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INVESTIGATIONS OF THE POSSIBLE ROLE OF PROTEASES IN ALTERING SURFACE PROTEINS OF VIRALLY TRANSFORMED HAMSTER FIBROBLASTS

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Virally transformed fibroblasts have on their surfaces zero or reduced amounts of a large external transformation-sensitive (LETS) glycoprotein. This protein is extremely sensitive to proteolysis. When prelabeled normal fibroblasts are cocultivated with transformed cells, the LETS glycoprotein of the normal cells shows an increased rate of turnover. Experiments are described which investigate the possibility that this phenomenon and the absence of LETS glycoprotein are due to proteolysis by the transformed cells. In particular, the role of plasminogen activation is examined by the use of protease inhibitors and plasminogen-depleted serum. It is concluded that activation of plasminogen is not required for the disappearance of the LETS glycoprotein although the involvement of other proteases cannot be ruled out. The role of proteases in affecting cell growth and behavior is discussed.

INTRODUCTION

In a previous paper (1) it was demonstrated, using lactoperoxidase-catalyzed iodination (2, 3) to label proteins exposed at the surface of cells, that the surfaces of virus-transformed fibroblasts lack one of the major exterior proteins detected on normal fibroblasts. This result has been confirmed for a variety of different cell types transformed by different viruses (4–6) and with an alternative external labeling technique specific for galactose residues (7–9) or immunological methods (10, 11). The proteins detected by the different methods appear to be identical (9, unpublished results). Subsequently it was shown, by metabolic labeling with sugar precursors, that normal cells synthesize and accumulate an external glycoprotein of electrophoretic mobility identical to this iodinatable protein, whereas transformed cells do not (6, 12, 13). Taken together these results provide evidence for a large, external transformation-sensitive (LETS) glycoprotein.

Since reduced levels of the LETS glycoprotein can be detected by metabolic labeling as well as by external probes, the difference between normal and transformed cells cannot be due merely to masking. Absence of the LETS glycoprotein must therefore be

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due either to a failure in its synthesis or insertion at the surface of the transformed cells, or to its continual loss or removal. Evidence from experiments involving cycloheximide demonstrates that synthesis of the LETS glycoprotein or of a precursor continues in transformed cells (14, Hynes and Bye, in preparation).

It was also shown that the LETS protein is very sensitive to proteolytic digestion (1, 4, 6). Since transformed cells produce proteolytic enzymes (15–19), it is conceivable that the absence of a major iodinatable protein from the surfaces of transformed cells is due to its being removed by proteases produced by the transformed cells (20) either directly or through activation of a serum proenzyme such as plasminogen (21–23). The experiments reported here attempt to investigate this possibility. Similar experiments have been reported briefly elsewhere (14).

METHODS

Cells and Culture Methods

Cells used were two hamster fibroblast cell lines, NIL8 (24) and BHK (25), and derivatives of these transformed by hamster sarcoma virus NIL8.HSV6 (26), and polyoma virus BHK.Py, respectively. These cells were kindly provided by Drs. I. A. Macpherson and G. D. Clarke. Transformed cells were isolated by their ability to grow in soft agar (27). All lines were grown in Dulbecco's modification of Eagle's medium (28) plus 10% calf serum and subcultured by trypsinization and seeding in plastic Petri dishes (Nunc).

Lactoperoxidase-Catalyzed Iodination

This was performed on cell monolayers as described previously (1). Cells were washed three times with phosphate-buffered saline, pH 7.2, and labeled in the same buffer plus 5 mM glucose, 400 μCi/ml carrier-free sodium [^{125}I] iodide, 20 μg/ml lactoperoxidase, and 0.1 U/ml glucose oxidase. Incubation was for 10 min at room temperature. The reaction was stopped by addition of phosphate-buffered iodide and the monolayers washed twice more with the same buffer. For harvest, cells were scraped into phosphate-buffered iodide plus 2 mM phenyl methyl sulfonyl fluoride to inhibit proteases, concentrated by centrifugation, and dissolved by boiling in electrophoresis sample buffer containing 2% Na dodecyl suplhate (SDS) and 2 mM phenyl methyl sulfonyl fluoride. Samples were made 0.1 M with dithiothreitol and boiled again before electrophoresis.

Polyacrylamide Gel Electrophoresis

This was performed with the SDS buffers described by Laemmli (29). Electrophoresis was in vertical slabs and 12 samples were run in parallel. Gels were stained with Coomassie Brilliant blue if necessary. For detection of radioactivity, gel slabs were dried down onto paper (30) and autoradiographs made on Kodirex X-ray film.

Fibrinogen Preparation and Fibrinolysis Assay

Fibrinogen was labeled by lactoperoxidase-catalyzed iodination and the fibrinolysis assay on plates was performed as described by Unkeless et al. (15), except that 20 μg

fibrinogen/cm^2 of plate was used instead of 10 μg/cm^2.

Plasminogen-Depleted Serum

This was prepared by running calf serum over lysyl-Sepharose until no further material bound to the column (21, 22, 31). Bound material was eluted from the column with ϵ-amino caproic acid. This bound material migrated as a single band on SDS-polyacrylamide gels (mol wt 90–95 $\times$ 10^3, coincident with plasminogen). One preparation was a kind gift from Dr. J. Quigley.

Radiochemicals

Carrier-free sodium [^{125}I] iodide and L-leucine-^{14}C(U) (342 Ci/mole) were purchased from the Radiochemical Centre, Amersham, Bucks, England.

Enzymes and Inhibitors

Lactoperoxidase, tosyl arginine methylester (TAME), tosyl phenylalanine chloromethane (TPCK), and tosyl lysyl chloromethane (TLCK) were from Calbiochem; glucose oxidase and lima bean and pancreatic trypsin inhibitors from Worthington Biochemicals; human plasminogen from Kabi, Stockholm; Streptokinase, Behringwerke AG; twice-crystallized trypsin, Koch Light Labs.; soyabean, pancreatic, and ovomucoid trypsin inhibitors, ϵ-amino caproic acid, twice-crystallized trypsin, and phenyl methyl sulphonyl fluroide from Sigma, London. Nitrophenyl-p-guanidino benzoate (NPGB) was a gift from Dr. J. Unkeless, antipain and leupeptin were gifts from Dr. A. McIlhinney, and Trasylol (10,000 KIU/ml) was a gift from Bayer Pharmaceuticals Ltd.

RESULTS

Effect of Cocultivation with Transformed Cells on the Surfaces of Normal Cells

Normal cells (NIL8 or BHK) were iodinated with lactoperoxidase and washed, and medium was returned to the dishes. They were either left simply in medium with or without serum, or else additional normal or transformed cells (NIL8, HSV, or BHK-Py) were plated on top of the iodinated monolayer, either with or without serum. The dishes were then returned to the incubator and at various times the medium was removed and the monolayer washed, harvested, solubilized, and run on SDS-polyacrylamide gels. As shown elsewhere, the proteins detected in this manner are external, i.e. they extend beyond the plasma membrane (1–3). The results in Figs. 1 and 2 show that when transformed cells were plated on top of the labeled normal ones and left for 24 hr, there was a greater loss from the cell layer of the high molecular weight iodinated LETS glycoprotein than was observed in controls. Addition of unlabeled normal cells had no effect (Figs. 1, 2). It should also be noted that loss of prelabeled LETS glycoprotein occurred without a requirement for added serum. Furthermore, loss of the LETS glycoprotein occurred in several different sera (calf, hamster, and chicken; Fig. 1). Other relevant facts are: (a) neither of the transformed lines used produces the transforming virus concerned, so the effect is not due to cross infection; (b) the overlying cells were prepared as a suspension with EDTA without any proteolytic enzymes – less than 10% were permeable to trypan

blue. At shorter times (e.g., 5 hr) loss of LETS glycoprotein label was not complete but a difference could already be seen between addition of transformed and normal cells (data not shown). NIL8.HSV cells produced more complete loss of prelabeled LETS protein than did BHK.Py whether acting on NIL8 or BHK cells.

Thus, cocultivation of prelabeled normal cells with transformed cells leads to an increased rate of disappearance of the major iodinated protein of the normal cells. This loss appears to be at least partially specific for this one protein, resulting in an iodination profile similar to that observed for transformed cells. We cannot rule out the possibility that the transformed cells are more fragile and, on suspension using EDTA, they nonspecifically leak more proteases than do normal cells under the same conditions, although there was no difference in the degree of permeability to trypan blue. In fact, transformed cells are detached from dishes far more readily than are normal ones and, consequently, are generally subjected to less vigorous pipetting to obtain single cell suspensions.

Unkeless et al. (15) demonstrated that there was no increased leakage of lysosomal hydrolases by transformed cells. In any case, leakage of proteases is one form of any model suggesting that proteases are involved in alterations to transformed cell surfaces.

Fibrinolysis by Transformed Cells

In order to confirm that the transformed cells used in the experiment shown in Figs. 1 and 2 were, in fact, producing proteolytic enzymes, we assayed them according to the fibrinolysis procedure of Unkeless et al. (15). Both transformed cell types were more effective than their normal counterparts in releasing [^{125}I] fibrin from the dishes. In 24 hr. BHK cells removed 16–25% of the [^{125}I] fibrin compared with 75–80% for BHK.Py, while NIL8 cells removed 25% compared with 100% for NIL8.HSV. This fibrinolysis was inhibited by certain inhibitors of proteolytic enzymes (Table I): pancreatic trypsin inhibitor was most effective. After 22-hr incubations, ϵ-aminocaproic acid and soyabean trypsin inhibitor were effective in inhibiting fibrinolysis by both cell types. Fibrinolysis by NIL8.HSV cells was more rapid than by BHK.Py so that by 22 hr most of the [^{125}I] fibrin had been removed by control NIL8.HSV cells, and by 48 hr, those in these two inhibitors had caught up since the inhibition was not complete. Thus, the same three inhibitors were effective in inhibiting fibrinolysis by both cell types, in agreement with the previous results of Unkeless et al. (15).

Effects of Protease Inhibitors on Cells

The inhibitors listed in Table II were tested for their ability to inhibit growth of NIL8.HSV or BHK.Py cells and cause reversion of their morphology to normal. The morphology of NIL8 cells is flat, whereas NIL8.HSV cells are refractile and grow in suspension as well as attached to the dish. BHK.Py cells grow in a random multilayered fashion, as opposed to the orientated growth pattern of BHK. Thus, reversion to normal phenotype would have been detected readily in either case. None of these inhibitors produced such a reversion. Cells cultivated in these inhibitors for periods of one to several days were iodinated and analyzed on SDS-polyacrylamide gels as shown in Fig. 3. In no case did labeling of the LETS glycoprotein occur on transformed cells. Some low molecular weight protease inhibitors (TPCK and TLCK) were toxic to the NIL8.HSV cells, and their

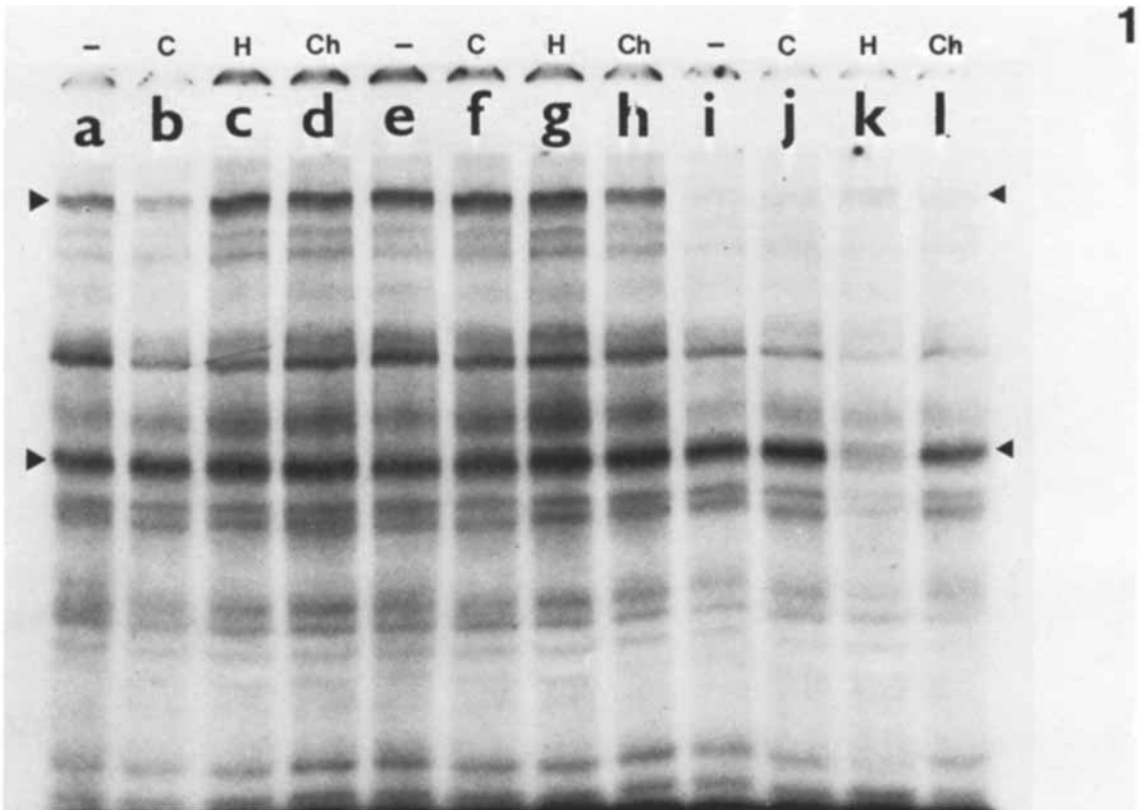

Fig. 1. Cocultivation of iodinated NIL8 cells with NIL8.HSV cells. Monolayers of NIL8 were iodinated, washed, and overlaid with 10^6 cells obtained in suspension with 0.02% EDTA, or without additional cells. All dishes had 2 ml of medium either without serum or with 10% serum as indicated. 24 hr later, the medium was removed and the cells were washed and analyzed by SDS-polyacrylamide gel electrophoresis. The figure is an autoradiograph of a 6.5% gel. Arrows mark the LETS glycoprotein (near top of gel) and lactoperoxidase (85,000 daltons, in the middle of the gel). The enzyme was detected in this experiment since the cells were dissolved directly from the dish rather than being scraped and pelleted. Additions: a–d, no cells; e–h, NIL8 cells; i–l, NIL8-HSV cells. Added serum as marked: calf, C; hamster, H; chicken, Ch. Samples represent equal fractions of the material from each dish.

effects on iodination therefore difficult to analyze. Trasylol (100 KIU/ml) and antipain and leupeptin (100 μg/ml), all of which inhibit plasmin, were also without effect on the morphology or growth of NIL8.HSV. Their effects on BHK.Py and on iodination profile were not tested.

Several of these inhibitors were also tested to see whether they inhibited the loss of prelabeled LETS glycoprotein from normal cells cocultivated with transformed cells (Fig. 4 and Table III). All those tested were ineffective. In particular, pancreatic trypsin inhibitor and ϵ-aminocaproic acid, which block fibrinolysis (Table I) and inhibit plasmin (Unkeless et al., 1973) failed to block loss of LETS glycoprotein in either cell type. Soyabean trypsin inhibitor did not block removal by BHK.Py; its effect on removal by NIL8.HSV was not tested.

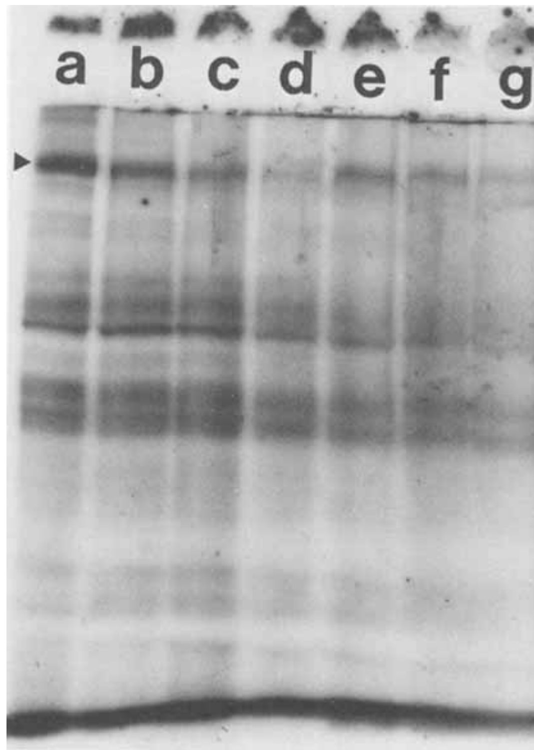

Fig. 2. Cocultivation of iodinated BHK cells with BHK.Py cells. Procedures as figure 1. (a) No incubation; (b–d) no added serum; cells overlaid as below: (b) none; (c) BHK; (d) BHK.Py. (e–g) 10% calf serum; cells overlaid as below; (e) none; (f) BHK; (g) BHK.Py. Arrow marks position of the LETS glycoprotein.

Effect of Plasminogen-Depleted Serum

Plasminogen can be removed from serum by affinity chromatography on lysine-Sepharose (see Materials and Methods). NIL8 and NIL8.HSV cells were cultured for two passages in such serum and parallel cultures in plasminogen-depleted serum to which purified plasminogen had been added back (reconstituted serum). No differences in pattern or rate of growth were noticed between depleted and reconstituted serum-containing dishes in either case. Dishes of each type were iodinated and some labeled NIL8 dishes were overlaid with NIL8 or NIL8.HSV cells for incubation overnight before harvest. This post-incubation was done in both the presence and the absence of plasminogen. In the latter case, both labeled and overlaid cells had been grown for two passages in the absence of plasminogen.

Iodinated samples were then analyzed on SDS-polyacrylamide gels. No differences were detectable dependent on the presence of plasminogen. In particular, NIL8.HSV cul-

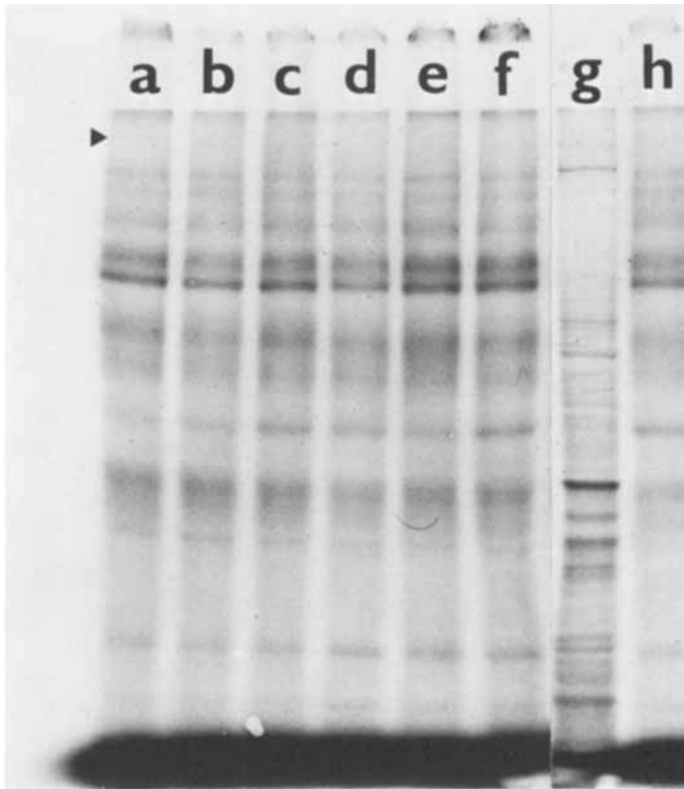

Fig. 3. Cultivation of NIL8.HSV cells in protease inhibitors. Cells were cultured for 2 days with added inhibitors as follows: (a) PMSF, 50 μg/ml; (b) TAME, 50 μg/ml; (c) ε-amino caproic acid, 500 μg/ml; (d) soybean trypsin inhibitor, 200 μg/ml; (e) lima bean trypsin inhibitor, 200 μg/ml; (f) ovomucoid, 200 μg/ml; (g) [^{14}C] leucine-labeled marker; (h) control iodinated NIL8.HSV. All samples were iodinated at confluence and equal numbers of cells applied to the gel, which was 6.5% acrylamide. Arrow marks position of migration of the LETS glycoprotein in NIL.8 control cells.

tivated in its absence still had no iodinatable LETS glycoprotein, and loss of this protein from NIL8 cells was accelerated by the presence of NIL8.HSV cells whether or not plasminogen was present (Fig. 5).

DISCUSSION

The results reported here show that virus-transformed cells cocultivated with prelabeled normal cells cause an accelerated rate of turnover of a major external protein. This protein is absent from the surfaces of the transformed cells themselves, so that the change produced in the normal cells by cocultivation with transformed cells is in the direction of transformation. The transformed cells release proteolytic enzymes as reported here and elsewhere and the major external protein in question is known to be very sensitive to proteolysis (see Introduction). A simple interpretation of all these results would

TABLE I. Fibrinolysis by transformed cells

	BHK.Py				NIL8.HSV			
	22 hr		48 hr		22 hr		48 hr	
Inhibitor added (concentration; μg/ml)	cpm	% inhibition	cpm	% inhibition	cpm	% inhibition	cpm	% inhibition
None	1,357	–	1,892	–	1,865	–	1,996	–
ϵ-Aminocaproic acid (1,000)	130	90	398	79	610	67	1,529	24
Soybean trypsin inhibitor (500)	113	92	192	90	1,128	40	1,871	6
Pancreatic trypsin inhibitor (500)	135	90	154	92	169	91	267	87
Ovomucoid trypsin inhibitor (500)	1,110	18	1,888	0	1,979	0	1,929	3
Tosyl lysine chloromethane, TLCK (100)	816	40	1,875	1	1,871	0	1,987	0

2.10^6 cells prepared as single-cell suspensions with 0.02% EDTA were plated in 5-cm dishes coated with [^{125}I] fibrin. Each dish contained 5 ml of medium containing 10% calf serum. Incubation was at 37°C for 48 hr. 100-μl aliquots were removed and counted.

be that transformed cell proteases are responsible for the removal of the LETS glycoprotein from the cells. However, other interpretations are equally possible.

We have attempted to produce accelerated loss of iodinated LETS glycoprotein from the surfaces of normal cells, using media conditioned in various ways by transformed cells. No consistent increase in turnover of prelabeled LETS glycoprotein in response to addition of such conditioned media has been observed, although these media do carry out fibrinolysis (unpublished results). This result could be due to inadequate concentration or instability of a putative protease or to the fact that it is cell bound. We are at present unable to decide among these possibilities or to prove that a protease is in fact involved. It is known that confluent resting cells, such as those used in the mixing experiments described here, have high levels of the LETS glycoprotein on their surfaces and that after stimulation of resting cells by addition of serum, the external level of the LETS glycoprotein falls (32). Therefore, effectors not known to involve proteolysis can lead to alterations in levels of surface proteins and could be involved in the results described here.

The results of the experiments using protease inhibitors are inconclusive in deciding whether or not proteolysis is involved in causing loss of the LETS glycoprotein. None of the inhibitors tested was effective in causing reappearance of this protein on the surfaces of transformed cells (Fig. 3, Table II) or in blocking the accelerated loss of prelabeled LETS glycoprotein in the mixing experiments (Fig. 4, Table III). This could merely mean that none of the inhibitors tested was effective against the relevant protease(s) or that proteases are not involved.

Possibility of Involvement of Plasminogen Activation

One protease which is a candidate for a role in altering surface proteins during transformation is plasmin, produced after activation of plasminogen by activators released by transformed cells (15, 16, 19–23, 33–35). In direct tests on NIL8 cells, human plasminogen, when activated by streptokinase, is able to remove this polypeptide from the surfaces of the cells (14). Complete removal is produced by incubation for 1 hr at 37°C with a concentration of plasminogen (70 μg/ml) equivalent to that present in 10% calf serum if it is assumed that plasminogen comprises 1% of serum protein (22). Therefore, plasmin, produced by activation of serum plasminogen, is potentially responsible for the accelerated loss of LETS glycoprotein in the presence of transformed cells and for its absence from the transformed cells themselves. However, several lines of evidence suggest that it is not required. (1) Loss of prelabeled LETS glycoprotein in the mixing experiments is not dependent on added serum (Fig. 1) and occurs equally well in sera (such as chicken) whose plasminogen is not activated by heterospecific factors (22). (2) Loss of LETS glycoprotein is not blocked by inhibitors which do block fibrinolysis (compare Table I and III and Fig. 4). (3) Removal is not produced by conditioned media which do lead to fibrinolysis. (4) Loss occurs equally well in plasminogen-depleted serum (Fig. 5) and transformed NIL8-HSV cells cultured in plasminogen-depleted serum still lack the LETS glycoprotein on their surfaces.

This evidence renders unlikely a requirement for activation of plasminogen to cause loss of the LETS glycoprotein: this conclusion is subject to the reservations that plasminogen involved in affecting cell surface proteins might be inaccessible to inhibitors and that the plasminogen-depleted serum might retain sufficient activity to affect cell surface pro-

teins, although fibrinolysis was markedly depressed.

Chicken cell plasminogen activator is inhibited by arginine analogs (33). If the same is true of the hamster cell activator, then failure of TAME and NPGB to affect levels of the LETS glycoprotein (Tables II and III, Fig. 4) argue against a role for the plasminogen activator in causing the absence of this protein. The same arguments about accessibility of the enzyme to inhibitors apply as in the case of plasminogen. We have reported elsewhere that certain cell types showing high levels of fibrinolysis, presumably involving production of plasminogen activator and activation of plasminogen, also have high levels of the LETS glycoprotein (36). This result also argues against these two proteases being sufficient to cause loss of the LETS glycoprotein. It is at present unclear how this protein, which is very protease sensitive, continues to survive in a proteolytic milieu on the surfaces of these cells.

Possible Role of Proteases in Growth and Morphology

Our experiments were mainly directed at the question of a relationship between proteolysis and absence of the LETS glycoprotein. It is not clear whether this protein plays any role in the altered growth behavior and morphology of transformed cells. However, it has been suggested many times that proteases might be so involved. Several of our results bear on this question.

Evidence has been presented by others that added proteases alter many properties of normal cells toward those of transformed cells (reviewed in reference 20). In particular they stimulate growth (37–39) although this result has been disputed (40). Reciprocally, protease inhibitors inhibit growth of transformed cells (41, 42). However, it is now becoming clear that this growth inhibition is not specific for transformed cells (43–45) and does not lead to G_O arrest as originally suggested (46, 47). Several of the protease inhibitors used in these experiments have side effects such as inhibition of protein synthesis (43–45), binding to glutathione (48), and inhibition of RNA synthesis (49). This renders interpretation of the results difficult.

With particular reference to plasminogen, it has been shown that this protein is required for a series of morphological changes characteristic of transformed cells for migration and for growth in agar but apparently not for increased growth in culture (21, 34). Inhibition of fibrinolytic activity did not restore density-dependent growth inhibition to SV3T3 cells (50). However, there exists a correlation between fibrinolytic activity of cells, their ability to grow in soft agar, and their tumorigenicity (35, 51, 52).

In our experiments we did not observe any effects of the protease inhibitors tested on the growth or morphology of transformed hamster cells, with the exception of those which were toxic. These inhibitors (e.g., TLCK) produced variable effects sometimes including growth inhibition and flattened morphology but always associated with the loss of cell viability. The results depended on the inhibitor to cell ratio and on the time of addition of the TLCK. Similar results have been obtained by others (43, 44). The macromolecular inhibitors, including those active against plasmin, did not inhibit growth, which is largely in agreement with previous reports with the exception of ovomucoid (cf. reference 42). Similarly, plasminogen depletion of serum did not lead to restriction of growth. More surprisingly, we did not observe morphological changes consequent upon depletion

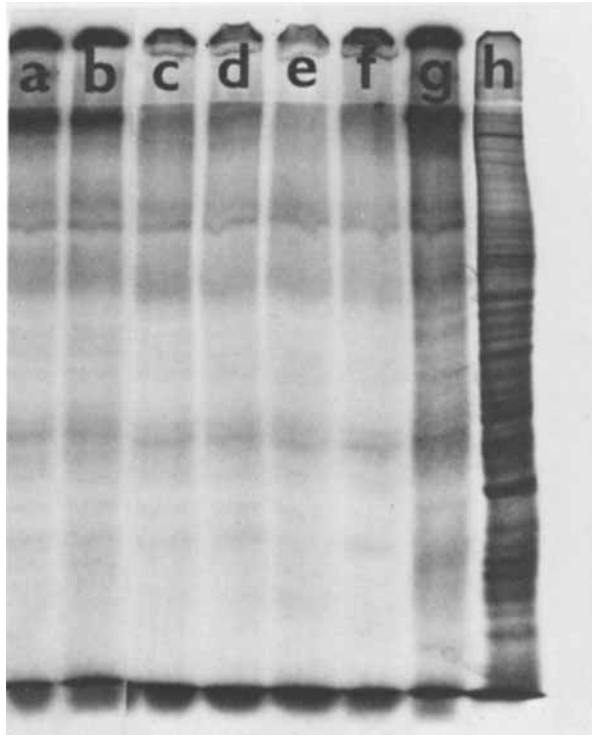

Fig. 4. Cocultivation of iodinated NIL8 cells with 3 × 10^6 cells in the presence of protease inhibitors. All dishes contained 4 ml 10% calf serum and after 24 hr cells were harvested by scraping. Equal fractions were applied to the gel. The figure is an autoradiograph of a 7.5% gel. Arrow marks position of the LETS glycoprotein. Additions were as follows: (a) none; (b) NIL8 cells; (c) NIL8.HSV cells; (d) NIL8. HSV cells, 10^{-4} M NPGB; (e) NIL8.HSV cells, 1 mg/ml ϵ-aminocaproic acid; (f) NIL8.HSV cells, 500 μg/ml pancreatic trypsin inhibitor; (g) control, no incubation; (h) [^{14}C]leucine-labeled marker cells.

or inhibition of plasminogen. This may be related to the fact that our experiments were performed in calf serum which contains inhibitors of the plasminogen-dependent morphological changes (34). Therefore, the transformed morphology exhibited by our cells may be unrelated to that shown by others to be plasminogen mediated.

It is obvious from this discussion that the involvement of protease in the changes occurring on transformation is complex and in need of further investigation. The hypothesis that proteases affect transformed cell behavior by acting on the cell surface remains an attractive one but will need investigating using purified enzymes and inhibitors and antibodies effective against them.

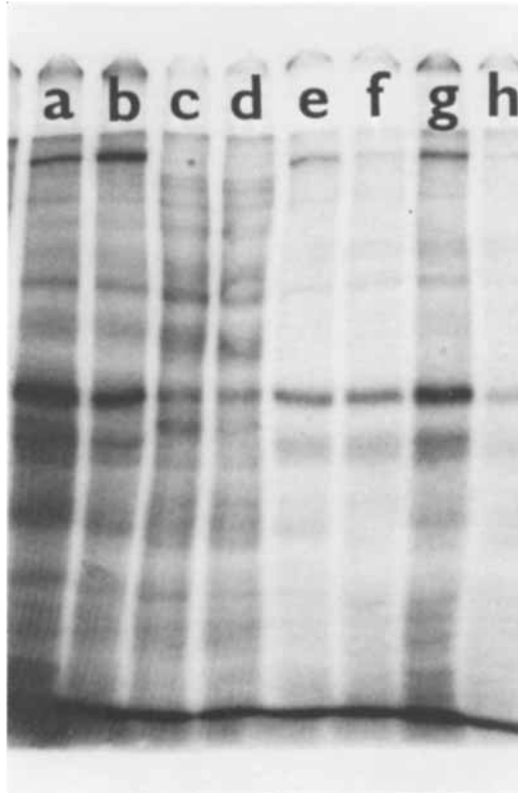

Fig. 5. Effects of plasminogen. Cells cultivated in presence or absence of plasminogen and iodinated, then either harvested directly, (a–d) or overlaid with other cells as in Fig. 1. Volume of medium with 10% serum, 1 ml in 30-mm dishes. Cells were dissolved directly from dishes leading to the appearance of labeled lactoperoxidase on the gel (arrow). (a) NIL.8, plasminogen-depleted serum; (b) NIL.8, reconstituted serum; (c) NIL.8-HSV, plasminogen-depleted serum; (d) NIL.8-HSV, reconstituted serum; (e) NIL.8, overlaid with NIL.8 in plasminogen-depleted serum; (f) NIL.8 overlaid with NIL.8-HSV in plasminogen-depleted serum; (g) NIL.8, overlaid with NIL.8 in reconstituted serum; (h) NIL.8, overlaid with NIL.8-HSV in reconstituted serum. Samples (e–h) represent equal fractions of the material present in each dish.

TABLE II. Protease inhibitors which failed to affect growth or presence of the LETS glycoprotein on transformed cells

Inhibitor	Highest concentration tested: μg/ml	
	NIL.HSV	BHK.Py
Soybean trypsin inhibitor	200	500
Lima bean trypsin inhibitor	200	
Ovomucoid	200	1,000
PMSF	50	
TAME	50	
ε-Aminocaproic acid	1,000	1,000
Pancreatic trypsin inhibitor	100	500
Tosyl lysine chloromethane (TLCK)	Toxic 50 μg/ml	100
Tosyl phenylalanine chloromethane (TPCK)	Toxic 50 μg/ml	

Inhibitors were added at various times after seeding and examined for effects on growth and morphology. Iodination was performed when cells had reached confluence.

TABLE III. Protease inhibitors which failed to affect loss of prelabeled LETS glycoprotein from normal cells cocultivated with transformed cells

Inhibitor	Highest concentration tested: μg/ml	
	NIL.HSV	BHK.Py
ε-Aminocaproic acid	1,000	1,000
Soybean trypsin inhibitor		500
Pancreatic trypsin inhibitor	500	500
Ovomucoid		500
TLCK		100
NPGB	10^{-4} M	

Procedures were as described in the text and in legends to Figs. 1 and 4.

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