Biochimica et Biophysica Acta, 471 (1977) 16—24

BBA 77850

EFFECTS OF CYTOCHALASIN B AND COLCHICINE ON ATTACHMENT OF A MAJOR SURFACE PROTEIN OF FIBROBLASTS

IQBAL UNNISA ALI and RICHARD O. HYNES

Center for Cancer Research and Department of Biology, Massachusetts Institute of Technology, Cambridge, Mass. 02139 (U.S.A.)

(Received April 13th,1977)

Summary

We have investigated the effects of the drugs cytochalasin B and colchicine on the surface levels of the large, external, transformation-sensitive (LETS) glycoprotein. Colchicine neither removed LETS protein from the surface, nor inhibited its regeneration after removal by mild trypsinization. Cells treated with cytochalasin B, however, showed both a 2—3-fold increase in the turnover rate of their surface LETS protein and a marked inhibition in its regeneration. Inhibition of regeneration was not due to inhibition of synthesis or transport to the surface. In fact, in the presence of cytochalasin B, increased quantities of LETS protein were released into the medium. The results are consistent with the idea of an association of LETS protein with the actin-containing microfilaments. However, other possible explanations, such as effects on cellular morphology or on transport of sugar precursors cannot yet be excluded.

Introduction

There is much speculation that membrane receptors may have a direct or indirect association with the cytoskeletal elements of the cell. This possibility has been suggested since surface phenomena, such as patching and capping of lectin and antibody receptors and agglutination, are affected by drugs which cause alterations in the organization of microfilaments and microtubules [1—4].

In this investigation we have examined the possibility of interconnections between a major surface glycoprotein (LETS protein) of normal fibroblasts and components of the cellular cytoskeletal system. Several observations suggest such an association.

(1) Upon transformation, LETS glycoprotein is lost from the cell surface [5] and the organization of the cytoskeletal system inside the cells is disrupted [6,7]. Both alterations are observed in cells infected with viral mutants temperature sensitive for transformation [8—11].

(2) Exponentially growing normal cells have lesser amounts of LETS protein [12] and of submembranous microfilaments than do growth-arrested cells [13].

(3) LETS protein is extremely sensitive to proteases and is removed from the cell surface by mild proteolysis [14]. Susceptibility of microfilament bundles to proteolysis has also been reported, although higher concentrations of protease and greater lengths of time may be required than are necessary to remove LETS protein from the surface [15,16].

(4) Extraction of cells with the nonionic detergent NP40 leaves the nucleus surrounded by a skeleton which consists predominantly of LETS protein, actin, myosin, and a protein of molecular weight 58 000 [16].

(5) Finally, upon addition of LETS proten isolated from normal cells to transformed cells, they regain actin cables [17].

With these correlations in mind, we have used the drugs cytochalasin B and colchicine which are known to disassemble cytoskeletal components and studied their effects on the amount of LETS protein present on the cell surface.

Materials and Methods

Materials

[^{35}S]Methionine (specific activity 500 Ci/mmol) and carrier-free sodium [^{125}I]iodide were purchasesd from New England Nuclear and Amersham/Searle, respectively. Cytochalasin B was a product of Aldrich Chemical Co. and colchicine was obtained from Sigma. Stock solutions of colchicine (1 mg/ml) and cytochalasin B (1 mg/ml in ethanol) were stored at 4°C.

Methods

Cells and growth conditions. Cells of the hamster fibroblast line, NIL8, were cultured in Dulbecco's modified Eagle's medium + 5% fetal calf serum at 37°C in an atmosphere of 95% air/5% CO_2. The cells used in all the experiments reported here were seeded at $5 \cdot 10^4$/3.5 cm dish in 2 ml of medium and were grown for 5 days.

Secondary chicken embryo fibroblasts were seeded at 10^6 cells/3.5 cm dish and were grown in 2 ml of Dulbecco's modified Eagle's medium + 5% calf serum for 2 days at 39°C in a 5% CO_2 incubator.

Turnover rate. Confluent cultures of NIL fibroblasts were either treated with different concentrations (see figure legends) of cytochalasin B or colchicine and then iodinated using the enzyme lactoperoxidase as described previously [14], or iodinated monolayers were incubated with 2 ml of fresh growth medium containing cytochalasin B or colchicine for different time periods. Medium was then collected and cells were lysed with sodium dodecyl sulfate after washing with phosphate-buffered saline. Cell lysates and media were analyzed on sodium dodecyl sulfate polyacrylamide gels.

Regeneration of LETS protein. LETS protein was removed from the surface by subjecting the cells to mild trypsinization using 1 μg/ml trypsin for 10 min. The reaction was stopped by adding 10 μg/ml soybean trypsin inhibitor. Cells were then incubated in 2 ml of fresh growth medium without or with different

concentrations of colchicine or cytochalasin B for 24 h. The reappearance of LETS protein was monitored by lactoperoxidase catalysed iodination followed by sodium dodecyl sulfate gel electrophoresis.

For metabolic labelling, immediately after trypsinization 2 ml of growth medium containing 10 μCi [^{35}S]methionine/ml was added. The concentration of methionine in the medium was reduced to 10% of normal. Half of the cultures received 5 μg/ml cytochalasin B in the labelling medium. After 4, 8, 16 and 24 h cell lysates and media from control and cytochalasin B-treated cultures were collected and analyzed on sodium dodecyl sulfate polyacrylamide gels.

The rate of protein synthesis was measured by counting the acid insoluble radioactivity. Protein concentration was determined by the method of Lowry et al. [18].

Results

Turnover of LETS protein in the presence of cytochalasin B or colchicine

Cultures of confluent, growth-arrested NIL8 cells were treated with different concentrations of either colchicine or cytochalasin B, drugs known to disrupt microtubules and microfilaments, respectively, for 24 h and then iodinated using the enzyme lactoperoxidase. As is evident from Fig. 1, cells treated with 2.5 and 5 μg/ml cytochalasin B had greatly reduced amounts of LETS protein and of other surface proteins (Fig. 1, b and c). Incubation of cells with 2.5, 5

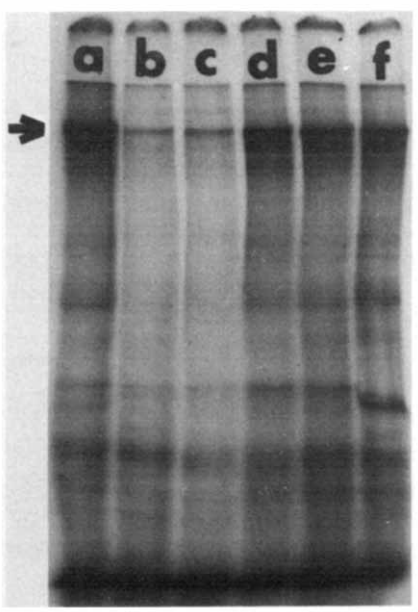

Fig. 1. Effect of cytochalasin B and colchicine on surface levels of LETS protein. Confluent monolayers of NIL cells were incubated with cytochalasin B or colchicine for 24 h and then iodinated. a, Control; b, 2.5 μg/ml cytochalasin B; c, 5 μg/ml cytochalasin B; d, 2.5 μg/ml colchicine; e, 5 μg/ml colchicine; f, 10 μg/ml colchicine. All samples were normalized for protein. Arrow marks position of LETS protein.

TABLE I

TURNOVER OF LETS PROTEIN IN THE PRESENCE OF CYTOCHALASIN B AND COLCHICINE

NIL8 cells were iodinated and returned to culture with additions as shown. After 4 h, medium and cells were harvested and analyzed on gels. LETS protein was quantitated by cutting the LETS band from the gels and counting it; background radioactivity was subtracted in each case. Total counts (cells + medium) in all cases were comparable to control (±20%).

Treatment of 4 h	Percent LETS protein	
	Cells	Medium
Control	83	17
Colchicine 10 μg/ml	78	22
Cytochalasin B 10 μg/ml	53	47
Cytochalasin B 10 μg/ml + colchicine 10 μg/ml	61	39
Cytochalasin B 20 μg/ml	40	60
Cytochalasin B 20 μg/ml + colchicine 10 μg/ml	35	65

and 10 μg/ml colchicine was, however, without any significant effect on LETS protein (Fig. 1, d–f).

In order to determine the effects of the drugs on the turnover of LETS protein, confluent monolayers of NIL cells were iodinated and kept in fresh medium containing either colchicine or cytochalasin B. After 4 h, the cell lysates and media were analyzed on SDS gels. Colchicine at 10 μg/ml, a concentration high enough to disrupt the microtubules [16], did not enhance the turnover of LETS protein. However, cells incubated with 10 or 20 μg/ml cytochalasin B had reduced amounts of LETS protein on their surfaces as compared to control untreated cells. There was a simultaneous increase in the amount of LETS protein in the medium (Table I). Incubation of cells with cytochalasin B and colchicine had no additional effect on the removal of LETS protein from the surface.

When the kinetics of removal of LETS protein from the cell surface in the presence of cytochalasin B was followed as a function of time, an initial 3-fold

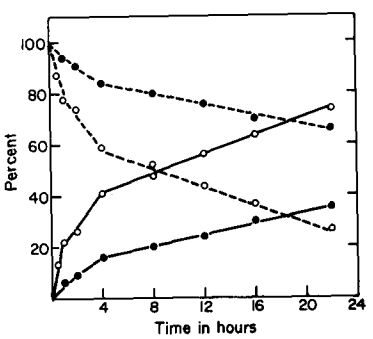

Fig. 2. Kinetics of removal of LETS protein from the cell surface in the presence of cytochalasin B. Iodinated cells were incubated in the absence or presence of 10 μg/ml cytochalasin B. The amount of LETS protein on the cells and in the medium was determined by analyzing the samples on gels at the indicated times. Quantitation was as described in Table I. ●------●, LETS protein associated with control cells; ○- - - -○, LETS protein associated with cells treated with 10 μg/ml cytochalasin B; ●———●, LETS protein released into the medium of control cells; ○———○, LETS protein released into the medium of cells treated with 10 μg/ml cytochalasin B.

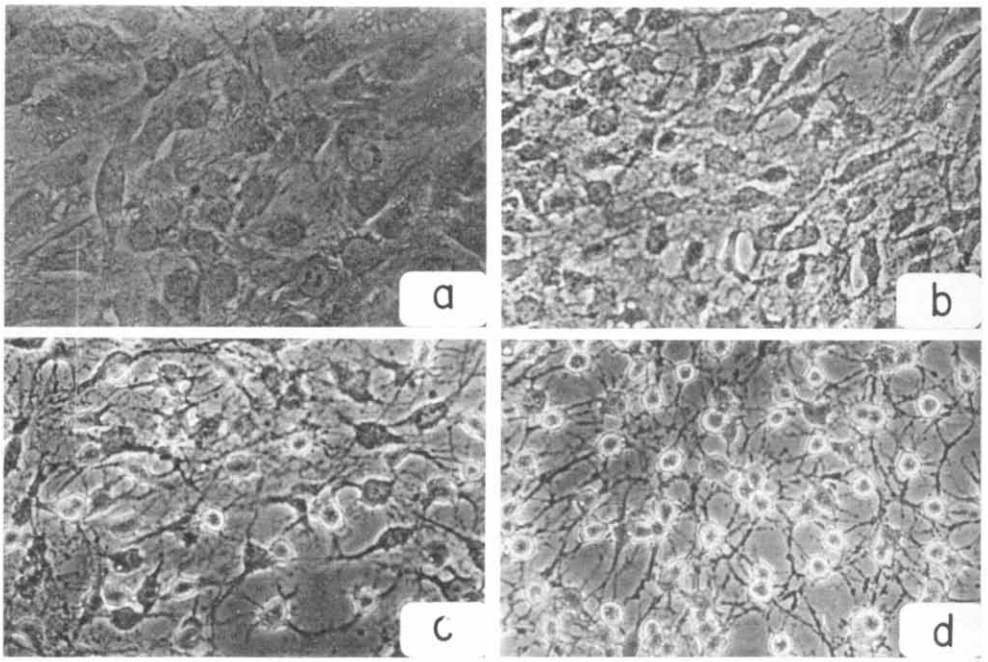

Fig. 3. Morphology of NIL cells treated with 10 μg/ml cytochalasin B for different time periods. a, Untreated control; b, 30 min; c, 12 h; d, 22 h.

increase was observed from 30 min to 4 h. Cytochalasin B-treated cells continued to release increased amounts of LETS protein into the medium for 22 h (Fig. 2).

Fig. 3 shows morphology of the cells treated with 10 μg/ml cytochalasin B. After 30 min, the cells were retracted and arborized (Fig. 3b). Such cells retained about 85% of the LETS protein. 12 h after addition of cytochalasin B, arborization had progressed still further (Fig. 3c). At this point, 50% of the iodinated LETS protein could still be detected on the cells. After 22 h, each cell was converted to a round central body from which extensive branched processes projected. Such cells were still firmly attached to the dish and very few detached cells were observed in the medium. The amount of prelabelled LETS protein on these cells dropped to about 25% as compared to 65% in control cells.

The possibility that the enhanced removal of LETS protein from the surface of cytochalasin B-treated cells might be due to the activation or release of proteases seems unlikely for two reasons. First, protease treatment generally leads to release of fragments of LETS protein in the medium (ref. 14 and Mahdavi, V. and Hynes, R.O. (1977), unpublished); no degradation products of LETS protein were found in the medium of drug-treated cells. Second, the decrease in the amount of surface LETS protein was accompanied by a simultaneous increase in the medium (Table I and Fig. 2).

Regeneration of LETS protein in the presence of cytochalasin B or colchicine

When LETS protein was removed from the cell surface by protease treat-

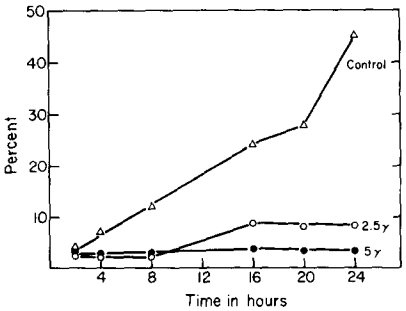

Fig. 4. Time course of regeneration of LETS protein in the presence of cytochalasin B. Cultures of NIL cells were treated with 1 μg/ml trypsin for 10 min at room temperature and incubated in fresh growth medium without or with 2.5 and 5 μg/ml cytochalasin B. The regeneration of LETS protein was monitored by iodination followed by analysis on gels. Quantitation was as in Table I. Percentage recovery in controls at 24 h varied from 45—80% in different experiments.

ment, a substantial amount was restored within 24 h (Fig. 4). This regeneration of LETS protein was unaffected by the presence of colchicine (data not shown), but was inhibited to a large extent by cytochalasin B (Fig. 4).

Transport of LETS protein to the surface in the presence of cytochalasin B

One possible explanation for reduced regeneration of LETS protein is that

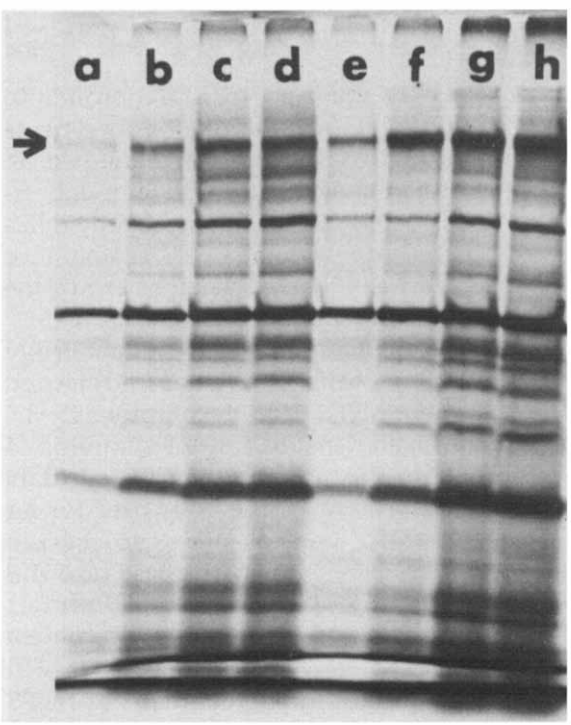

Fig. 5. Increased release of LETS protein in the medium of cells treated with cytochalasin B. Cultures of NIL cells were treated with 1 μg/ml trypsin for 10 min at room temperature and then incubated in medium containing 10 μg/ml [^{35}S]methionine without or with 5 μg/ml cytochalasin B. Equal aliquots of media were analyzed on gels at times indicated. Media from: a—d, control cells after 4, 8, 16 and 24 h; e—h, cytochalasin B-treated cells after 4, 8, 16 and 24 h. Arrow shows position of LETS protein.

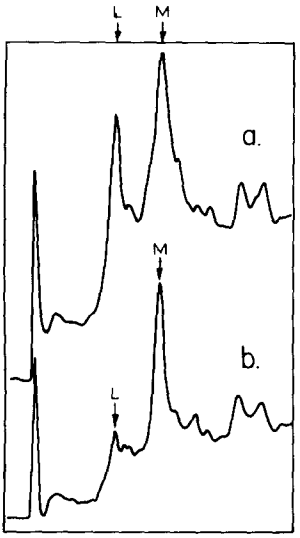

Fig. 6. Decreased level of LETS protein in cells treated with cytochalasin B. Scans of autoradiograms from gels of chick embryo fibroblasts. a, Control; b, 5 μg/ml cytochalasin B-treated cells analyzed 16 h after trypsinization and labelling with [^{35}S]methionine as described in Fig. 5. Profiles are lined up on interface between stacking and running gels. Arrows mark positions of LETS protein (L) and myosin (M).

cytochalasin B could block the synthesis and/or transport of LETS protein to the outer surface thus resulting in inhibition of its restoration. To investigate this possibility, the synthesis and secretion of LETS protein were studied by metabolic labelling with [^{35}S]methionine. Fig. 5 shows that the rate of appearance of LETS protein in the medium of NIL8 cells was not inhibited by cytochalasin B; in fact it was increased. This increased release of LETS protein into the medium in the presence of cytochalasin B was observed whether or not the cells were treated with trypsin prior to labelling (data not shown).

It was difficult to quantitate cell-bound LETS protein in metabolically labelled NIL8 cells since it is a relatively minor band. We therefore repeated the experiments in chicken embryo cells where LETS protein comprises 2—3% of the total cellular protein. Although the instantaneous rate of synthesis of LETS protein, as measured in a 15 min pulse, was not greatly affected (data not shown), accumulation of labelled LETS protein in the cells over longer periods was inhibited (Fig. 6). However, both control and cytochalasin B-treated cells had similar amounts of myosin label. As in the case of NIL8 cells, increased release (about 3—4 fold) of newly synthesized LETS protein into the medium was observed, whereas overall protein synthesis was inhibited by only 10—15%.

These results indicate that cytochalasin B does not inhibit synthesis of LETS protein or its transport to the cell surface. Rather, LETS protein apparently fails to bind normally at the cell surface; however, it is released apparently intact into the medium in greater amounts than usual. These experiments also confirm that cytochalasin B does not lead to increased degradation of LETS protein by causing, for instance, release of lysosomal enzymes.

Discussion

The present results demonstrate that levels of colchicine which arrest cells in mitosis [12] and disassemble their microtubules [16] have no effect on the turnover rate or regeneration of LETS protein. It is reported elsewhere that colchicine has no effect on the pattern of LETS fibrils detected by immunofluorescence [16]. Taken together these results argue against a connection between LETS protein and microtubules.

In contrast with colchicine, cytochalasin B causes increased turnover of LETS protein from the cell surface and inhibits regeneration of surface LETS protein after trypsin treatment. These effects are not due to inhibition either of synthesis of LETS protein or of its transport to the exterior of the cell. In fact, increased amounts of LETS protein accumulate in the medium of cytochalasin B treated cells. It seems clear that the effect of cytochalasin B is on the attachment of LETS protein at the cell surface.

Several different interpretations can be offered for the effects of cytochalasin B on the surface levels of LETS protein. This drug is known to disassemble actin microfilaments, although it is unclear whether this is due to a direct interaction with the components of the microfilaments [19,20] or an indirect result of its effect on the cell membrane [21,22], altering attachment and/or assembly of microfilaments. Cytochalasin B causes disassembly of the actin microfilament "cables" both in this system [16] and in others [23,24]. A simple interpretation of the results described here is that disassembly of microfilaments by cytochalasin B leads to detachment of LETS protein from the cell surface. However, since cytochalasin B has a variety of other effects upon cells [25], this interpretation remains tentative. For instance, the release of LETS protein may be a consequence of the rounding-up of cells in cytochalasin B rather than a direct effect of the disruption of microfilaments. Furthermore, cytochalasin B inhibits sugar transport [25,26] and reduces glucosamine incorporation by the cells used in the present study (unpublished data). Although there is no evidence for altered glycosylation of LETS protein caused by cytochalasin B in the present experiments, such a possibility cannot be ruled out as a contributory factor to some of the results described above.

Accepting these reservations, the effects of LETS protein on the cytoskeleton [17] and, reciprocally, of drugs which affect cytoskeletal structures on LETS protein, suggest that further investigation of possible connections will be of interest. A definitive solution will require direct analysis of protein-protein interactions at the cell surface.

Acknowledgements

This research was supported by grants from the National Institutes of Health R01-CA-17007 and P01-CA140-051.

References

1 Nicolson, G.L. (1976) Biochim. Biophys. Acta 457, 57—108
2 Ukena, T.E., Borysenko, J.Z., Karnovsky, M.J. and Berlin, R.D. (1974) J. Cell Biol. 61, 70—82
3 Yin, H.H., Ukena, T.E. and Berlin, R.D. (1972) Science 178, 867—868

4 Nicolson, G.L. (1974) Int. Rev. Cytol. 39, 89—190
5 Hynes, R.O. (1976) Biochim. Biophys. Acta 458, 73—107
6 Pollack, R., Osborn, M. and Weber, K. (1975) Proc. Natl. Acad. Sci. U.S. 72, 994—998
7 Brinkley, B., Fuller, G. and Highfield, D. (1975) Proc. Natl. Acad. Sci. U.S. 72, 4981—4985
8 Hynes, R.O. and Wyke, J. (1975) Virology 64, 492—504
9 Edelman, G.M. and Yahara, I. (1976) Proc. Natl. Acad. Sci. U.S. 73, 2047—2051
10 Ash, J.F., Vogt, P.K. and Singer, S.J. (1976) Proc. Natl. Acad. Sci. U.S. 73, 3603—3607
11 Wang, E. and Goldberg, A.R. (1976) Proc. Natl. Acad. Sci. U.S. 73, 4065—4069
12 Hynes, R.O. and Bye, J. (1974) Cell 3, 113—120
13 McNutt, N.S., Culp, L.A. and Black, P.H. (1973) J. Cell Biol. 56, 413—428
14 Hynes, R.O. (1973) Proc. Natl. Acad. Sci. U.S. 70, 3170—3174
15 Pollack, R. and Rifkin, D. (1975) Cell 6, 495—506
16 Mautner, V. and Hynes, R.O. (1977) J. Cell Biol., in press
17 Ali, I.U., Mautner, V., Lanza, R. and Hynes, R.O. (1977) Cell, 11, 115—126
18 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265—275
19 Spudich, J.A. (1972) Cold Spring Harbor Symp. Quant. Biol. 37, 585—593
20 Spudich, J.A. and Lin, S. (1972) Proc. Natl. Acad. Sci. U.S. 69, 442—446
21 Spooner, B.S. (1973) Dev. Biol. 35, f-13—18
22 Everhart, L.P. and Rubin, R.W. (1974) J. Cell Biol. 60, 442—447
23 Pollack, R. and Rifkin, D.B. (1976) in Cell Motility (Goldman, R., Pollard, T. and Rosenbaum, J. eds.), Cold Spring Harbor Conferences on Cell Proliferation, Vol. 3, pp. 389—401
24 Weber, K., Rathke, P.C., Osborn, M. and Franke, W.W. (1976) Exp. Cell Res. 102, 285—297
25 Pollard, T.D. and Weihing, R.R. (1974) Crit. Rev. Biochem. 2, 1—65
26 Kletzien, R.F., Perdue, J.F. and Springer, A. (1972) J. Biol. Chem. 247, 2964—2966