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Synthesis, Secretion, and Attachment of LETS Glycoprotein in Normal and Transformed Cells

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LETS glycoprotein is a surface glycoprotein which is absent or greatly diminished on the surfaces of transformed cells. Normal cells secrete large amounts of this protein into the medium; transformed cell medium contains much less. The difference is not due to degradation of the soluble LETS protein. Biosynthesis of LETS protein can be studied by analysis of cell extracts by detergent extraction and immune precipitation and appears to proceed in transformed cells at a reduced rate compared with normal cells. Addition of inhibitors of protein synthesis to transformed cell cultures causes the small amount of LETS protein in the medium to attach to the cells. Addition of normal conditioned medium, which contains LETS protein, to transformed cells alters their morphology towards normal. Addition of purified LETS protein to transformed cells causes the cells to attach, spread, align with one another, and regain actin cables. The results indicate that LETS protein can exchange between cell surface and medium and that it affects cellular adhesion, morphology, and cytoskeleton.

Key words: LETS protein, biosynthesis, adhesion, transformation, cytoskeleton

Much recent research has focused on the question of surface changes in cells transformed by viruses or chemicals. In particular, changes in surface proteins have been reported (1) and the most prominent of these is the loss or reduction of a large external transformation-sensitive (LETS) glycoprotein from the surfaces of transformed cells (2). LETS protein is a major surface protein of fibroblasts and myoblasts and it varies in amount on normal cells depending on their growth state and position in the cell cycle (3).

The absence of LETS protein from the surfaces of transformed cells raises the question of whether this is as a result of reduced or altered synthesis or of other factors such as degradation. Since LETS protein is known to be very sensitive to proteases (1, 2), the latter possibility has received some attention but remains unproven (4). It is known that prelabeled LETS protein turns over into the medium if cells are returned to culture

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(4–6), so it is possible that some alteration in the rate of turnover occurs on transformation such that the balance between LETS protein on the cells and in the culture medium is altered. The question also arises as to whether LETS protein in the medium can bind to cells. Indeed it is not clear that this is not the normal route by which it reaches the cell surface, as is the case for collagen (7). If this were the case, then reduced attachment could provide an explanation for the reduced surface levels of LETS protein in transformed cells.

In this paper, we collect together several experiments bearing on the questions of synthesis, secretion, and attachment of LETS protein in normal and transformed cells and discuss their implications for the understanding of the transformation-induced reduction in surface levels of this protein.

METHODS

Cells and Culture Conditions

The cells used were a normal hamster fibroblast line, NIL8, and its derivative, NIL8-HSV, transformed by hamster sarcoma virus (8). Cells were cultured in Dulbecco's modified Eagle's medium plus 5% fetal calf serum. For labeling with [^{35}S] methionine (New England Nuclear Corporation, Boston, Massachusetts, 22 Ci/mmole) the methionine concentration was reduced to 10% of normal.

Lactoperoxidase Iodination and Gel Electrophoresis

Iodination was performed on monolayers as described (2). Sodium dodecyl sulfate-(SDS)-polyacrylamide slab gels were run in the buffers described by Laemmli (9). Gels were dried and autoradiographed on Kodak x-ray film (NS-2T or RP-R2). For detection of [^{35}S] methionine, gels were impregnated with PPO prior to drying (10).

Purification of LETS Protein

LETS protein was purified from conditioned medium by ammonium sulfate precipitation and Sephadex G200 chromatography or from cells by urea extraction as described elsewhere (11, 12). For preparation of antisera, LETS protein was further purified on preparative SDS-polyacrylamide gels before injection.

Immunofluorescence

Cells grown on coverslips were stained for LETS protein or actin using indirect immunofluorescence as described elsewhere (13, 14). For staining surface proteins, cells were fixed with formaldehyde alone; for staining internal proteins, they were also permeabilized with acetone. Slides were viewed and photographed on a Zeiss microscope equipped with epifluorescent illumination.

Immune Precipitation

Cells labeled with [^{35}S] methionine were lysed in 2% deoxycholate, 0.05 M NaCl, 0.02 M Tris-HCl, pH 8.3 at 4°C, vortexed, and centrifuged at 10,000 × g for 10 min. Aliquots of the supernatent were incubated with 5–10 μl antiserum to LETS protein or with preimmune serum for 1 h at 37°C. Fifty to one hundred milliliters of goat antirabbit immunoglobulin (Cappel Labs, Cochraneville, Pennsylvania) were added and the incubation continued for 1 h at 37°C and then overnight at 4°C. The precipitates were collected and washed 3 times by centrifugation and then dissolved for SDS-polyacrylamide gel electrophoresis.

RESULTS

LETS Protein Synthesized by Transformed Cells

In an earlier paper we reported that chicken cells transformed by temperature-sensitive Rous sarcoma virus at the permissive temperature could regain surface LETS protein on shift to restrictive temperature even in the absence of protein synthesis (15). This result suggested that the transformed cells synthesize LETS protein but for some reason it is not found on the cell surface. No temperature-sensitive mutants were available in the NIL8 hamster system but we observed that addition of cycloheximide or puromycin to transformed NIL8-HSV cells led to the appearance of LETS protein on the surfaces of these cells and to some flattening of the cells (4). Although the explanation for this effect of inhibitors of protein synthesis remains obscure, it suggests the idea that transformed cell cultures contain a pool of LETS protein. Since the transformed hamster line is an established cloned line we have pursued this result in this system.

It turns out that the pool of LETS protein in these cultures is in the medium. This conclusion arises from experiments such as that shown in Fig. 1. If cycloheximide (2 or 20 μg/ml which inhibit protein synthesis $\geqslant$ 98% and 95%, respectively) was added to cultures of NIL8-HSV without medium change then, 24 h later, small amounts of LETS

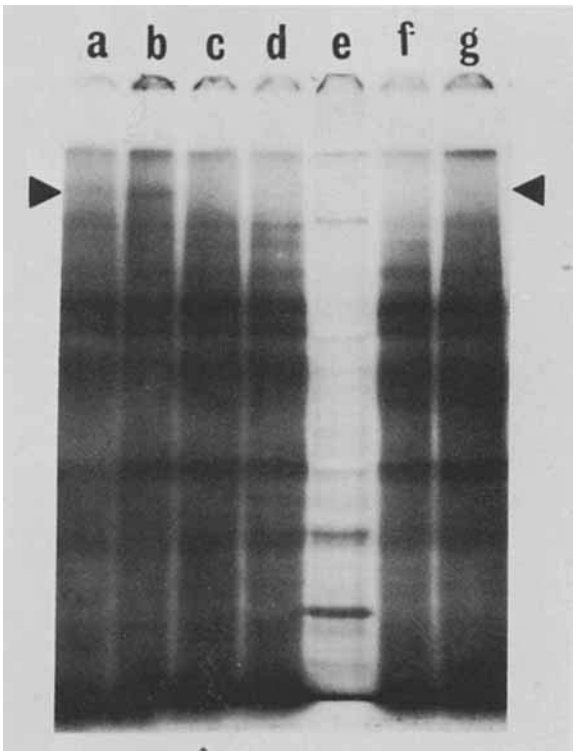

Fig. 1. Effect of cycloheximide on binding of LETS protein to transformed cells. NIL8-HSV cultures were iodinated 24 h after treatments as below and equal amounts of protein analyzed on an SDS-polyacrylamide slab. a, b) 2 and 20 μg/ml cycloheximide added to the medium. c,d) 2 and 20 μg/ml cycloheximide added to fresh medium. e) Metabolically labeled cell lysate run as molecular weight marker. Major bands at 200, 125, 65, 58, and 42 thousand daltons. f, g) Controls: medium changed or not, respectively. Arrow marks position of LETS protein.

protein could be detected on their surfaces by iodination (Fig. 1a, b). However, if the cells were changed to fresh medium at the time of addition of the cycloheximide, then no LETS protein appeared on the cells (Fig. 1c, d): their iodination patterns remained like those of controls (Fig. 1f, g).

This result suggested that the transformed cells were secreting LETS protein into the medium. A metabolic labeling study confirmed this conclusion. Figure 2a shows that NIL8-HSV medium after 24 h of labeling contains a labeled band comigrating with the LETS protein in conditioned medium of normal NIL8 cells (Fig. 2b), although much less was present in the transformed cell culture. Other differences were observed between the labeling patterns of the 2 cell types. NIL8 cells contained prominent bands at 185,000, 130,000, ~100,000, and around the position of the serum albumin. In contrast, the most prominent labeling in the transformed cell culture was of a doublet at about 60,000 daltons.

To test whether the reduced amount of LETS protein and other larger proteins in the NIL8-HSV medium might be due to degradation, prelabeled NIL8 conditioned medium was mixed with fresh unlabeled conditioned medium from normal or transformed cells and incubated. No degradation was observed (Fig. 2c, d, e). A similar result was obtained when prelabeled NIL8 conditioned medium was incubated with NIL8-HSV cells (data not shown) suggesting that degradation was not the explanation for the differing patterns.

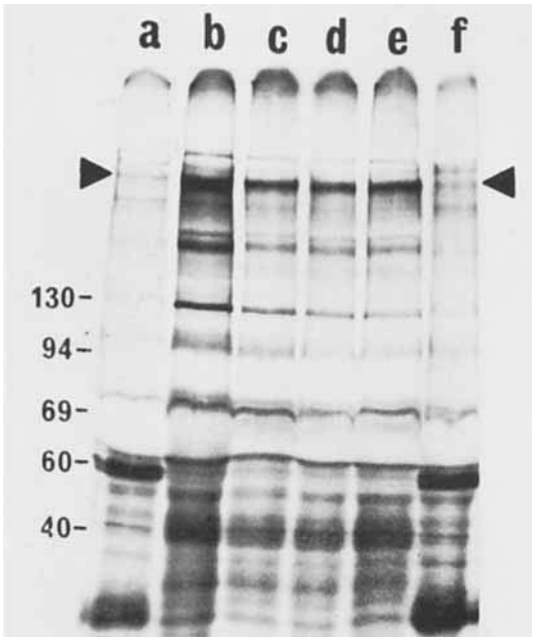

Fig. 2. Metabolically labeled conditioned medium. Analyzed on an SDS-polyacrylamide slab gel. a,b,f) Conditioned medium (65 μl) of NIL8-HSV (a, f) and NIL8 (b) cells after 24 h labeling with [^{35}S] methionine. c–e) Equal aliquots (50 μl) of ^{35}S-labeled NIL8 conditioned medium incubated for 18 h alone (c) or with equal volumes of unlabeled conditioned medium from NIL8 (d) or NIL8-HSV (e) before loading onto gel. Markers were β-galactosidase (130), phosphorylase A (94), bovine serum albumin (69), catalase (60), and creatine kinase (40) as shown on left. Arrow marks position of LETS protein.

Process of Synthesis of LETS Protein

Since it appeared that transformed cells released much less LETS protein into the medium we investigated the early stages of synthesis inside the cells. Figure 3 shows a pulse-chase experiment in NIL8 cells. After a 20 min pulse, LETS protein could be clearly seen in the profile (track 1) and the majority of it was resistant to trypsin (track 4). After a 60 or 120 min chase, LETS protein was still present (tracks 2, 3) but now much of it was sensitive to trypsin treatment of intact cells (tracks 5, 6). Other changes in profile were also observed: a band at 185,000 daltons increased in size during the chase and bands at 145,000 and 65,000 disappeared. One of these proteins is presumably related to the rapidly labeled protein reported by Kuusela et al (16) in chicken cells. None of these proteins was present at the surface after the pulse as judged by their insensitivity to trypsin (track 4). In other experiments of the same sort, it was found that LETS protein was largely insensitive to trypsin treatment up to about 50–60 min of labeling or chase, suggesting that during this period it was being processed to the cell surface. This long processing time is presumably related to the time taken to add the carbohydrate residues and to transport the protein from the rough endoplasmic reticulum (ER) to the surface. In this context, it is interesting to note that in chicken cells there is a minor band comigrating with LETS protein which labels with glucosamine but not with fucose and is trypsin-insensitive, whereas the surface LETS protein which is sensitive to trypsin labels with both sugars (5, 17).

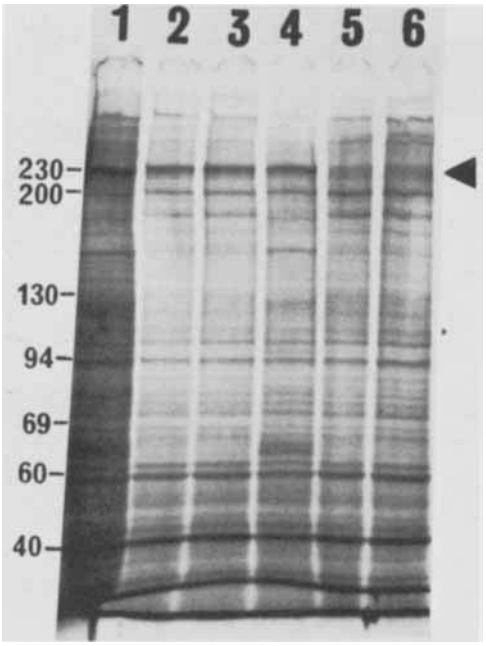

Fig. 3. Pulse-chase labeling of NIL8 cells. Analysis of total SDS lysates on SDS-polyacylamide slab gel. 1) 20 min pulse label. 2) 20 min pulse, 60 min chase. 3) 20 min pulse, 120 min chase. 4–6) As 1–3 respectively but treated at the end of the incubation with trypsin (10 μg/ml, 10 min, room temperature stopped with excess soyabean trypsin inhibitor). Markers on left as Fig. 2 plus myosin and LETS proteins at 200,000 and 230,000 daltons respectively.

To characterize further the course of synthesis and processing of LETS protein, cells were fractionated by detergent extraction. The majority of the iodinatable surface LETS protein is insoluble in deoxycholate (18). However, after a short pulse or a pulse followed by a chase, material comigrating with LETS protein and precipitable by specific antisera was detectable in the deoxycholate soluble material. Analysis of a pulse-chase experiment in this way is shown in Fig. 4. Both normal and transformed cells show synthesis

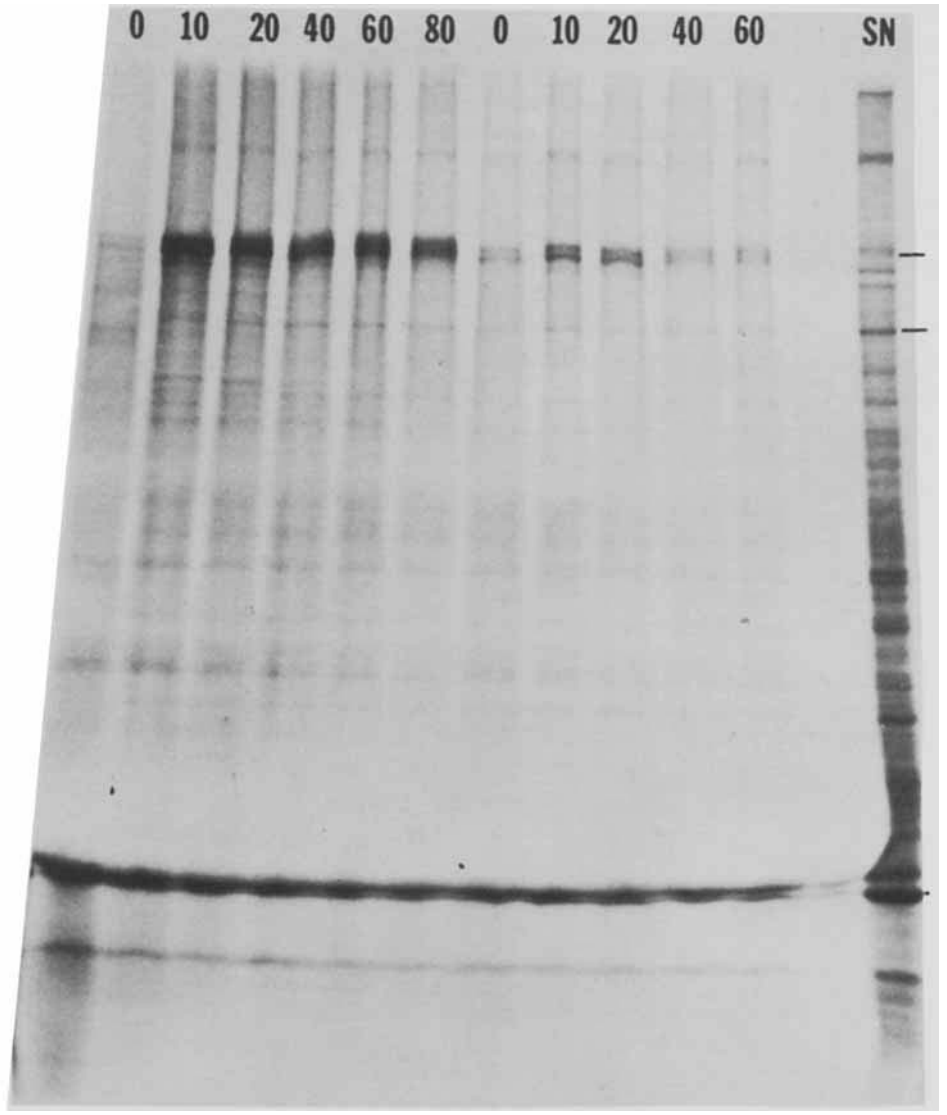

Fig. 4. Analysis of anti-LETS protein immune precipitates of deoxycholate-soluble cell extracts on a 5–10% gradient gel. SN) Total extract. All other samples are immune precipitates (see Materials and Methods). Comparison of the immune precipitates with the total extract shows the selectivity of the immune precipitation. Actin and other minor bands observed in the immune precipitates were also seen in precipitation with preimmune serum (not shown). Cells were pulse labeled for 10 min with [^{35}S] methionine and chased for periods of time shown (min) before harvesting. NIL8 cells on left. NIL8-HSV cells on right. Marks at right indicate positions of LETS protein, myosin, and actin.

of a doublet band comigrating with surface-labeled LETS protein and precipitable by antibody. In both cell types, more of this material was evident after 10 min of chase than immediately after the 10 min pulse. Thereafter, the amount of label precipitable in this band decreased with increasing times of chase, in both cell types. Gel analysis of the deoxycholate-insoluble material (without immune precipitation) showed a labeled doublet band comigrating with surface-labeled LETS protein which increased in amount with time of chase (Fig. 5). These results are consistent with synthesis of LETS protein in some precursor form not identified as yet, followed by a time-dependent processing through a deoxycholate-soluble form to a deoxycholate-insoluble form. This process appears to occur in both normal and transformed cells. Quantitation of autoradiograms such as those in Figs. 4 and 5, followed by correction for total incorporation of radioactivity in each cell sample gave estimates of the rate of LETS protein synthesis relative

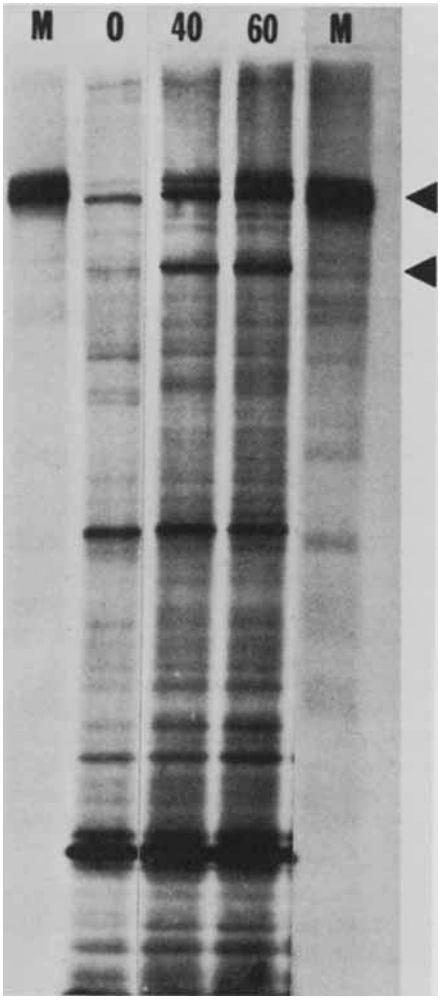

Fig. 5. Deoxycholate insoluble material from pulse-chase labeled NIL8 cells analyzed on 5–10% gradient gel. M) Iodinated NIL cell marker showing LETS protein doublet. Other samples are cells labeled for 10 min with [^{35}S]methionine and chased for periods of time shown (min) before harvesting. Arrows mark position of LETS protein and myosin.

to total protein synthesis. Different experiments gave relative synthesis rates of the putative deoxycholate soluble precursor form of LETS protein by transformed cells which were approximately 10% of those in normal cells. The rate of accumulation of label in the comigrating deoxycholate insoluble band was similarly reduced in transformed cells. This reduction must be contrasted with the very low levels of LETS protein found at the surface and in the medium of transformed cells, roughly estimated at $\leqslant 1\%$ and $\sim 5\%$, respectively.

The proviso must be made that it is not proven that the doublet band quantitated in these experiments is in fact LETS protein or a precursor to it. In support of this hypothesis is the comigration, in particular the fact that a comigrating doublet is observed on the gradient gels used here both for the iodinated surface protein (Fig. 5M) and for the various metabolically labeled samples. Furthermore, a specific antiserum prepared against LETS protein isolated from conditioned medium specifically precipitates these 2 labeled intracellular bands from the total deoxycholate supernatent (Fig. 4). Final proof of relatedness will depend upon peptide fingerprinting studies in progress.

Assuming for the present that the labeled bands do represent LETS protein in the course of intracellular synthesis and processing, these results suggest that reduced synthesis in the transformed cells is not by itself a sufficient explanation for the low levels of LETS protein in external positions (surface and medium) and suggest that some other alteration must exist, perhaps in the processing or transport to the cell surface or in retention at the surface.

Properties of LETS Protein in the Medium

Since in the cycloheximide experiment described earlier, it was found that LETS protein in the medium could bind to cells it is of interest to consider the effect of increasing the amount of LETS protein in the medium of NIL8-HSV cells. The simplest way to do this is to transfer these cells into medium conditioned by the growth of NIL8 cells which as shown earlier (Fig. 2) secrete intact LETS protein into the medium. Figure 6 shows the result of this experiment. NIL8-HSV cells normally grow as rather rounded, refractile cells and also grow detached in the culture medium (Fig. 6a). The floating cells are viable and will regenerate a similar mixed culture if passaged on their own. When NIL8 conditioned medium is added to NIL8-HSV cells, the number of floating cells is greatly reduced and the attached cells become more flattened and show a tendency to line up with each other more like normal cells (Fig. 6b, c). In contrast, cultures changed to fresh medium or medium conditioned by NIL8-HSV cells remain as controls (Fig. 6a, d). This result suggested that the LETS protein secreted by NIL8 cells could affect the properties of transformed cells. However, other components of the conditioned medium could equally well be responsible.

Accordingly, LETS protein was purified from NIL8 conditioned medium as described elsewhere (11) and added to cultures of NIL8-HSV cells. The result is shown in Fig. 7b, c. All the floaters attached to the dishes and the attached cells spread, elongated, and aligned with one another. A similar result was obtained with LETS protein extracted from cells with urea (Fig. 7d) as reported elsewhere by Yamada et al (12) and confirmed by us.[1] Thus, both LETS protein from the surface of NIL8 cells and in their conditioned medium is able to affect the adhesion and morphology of transformed cells.

[1] In these experiments much greater amounts of LETS protein were detected on the surface by iodination (11) than were observed in the cycloheximide experiments discussed earlier.

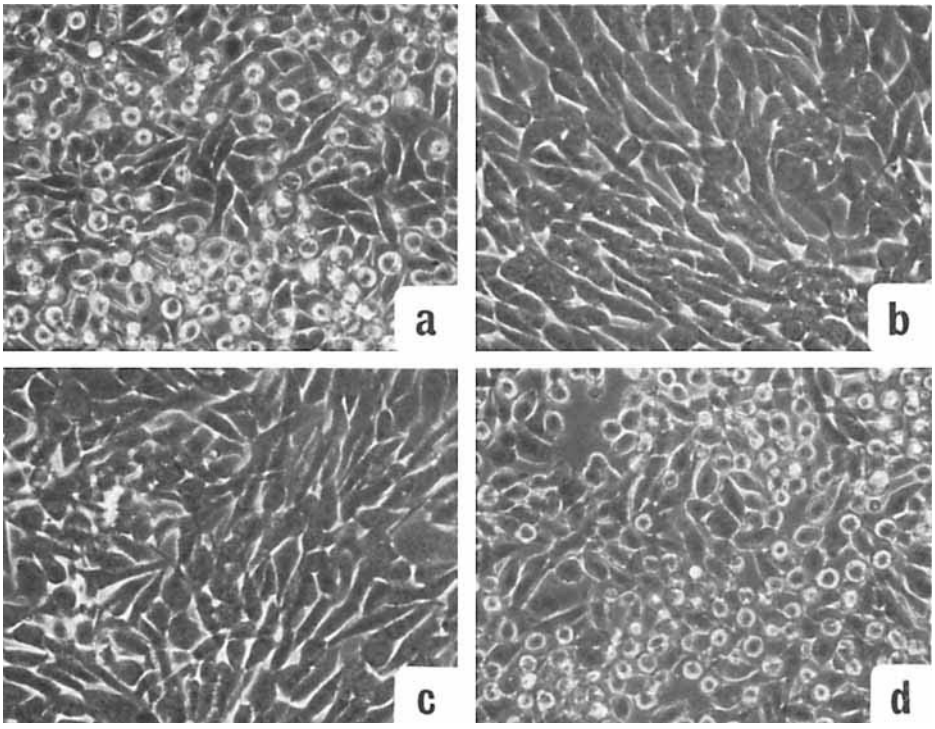

Fig. 6. Effects of conditioned medium on transformed cells. NIL8-HSV cells were cultured for 3 days and then changed for 2 more days into a) fresh medium or to medium conditioned for 5 days by b, c) NIL8 cells or d) NIL8-HSV cells. Note the attachment of floaters and flattening and alignment of cells in (b) and (c). Unchanged controls looked like (a) and (d). Phase contrast, approximately 200 ×.

Immunofluorescence studies show that added LETS protein binds to NIL8-HSV cells in a fibrillar network similar to that observed on normal cells (Fig. 8b and Refs. 11, 14, 19). Furthermore in addition to its effects on cell shape described above, addition of LETS protein to transformed cells leads to the appearance of actin cables within the cells (Fig. 8d) whereas these are absent from control NIL8-HSV cells (Fig. 8c).

All these effects of LETS protein on transformed cells are blocked or reversed by treatment with low levels of trypsin, such as cleave LETS protein, and are unaffected by chondroitinase ABC or hyaluronidase (11). Furthermore, preincubation of the LETS protein preparation with specific antisera to LETS protein blocks the effect (11).

These results suggest that several of the properties of transformed cells (reduced adhesion of cells, morphology and absence of actin cables) are a result of their low levels of LETS protein and that these properties can be altered towards normal by addition of LETS protein from normal cells or their conditioned medium.

DISCUSSION

The data presented indicate that both normal and transformed cells release LETS protein into the culture medium. Vaheri and Ruoslahti (20) have reported the detection of material immunologically cross-reactive with LETS protein in the medium of normal

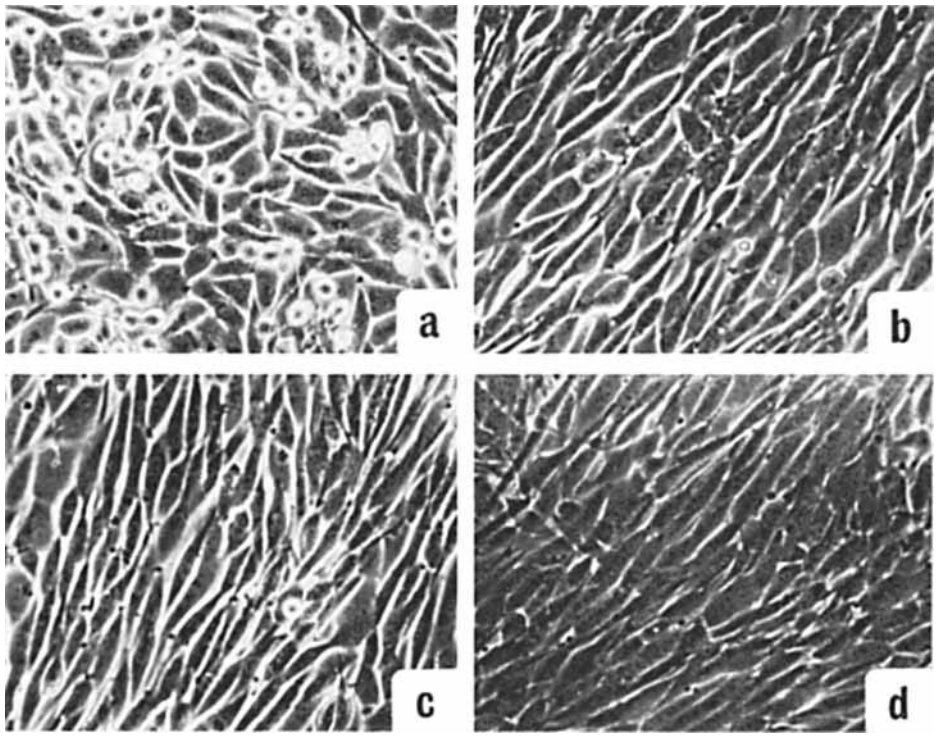

Fig. 7. Effects of purified LETS protein on transformed cells. Two-day-old cultures of NIL8-HSV cells received additions as below and were photographed 24 h later. a) Buffer alone. b) 30 μg/ml of LETS protein purified from conditioned medium of NIL8 cells as described (11). c) 40 μg/ml of LETS protein purified from conditioned medium and further fractionated on Sephadex. G-200. d) 100 μg/ml of LETS protein extracted with urea from chick embryo fibroblasts (12). Panel (d) was from a separate experiment from (a)–(c). Phase contrast, approximate magnification, 200 ×.

and transformed cells. The present results show that both cell types secrete apparently intact LETS protein which is capable of binding to cells. The transformed cells studied have markedly reduced quantities of LETS protein in their conditioned medium as well as on their surfaces, when compared with normal cells. There is no evidence that this is due to degradation of material secreted into the medium, since added NIL8 conditioned medium is not degraded and purified LETS protein added to the cells binds to them and alters many of their properties towards those of normal cells.

Studies of the biosynthesis of LETS protein show a reduced rate of synthesis in the transformed cells. The reduction in synthesis rate observed, while considerable, was not as great as that in the levels of LETS protein on the surface or in the medium of transformed cells. This discrepancy suggests that while reduced synthesis of LETS protein contributes to the reduction in surface levels of LETS protein in the transformed cells, it is not the complete explanation. Increased turnover (5, 15) into the medium also appears to contribute, although the reason for this is as yet unclear. Possibilities include synthesis of an altered form of LETS protein by the transformed cells or alteration in other components at the transformed cell surface which affect retention of LETS.

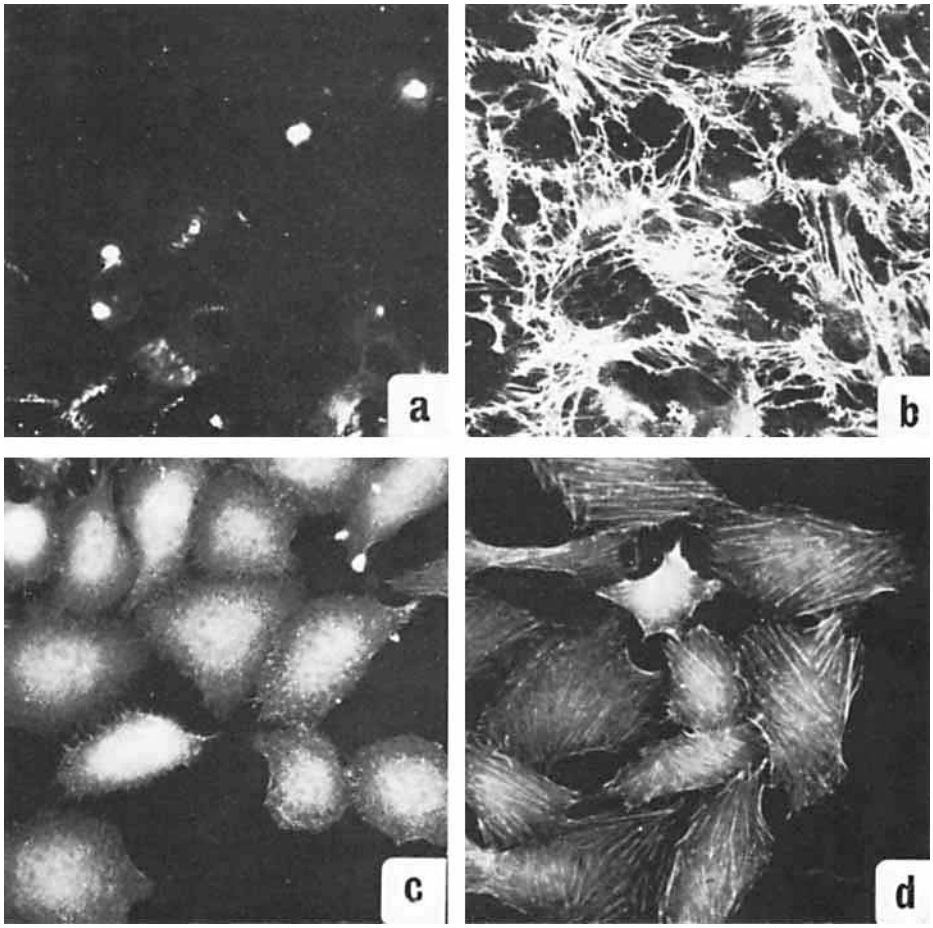

Fig. 8. Immunofluorescent staining of NIL8-HSV cells treated or not with 100 μg/ml LETS protein from chicken cells and fixed 24 h later. a) No LETS protein added, stained for LETS protein. b) LETS protein added, stained for LETS protein. c) No LETS protein added, stained for actin. d) LETS protein added, stained for actin. Bar represents 25 μm.

The results presented also raise the question of the normal route of processing of LETS protein. Is it internal → surface → medium or internal → medium → surface? Evidence is available for exchange in both directions between cell surface and medium. It was reported previously that prelabeled LETS protein falls off into the medium (4, 5, 15), and it is reported here and elsewhere (6, 11, 12) that LETS protein added to the culture medium binds to cells. It is possible that an equilibrium exists between the 2 compartments.

Another possibility raised by the experiments reported here concerns the effect of protein synthesis inhibitors on the transformed phenotype. Ash et al (21) have reported that cycloheximide or puromycin treatment of transformed NRK cells leads to reversion of their morphology and of their arrangement of myosin filaments towards normal. We have shown that these same inhibitors lead to the reattachment of LETS protein onto the

surfaces of NIL8-HSV cells. Furthermore, the addition of exogenous LETS protein leads to reversion of morphology and of arrangement of actin towards normal. The parallels suggest that the 2 sets of observations may be related.

It is clear that the presence of LETS protein in the culture medium has profound effects on cellular properties and it seems likely that the absence of this protein from transformed cells and their medium is involved in their altered behavior in vitro.

ACKNOWLEDGMENTS

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NOTE ADDED IN PROOF

After submission of this manuscript, it was reported by Olden and Yamada (1977, Cell II, 957–969) that transformed chicken cells show reduced synthesis of LETS protein but that surface levels of this protein were reduced even further.

REFERENCES

1. Hynes RO: BBA Reviews on Cancer 458:73, 1976.
2. Hynes RO: Proc Natl Acad Sci USA 70:3170, 1973.
3. Hynes RO, Bye JM: Cell 3:113, 1974.
4. Hynes RO, Wyke JA, Bye JM, Humphryes KC, Pearlstein ES: In Reich E, Shaw E, Rifkin DB (eds): "Proteases and Biological Control." New York: Cold Spring Harbor Laboratory, 1975, pp 931–944.
5. Robbins PW, Wickus GG, Branton PE, Gaffney BJ, Hirschberg CB, Fuchs P, Blumberg PM: Cold Spring Harbor Symp Quant Biol 39:1173, 1974.
6. Yamada KM, Weston JA: Cell 5:75, 1975.
7. Goldberg B, Sherr CJ: Proc Natl Acad Sci USA 70:361, 1973.
8. Zavada J, Macpherson IA: Nature (London) 225:24, 1970.
9. Laemmli UK: Nature (London) 227:680, 1970.
10. Bonner WM, Laskey RA: Eur J Biochem 46:83, 1974.
11. Ali IU, Mautner VM, Lanza RP, Hynes RO: Cell 11:115, 1977.
12. Yamada KM, Yamada SS, Pastan I: Proc Natl Acad Sci USA 73:1217, 1976.
13. Lazarides E, Weber K: Proc Natl Acad Sci USA 71:2268, 1974.
14. Mautner VM, Hynes RO: J Cell Biol (in press).
15. Hynes RO, Wyke JA: Virology 64:492, 1975.
16. Kuusela P, Ruoslahti E, Vaheri A: Biochim Biophys Acta 379:295, 1975.
17. Critchley DR, Wyke JA, Hynes RO: Biochim Biophys Acta 436:335, 1976.
18. Hynes RO, Destree AT, Mautner VM: In Marchesi VT (ed): "Membranes and Neoplasia: New Approaches and Strategies." New York: Alan R Liss, Inc, 1976, pp 189–201.
19. Wartiovaara J, Linder E, Ruoslahti E, Vaheri A: J Exp Med 140:1522, 1975.
20. Vaheri A, Ruoslahti E: J Exp Med 142:530, 1975.
21. Ash JF, Vogt PK, Singer SJ: Proc Natl Acad Sci USA 73:3603, 1976.