (49) to reside on the cell surface.

MSA, purified recently some 35,000-fold relative to MSA in calf serum by Dulak & Temin (45, 46), is identified as a small protein (mol wt $\sim 10,000$), which is found to have the sulfation factor and nonsuppressible insulin-like activities of somatomedin. MSA, somatomedin, and insulin all compete for the same receptors [Van Wyk, quoted by Temin (2)]. It is therefore entirely possible that MSA and somatomedin are the same molecule.

The source of the recently purified MSA is itself of interest. A line of untransformed buffalo rat liver cells isolated by Coon (50) has the rare ability to grow in serum-free medium. Medium conditioned by the rat liver cells is the starting point of the purification. Thus, at least some cells can produce their own MSA.

MSA substitutes for serum, at a protein concentration 10^{-5} that of the serum, in inducing the incorporation of thymidine into chick embryo cell DNA as measured in a 1-hr pulse 12 hr after stimulation. It also substitutes for serum in supporting proliferation of untransformed chicken and duck embryo fibroblasts, but must be supplemented with 0.1% serum to support chick cells infected by Rous sarcoma virus (RSV).

VIRAL TRANSFORMATION

The transformation of animal cells in culture by oncogenic viruses initiates a broad spectrum of changes in cell behavior. For a review of transformation, see chapters 6 and 11 of reference 15. A comprehensive though not complete list of the properties distinguishing transformed cells from their untransformed parent cells is given in Table 1. The phenomenology of transformation is based largely on the study of infection of mouse, hamster, and monkey cells by the DNA-containing papova viruses SV40 and polyoma, and infection of chicken, mouse, and rat cells by the RNA tumor viruses, Rous (or avian) sarcoma virus (RSV), murine sarcoma virus (MSV), and associated RNA helper viruses, avian and murine leukemia virus (ALV and MLV). It is interesting that Table 1 applies to transformation by both DNA and RNA tumor viruses, and that more than half of the list is associated either directly or by implication with changes in the cell surface.

While it is known that in transformation the viral genome is retained and replicates in the cell nucleus (51-53), and is most probably integrated in the cell genome (54), no biochemical mechanism by which the transforming virus exerts its effect on the cell has yet been identified. For our present purpose of elucidating the effect of viral transformation on the cell surface and on proliferation, we will first describe

an experiment with SV40 that demonstrates the range and variability of viral effects, and then focus on the new experimental system of temperature-sensitive (*ts*) avian sarcoma viruses in interaction with chick fibroblasts, which has provided clear answers to a few basic questions on the nature of transformation.

The properties of transformed cells listed in Table 1 can in many cases be used for selection of transformants following viral infection. Thus, mouse 3T3 cells transformed by SV40 can be isolated by recloning from colonies observed to grow to higher than normal density after infection, in nutrient medium containing a standard 10% concentration of calf serum. For study of the frequency and range of expression of the various characters of the transformed phenotype, however, a nonselective assay is required; such a study has recently been carried out by Risser & Pollack (4). Following infection of 3T3 cells by SV40 virus at a concentration of 2×10^3 infectious units per cell, some 40 clones were picked at random, i.e. without regard to growth properties or morphology. If the random clones are assayed for frequency of transformation as defined by abnormally high saturation density in 10% serum, only 25% of the clones are transformed. Such a result has typically led in the past to the statement that SV40 transformation of 3T3 is a low frequency event. However, if transformation is defined as the expression of any of the ensemble of properties in Table 1, fully 90% of the clones are found to be transformed. The property of serum transformation, i.e. the ability of a clone to proliferate in 1% serum with the 30-hr doubling time observed for standard high density transformants, is especially interesting. In the present experiment, serum transformation is exhibited by all clones that are transformed in any other assay, and also by some clones that are normal by all other assays, and therefore may be considered to be the fundamental property of transformation. Other parameters of transformation in Table 1 show a broad range in degree of expression. Thus SV40 T-antigen is not detected by fluorescein-coupled antibodies or complement fixation in some 40% of the clones, and an additional 25% show mixed positive and negative fluorescent labeling from cell to cell of a clone.

We conclude from this experiment that the interaction of virus and cell in transformation is far from a simple all or none switch. However, the ability of transformed cells to grow in low serum may be primary, and it may be possible in the future to relate such a primary parameter of transformation to the structural and enzymatic effects discussed in the following sections.

The infection of chick fibroblasts by members of the rapidly growing family of *ts* mutants of avian sarcoma virus is a promising current system for discovering mechanisms of viral transformation [for review see Vogt et al (55)].

To recover *ts* mutants, virus stocks are mutagenized by treatment with nitrosoguanidine or by permitting replication in the presence of the RNA mutagens 5-azacytidine or 5-fluorouracil or the DNA mutagen 5-bromodeoxyuridine. Most mutant viruses have been isolated by random cloning and testing of infected cells. Since the first isolations by Martin (5) and Biquard & Vigier (56) in 1970, several dozen temperature-sensitive mutants have been prepared by ten or more groups (see reference 15). All of these mutants are permissive, i.e. cause transformation, at 35–37° C and are nonpermissive at 40–41° C. In general, temperature sensitivity is

believed to result from reduced conformational stability of an aberrant protein coded by a mutant gene. Thus we can already conclude that chick fibroblasts are transformed by avian sarcoma virus as a result of the positive action of a virally coded protein on the cell system.

For a class of *ts* viral mutants called T1 (57), maintenance of the transformed cell phenotype requires maintenance of the permissive temperature. Thus some viral gene products are required continuously for cell transformation. The following membrane-specific properties of transformed cells (Table 1) are among those made temperature sensitive by T1 mutants: increased rate of sugar uptake, increased agglutinability by plant lectins, changes in the amount of particular glycolipids and glycoproteins in the cell membrane, reduced concentration of cAMP, and production of fibrinolysin T (6).

The time course and requirements for RNA and protein synthesis in establishing the transformed phenotype on temperature shift down or the normal phenotype on shift up show interesting variations for the different T1 mutants. For some, 50% transformation occurs in 5-6 hr and requires protein synthesis but not RNA synthesis (58, 59). The return to normal phenotype occurs in the same time and requires no macromolecular synthesis at all (59). For the mutant of Bader (60) morphological transformation appears within 1 hr, even in the presence of inhibitors of protein synthesis. For the T5 mutant of Martin (61) the appearance of fibrinolysin T (6) seems to require both RNA and protein synthesis.

The genetics of the temperature-sensitive viruses has barely begun, but already Wyke (62) has divided a group of 11 T1 viruses into four complementation groups on the basis of their ability to complement one another in producing transformation at the nonpermissive temperature. This result, if taken at face value, implies at least four distinct viral genes for RSV transformation.

The *ts* avian sarcoma viruses so far discussed are temperature-sensitive for transformation but not for virus production. Some mutants are coordinately *ts*, however, and one such, *ts*334, has unusual transformation properties. It is temperature-sensitive for morphological transformation, as shown by the scanning electron micrographs of Figure 2, but not for growth in agar suspension culture (63). Thus the opportunity may exist for examining separately the genetic and biochemical basis for these two phenotypic characters.

AGGLUTINATION, SURFACE RECEPTORS, AND INTERNAL CONNECTIONS

Since the discovery by Ambrose et al (64) and Aub et al (65) of the preferential agglutinability of cells from spontaneous tumors by some plant lectins, Burger and co-workers (66, 67), Inbar & Sachs (68), and others have established a strong correlation between loss of density-dependent inhibition of growth for cells in culture and an increase in agglutinability. However, using the lectin concanavalin A (conA), labeled with ^{125}I or ^3H , Arndt-Jovin & Berg (69), Cline & Livingston (70), and Ozanne & Sambrook (71) found that increased agglutinability was not associated with a significant increase in the amount of lectin bound per cell. Nicolson

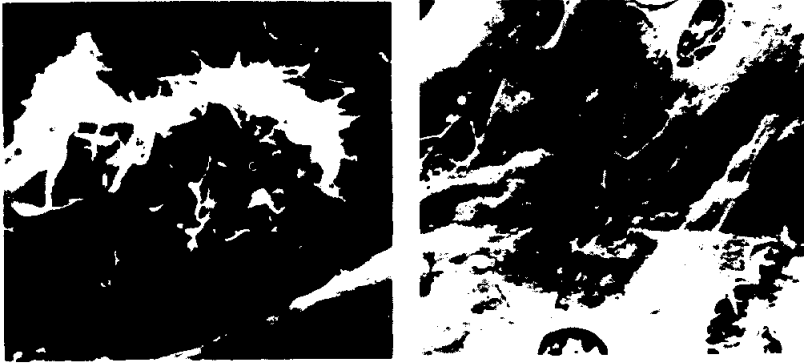


Figure 2 Chick cells infected with *rs334*, a temperature-sensitive mutant of avian sarcoma virus B77. (left) At permissive temperature (35° C), cell transformed; (right) at nonpermissive temperature (41° C), cells not transformed. Magnification $\times 5000$. Scanning electron micrographs by A. Boyde and R. Weiss. Used with permission from J. Tooze, Ed., *The Molecular Biology of Tumor Viruses* (15).

(72) pointed the way to resolution of this puzzle by electron microscopy. The distribution of conA bound to 3T3 cells and SV40-transformed SV3T3 cells was observed using ferritin-conjugated conA. On agglutinable cells conA was found in patches, and on nonagglutinable cells it was found randomly dispersed.

The question then arises whether conA receptors on agglutinable cells preexist in patches or whether the patches are produced by the addition of the conA. The work of Rosenblith et al (73) suggests the latter. These workers used electron microscopy of shadow-cast replicas of 3T3 and SV3T3 surfaces; the positions of conA receptor complexes were marked by hemocyanin, a molecule 350 Å in diameter which binds specifically to conA. They observed that aggregates are not formed on either 3T3 or SV3T3 surfaces at 4° C, and also not at 37° C when the cells are prefixed by 1% paraformaldehyde before the addition of conA. This result demonstrates that conA receptors are dispersed on both agglutinable and nonagglutinable cells before addition of conA, and that reduction of the temperature to 4° C or prefixation immobilizes the receptors.

At 37° C and without prefixation, agglutinable SV3T3 cells form patches while nonagglutinable 3T3 cells do not, as had been shown by Nicolson (72). These results, taken together, demonstrate that conA receptors of transformed cells have lateral mobility but that receptors of untransformed cells are not free to move, even in the absence of any experimental conditions designed to fix them.

The conclusions so far are independent of any model, but it is natural to make the further interpretation that patches are formed when laterally diffusing receptors approach each other closely enough to be cross linked by conA. Native conA has four binding sites for α -methylglucose-like receptors (74) and therefore can in principle form networks of receptors just as antibodies form networks with antigen.

The original interpretation of Nicolson (72), that agglutination of cells results from the concentrated cell-cell binding force which patches can provide, still holds. However, since the main interest in agglutination is in the insight it can provide about surface receptors, we do not need to insist on a connection between patches and agglutination and can instead work directly with the results concerning receptors of a single cell.

It is well known that 3T3 cells are made as agglutinable as SV3T3 cells by treatment with trypsin (75). This result is both explained and made more significant by further experimental results of Rosenblith et al (73). They observe that trypsinized 3T3s form patches of conA at 37° C, but not at 4° C or when prefixed. Thus trypsin appears to act by freeing 3T3 conA receptors of their normal constraints, as does transformation by SV40. The reciprocal question whether viral transformation frees conA receptors by proteolytic activity is not yet answered, but in view of the proteases found to be associated with transformed cells (see the next section), experiments to answer the question will undoubtedly be performed soon.

Note that the earlier view that trypsin produces agglutinability by exposing lectin receptors (which however disagreed with experiments showing no increase in lectin binding) may now be replaced by the idea that trypsin removes normal cellular restraints on receptor mobility. Since we have given evidence above that some membrane particles are free to move, the source of this restraint becomes of great interest. We will present a hypothesis below but first consider some parallel evidence from lymphocytes.

Taylor et al (76) discovered that when the immunoglobulin-bearing surface of the lymphocyte is exposed to fluorescein conjugated antiimmunoglobulin antibodies, the endogenous immunoglobulin, which we will call "receptor Ig," first aggregates into patches that are subsequently transported to one pole of the cell (to form a "cap") and then engulfed. Patch and cap formation has been confirmed and characterized further by de Petris & Raff (77), Edelman and co-workers (78-80, 82), and Unanue, Perkins & Karnovsky (81).

The plant agglutinin conA is also bound to lymphocyte surfaces. This fact has permitted a determination whether conA receptor sites on lymphocytes also cluster. In fact, if conA is added to lymphocytes at 4° C, excess conA washed away, and the cells brought to 37° C, conA forms patches and caps with its own receptors (78, 80). However, if conA is added at 21 or 37° C, then both its own patch and cap formation and that of receptor Ig are inhibited. Both inhibitions are surprising. The conA self-inhibition is surprising because the previous 4-37°C incubation experiment shows that conA receptors on lymphocytes can be mobile under some conditions. The cross inhibition between conA and receptor Ig is surprising because the two sets of receptors are certainly different and neither one occupies a large enough fraction of the membrane surface to make it likely that there can be any simple steric interference of one group with the other.

An additional observation by Edelman, Yahara & Wang (79) may point to an explanation: both the self and cross inhibitions are reversed by colchicine, colcemid, vinblastine, and vincristine—four reagents which promote the disassembly of microtubules (83).

Therefore, Edelman and co-workers (11) hypothesize that there is an interior cellular structure of microtubules bound to various groups of membrane receptor proteins and glycoproteins and that the binding can be modulated, i.e. promoted by some reagents or physical conditions and diminished by others. They suggest that conA may stimulate the internal structure to bind to both conA receptors and receptor Ig and that such binding prevents lateral mobility of both sets of receptors. How would the evidence for free conA receptors after 4–37° C incubation be explained? Microtubules can dissociate at low temperature (84) and may reassemble at 37° C only on a time scale of hours (85); therefore patch and cap formation of conA receptors may occur before the internal structure is restored.

The interesting possibility that the microtubular structure is involved in the first steps of mitogenesis arises as follows: some lectins such as conA are mitogenic and others such as wheat germ agglutinin are not. Preliminary experiments reported by Edelman (11) show that a number of mitogenic lectins inhibit receptor Ig cap formation and a number of nonmitogenic lectins do not. Thus the mitogenic lectins may be precisely those having receptors that can be attached to the internal immobilizing structure. In support of this view, mitogenesis by conA is inhibited by colchicine, vinblastine, and vincristine at low concentration (11).

A PROTEOLYTIC ACTIVITY QUITE GENERALLY ASSOCIATED WITH TRANSFORMED CELLS IN VITRO AND MALIGNANT TUMORS IN VIVO

Reich and co-workers (6, 7) have recently discovered a proteolytic activity which they have shown to be broadly associated with transformed cells in vitro and in vivo. An early and previously neglected clue leading to the discovery was found in the cell culture experiments of Fischer (87) where explanted tumors but not bits of normal tissue were observed to lyse a blood clot used as substrate. Repeating Fischer's experiments under conditions of modern cell culture, Unkeless et al (6) and Ossowski et al (7) found that virus-transformed fibroblasts but not normal fibroblasts hydrolyze a substrate of fibrin. Fibrinolysis is induced by both DNA- and RNA-transforming viruses but not by a wide range of cytolytic viruses or the nontransforming avian leukosis viruses or the temperate paramyxovirus SV5 (6, 86). As noted earlier, the production of fibrinolytic activity is temperature sensitive (6) in chick cells infected with Martin's *ts* mutant of avian sarcoma virus, T5 (61). The activity can be detected within 1 hr of shift to permissive temperature (86) and, therefore, is an early event in transformation. Fibrinolysis is observed to be associated with primary cultures of chemically induced mammary carcinomas, hepatomas, and skin cancers and with a variety of human and animal tumor cell lines (86). It is not associated with the single class of benign tumor so far tested, the rat mammary fibroadenoma (86).

Reich and co-workers determined (6, 7) that the fibrinolytic activity, which they name fibrinolysin T (for tumor), arises from the coordinate activity of two proteases. One, T_S, is present in all vertebrate sera (6, 7, 86) and has been identified by Quigley et al (88) as the known zymogen, plasminogen (mol wt 90,000). The second protease,

T_C , was shown to be present in trace amounts in transformed cells only. T_C has been partially purified and characterized by Unkeless et al (89) as an arginine-specific protease (mol wt. 38,000); it hydrolyzes a single peptide bond in plasminogen to generate the active fibrinolytic protease, plasmin (EC 3.4.4.14).

T_C from a given transformed cell type will not produce fibrinolysis with all sera. Thus, T_C (6) from RSV-infected chick embryo fibroblasts produces fibrinolysis with chicken and monkey sera but not with dog or hamster sera, while T_C (7) from SV40-transformed hamster cultures produces fibrinolysis with dog, hamster, and monkey sera but not with chicken or calf sera. The serum specificity is in part due to inhibitors of plasmin in some sera (86), but is also in part due to a specificity of interaction of the particular T_C with the plasminogen of the particular serum. Thus the failure of T_C from transformed hamster cells to produce fibrinolysis with chicken serum is explained by the observation (88) that plasminogen from chicken serum is not activated by hamster T_C .

While the interactions of T_C , T_S , and serum inhibitors are not yet fully understood, the existence of serum specificity can nevertheless be used as an experimental tool. For example, the spectrum of active sera (7) for mouse cells is the same whether the mouse cells are transformed by SV40 or MSV, but different from the spectrum for hamster cells transformed by SV40. T_C is therefore characteristic of the cell type and not of the transforming virus. Whether T_C is newly synthesized following transformation or merely activated is not currently known, but T_C is found in homogenates of transformed cells, tightly bound to one of the sedimentable membranous cellular particulates in the postnuclear fraction (86).

Initial studies by Ossowski et al (7, 90, 91) indicate that some of the parameters of transformation listed in Table 1 are determined, at least in part, by the activation of the fibrinolytic proteases. Reich and co-workers suggest that characteristic changes of cell morphology (7, 90), colony formation in soft agar (91), and enhanced cell mobility (91) all require the presence of an intact fibrinolytic system. They show also (91) that transformed cells produce receptors for plasmin which are not detectable in normal cultures. The relationship (if any) between fibrinolysis and saturation density of cultures is under study (86).

Considering a possible role for the fibrinolytic system in the formation and growth of tumors *in vivo*, Reich (86) reasons that the initiation of growth by either normal or transformed cells may require an increased level of plasma protein over that normally found in the extravascular, extracellular fluid compartment. He cites as an example an increase by nearly two orders of magnitude in the concentration of plasma protein in the cerebrospinal fluid of patients with brain tumors, and speculates that fibrinolysin T may produce effects of this type by increasing the permeability of the vascular system.

We may speculate further that fibrinolysin T acts on the surfaces of both tumor cells and nearby normal cells to disturb their normal growth control, perhaps by altering the mobilities of membrane receptors. If in addition some normal cells under such stimulus should be found to differentiate so as to express endogenous viral information and so generate more cell factor T_C , a positive feedback loop leading naturally to tumor growth would be shown to exist.

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