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CELL ADHESION AND ACQUISITION OF DETERGENT RESISTANCE BY THE CYTOSKELETON OF CULTURED CHICK FIBROBLASTS

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Summary

About 30% of the proteins of adherent cultured chick embryo fibroblasts are not solubilized by the non-ionic detergent Triton X-100 and remain firmly attached to the substratum. The insoluble residue contains a considerable part of the cell's cytoskeleton and its major constituents are large external transformation-sensitive (LETS) protein, the heavy chain of myosin, a 52 000 molecular weight protein and actin. Kinetic studies reveal that cytoskeleton insolubility in Triton is acquired either concurrently with cell adhesion or very closely with it. Neither cell adhesion nor binding of the Triton cytoskeleton to the substratum require de novo synthesis of protein. In the attempt to assess the role of LETS protein in cytoskeleton attachment, we find that trypsin-detached cells rapidly acquire Triton-insoluble cytoskeleton although their LETS protein content is about 15—20% of its level in long-term cultures. Removal of the great majority of LETS moelcules of adherent cultures by either urea or trypsin treatment does not affect the relative amount or composition of the anchored cytoskeletal proteins. Also, LETS protein of cultures exposed to cycloheximide for extended periods of time, is reduced to 10% of its maximum amount without much affecting the attachment and composition of the cytoskeleton. It is deduced that the great majority of LETS protein is not required for the attachment of the Triton cytoskeleton to the substratum.

Introduction

A considerable body of evidence indicates that eukaryotic cells possess an elaborate cytoplasmic structure which includes structural and contractile pro-

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Abbreviation: LETS protein, large external transformation-sensitive protein.

teins. This intracellular system, which has been designated the cell cytoskeleton, has been implicated in determination of cellular morphology, cell adhesion to the substratum, intracellular organelle movement and locomotion. Several authors have recently shown that extraction of monolayers of various cultured cells with non-ionic detergents such as Triton X-100 and NP-40 provides a simple and rapid way to isolate a major part of the cytoskeleton which retains its attachment to the substratum [1—4]. The detergent-insoluble cytoskeleton which adheres firmly to the substratum, consists largely of intermediate-size filaments and bundles of microfilaments [1—4]. The major protein components of this structure have been identified by a variety of methods. These elements are actin [1—3], a protein of 52 000—58 000 molecular weight [1—3], which is probably the major constituent of the 10-nm filaments [1,5], the heavy chain of myosin [1,3] and the large external transformation sensitive (LETS) protein (refs. 1, 3 and 4; for review on this protein see ref. 6). In this study we have addressed ourselves to two questions. First, in what way is the firm anchorage of the Triton cytoskeleton related to adhesion of cells to the substratum. Second, to what extent is LETS protein, which is probably a transmembrane element [7], and has been implicated in cell adhesion [8,9], involved in the linking of the cytoplasmic cytoskeleton to the substratum. We find that the cytoskeleton of cultured chick embryo fibroblasts is rendered insoluble in Triton either concomitantly with cell binding to the substratum or very closely with it. Both cell adhesion and acquirement of cytoskeletal resistance to Triton do not require de novo synthesis of protein. Our results indicate that up to 90% of LETS protein can be removed without considerably affecting either cell attachment or cytoskeleton insolubility in Triton.

Materials and Methods

Cell cultures. Normal chick fibroblasts were prepared from 10-day-old chicken embryos as described [10]. The primary cells were inoculated at a density of $3—4 \cdot 10^5$ cells/cm^2 in 100-mm plastic tissue culture dishes (Corning Glassworks, Corning, NY), which contained Dulbecco's modified Eagle's medium (Grand Island Biological Co., Grand Island, NY), including 10% fetal calf serum (Bio-Lab, Israel), 1.96 g/l $NaHCO_3$, $5 \cdot 10^4$ units/l penicillin and $5 \cdot 10^4$ μg/l streptomycin. The cultures were incubated routinely at 37°C in a closed 5% CO_2/95% air environment and fed with fresh growth medium every 2 days. After 40—50 h as primary culture, the cells were detached from the substratum with 0.25% trypsin and a secondary culture was established as above. After 3—5 similar passages, varied cell types contained in the original primary culture were diluted out and the cell population consisted exclusively of fibroblasts. Fibroblast cultures at passages 5—8 were routinely used for the experiments described in this communication.

Cell adhesion assay. The assay used in this study measures the rate of cell attachment to the polystyrene surface of a culture dish. Confluent fibroblasts were detached from the dish with 0.25% trypsin (1 : 250, Serva, Heidelberg) and washed. Viable cells were counted in a haemocytometer using the trypan blue exclusion test. The cells, resuspended in growth medium at approx. $5 \cdot 10^5$ cells/ml, were inoculated in a series of 60-mm tissue culture dishes at a

density of $7 \cdot 10^4$ cells/cm^2. The newly seeded cultures were incubated at 37° C and at various intervals plates were removed, the medium aspirated and non-adherent cells washed away by three gentle rinses with prewarmed Tris/glucose/magnesium/calcium buffer (Buffer I), which consisted of 25 mM Tris-HCl (pH 7.4) containing 137 mM NaCl, 5 mM KCl, 5 mM glucose, 0.5 mM $MgCl_2$ and 0.025 mM $CaCl_2$. The cells which remained bound were solubilized by 1% sodium dodecyl sulfate (SDS) in 50 mM Tris-HCl (pH 6.8), and the amount of cellular protein was determined using the method of Lowry et al. [11]. Number of adherent cells was calculated on the basis of the known cellular content of protein which is 0.28 ng protein/cell [12]. Direct counting of adherent cells after their dispersion with trypsin yielded results similar to those obtained by the above method.

Detergent extraction. A considerable part of the cell cytoskeleton of cultured cells is resistant to extraction with the non-ionic detergent Triton X-100 [1,2]. The detergent-insoluble cytoskeletal proteins of the cultured avian cells were isolated according to the procedure of Brown et al. [1]. Monolayers inoculated for various periods of time and under different growth conditions, were rinsed three times with Buffer I and incubated for 10 min at room temperature with 0.5% Triton X-100 (Koch-Light, England, polyetheylene-glycol-[9-10]-*p-tert*-octylphenol) in Buffer I (0.3 ml/60 mm plate). Maximum amount of non-cytoskeletal cell protein was extracted under these conditions (ref. 1 and our own observations). The supernatant solution was saved and the insoluble residue which remained on the dish was washed once with Buffer I and solubilized with 0.3 ml Buffer I to which 30 μl 10% SDS in 50 mM Tris-HCl (pH 6.8) were added. Amount of protein in the detergent extract and in the insoluble residue was determined according to the method of Lowry et al. [11]. For electrophoretic analysis, the supernatant fraction was made 1% SDS by adding to it 0.1 volume of 10% SDS in 50 mM Tris-HCl (pH 6.8), and both supernatant and the solubilized Triton-resistant residue were reduced with 0.025 volume of mercaptoethanol. Total cell proteins, which were obtained by lysis of monolayers with 1% SDS, were treated as the Triton-insoluble fraction prior to electrophoretic analysis.

SDS-polyacrylamide gel electrophoresis. Prior to electrophoresis, denatured and reduced total cell proteins or Triton-extractable and Triton-insoluble proteins were boiled for 3 min. Aliquots of 50—150 μg protein in 20—150 μl were applied onto 10 × 0.6 cm cylindrical gels of 0.1% SDS, 8.4% polyacrylamide. Electrophoresis was conducted at 0.6 mA/gel for about 18 h in 25 mM Tris, 1.92 mM glycine (pH 8.3), containing 0.1% SDS, and was stopped when a bromophenol blue marker reached the end of the gel. The electrophoretically separated protein bands were stained with Coomasie Blue according to the procedure of Fairbanks et al. [13]. Nominal molecular weights of the bands obtained were estimated by comparing their electrophoretic mobilities with those of marker proteins as follows: Heavy chain of myosin (M_r = 200 000) and actin (M_r = 42 000) extracted from rabbit myofibrils with Triton [14], LETS protein (M_r = 230 000) extracted from cultured chick fibroblasts with urea [15] and bovine serum albumin (67 000). Gels were scanned on a Gilford spectrophotometer. Peaks were cut out and weighed to estimate relative amounts of certain proteins in differently treated samples.

Radioiodination of surface proteins. Surface proteins of confluent fibroblasts were labeled with ^{125}I using the lactoperoxidase-catalyzed reaction as described by Hynes and Bye [16].

Determination of the rate of incorporation of [^{3}H]valine into protein. Rates of incorporation of tritium-labeled valine into proteins were measured in cycloheximide-treated cultures and in untreated cells according to the procedure of Kaftory et al. [12].

Results

Protein components of the Triton-insoluble cytoskeleton

Triton X-100, as well as other non-ionic detergents extracts about two-thirds of the proteins of cultured normal embryonic chick cells, whereas about 30% of the proteins remain insoluble and adhere firmly to the substratum [1,3,4]. The detergent-insoluble residue consists of the cell nucleus and filament bundles which constitute part of the cell cytoskeleton (refs. 1 and 2, and our unpublished scanning electron microscopical observations). We have first analyzed the protein composition of the detergent-insoluble cytoskeletal system of confluent cultured chick fibroblasts. Fig. 1 shows typical electropherograms of the total cell protein, of the detergent-extractable fraction and of the insoluble Triton cytoskeleton. The cytoskeletal residue (Fig. 1, lane C) contains four major proteins as well as numerous minor bands. Two proteins have apparent molecular weights of 42 000 and 200 000 and they coelectrophorese with muscle actin and with the heavy chain of myosin, respectively (electropherograms of marker proteins not shown). These bands, which seem to be identical to the cytoskeletal myosin and actin [1], constitute 2.5% and 24%, respectively, of the total protein of the Triton cytoskeleton. A third band of about 230 000 daltons coelectrophorese with marker LETS protein. Also, cell surface LETS protein which was radioiodinated prior to Triton extraction, quantitatively remained in the Triton-insoluble residue (not shown). This band, which seems therefore to represent LETS protein, constitutes about 10% of the total cytoskeletal proteins. The fourth major protein of the Triton cytoskeleton has an apparent molecular weight of 52 000. This component which has been described by several authors [1—3], is most probably the major protein of the 10-nm filaments [1,5]. The 52 000 dalton band, (henceforth designated the 52K protein) is presented in the Triton cytoskeleton at approximately equivalent amount to actin. In addition to the four major detergent-insoluble proteins, the Triton cytoskeleton includes low molecular weight proteins which comigrate with histones as well as numerous minor bands which are present in minute amounts. As seen in Fig. 1, lane B, considerable portions of actin, 52K protein and myosin are solubilized by Triton. In contrast, only a minor part of LETS protein is released by this detergent. The composition of the Triton-extractable fraction and of the insoluble residue as described here, is in full agreement with previous reports on the protein constituents of these fractions in normal chick fibroblasts [1,2] and mouse 3T3 cells [2,3].

Kinetics of fibroblast adhesion and attachment of the cytoskeleton to the substratum

A possible role of the cytoskeletal system is its participation in cell adhesion

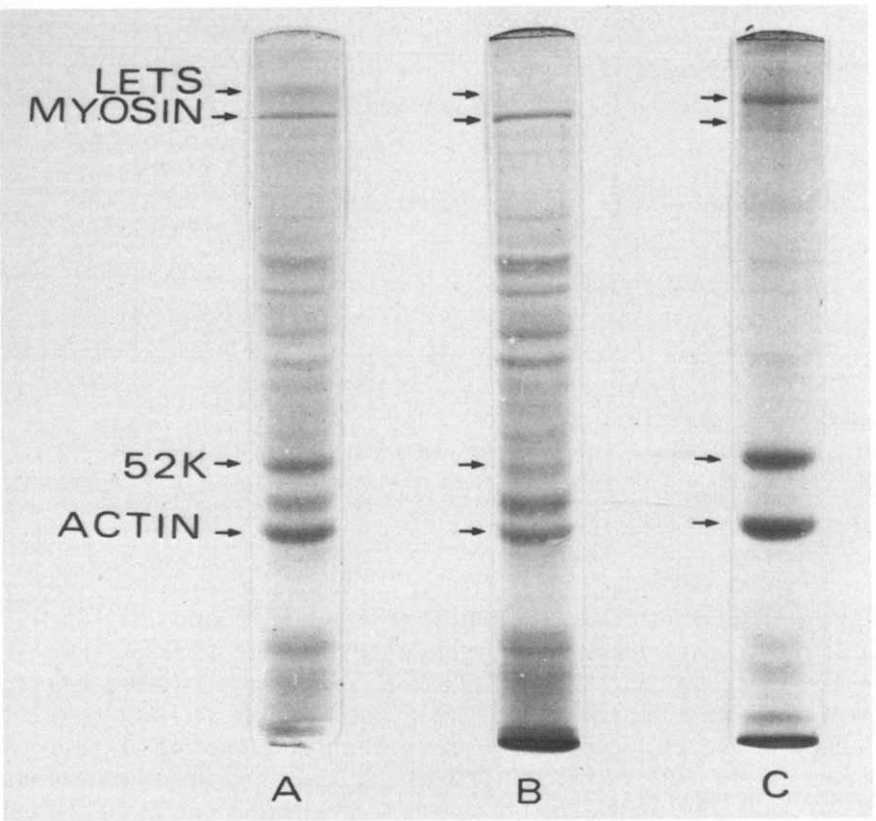

Fig. 1. Gel electrophoresis of total cell protein and of Triton extract and Triton-insoluble cytoskeleton. Total cell protein, Triton extract and detergent-insoluble residue were obtained from chick fibroblasts as described in the text. The three protein preparations were electrophoresed in 0.1% SDS-8.4% polyacrylamide gels as described under Materials and Methods. (A) Total cell protein, (B) Triton extract, (C) Triton cytoskeleton.

to the substratum. We have followed therefore, the kinetics of attachment of chick fibroblasts to the surface of the culture dish together with the kinetics of acquirement of the detergent insolubility by their cytoskeleton. Measurements of cell attachment and the proportion of the Triton-insoluble fraction were conducted as described under Materials and Methods. As shown in Fig. 2A, chick fibroblasts adhere initially very rapidly to the polystyrene surface and already after 10 min incubation, about 50% of the cell population become bound and cannot be detached from the substratum by mild rinsing with Buffer I. Following the initial phase of rapid attachment, the remaining cells adhere more slowly: 60 min after inoculation, about 75% of the cell population attach and at roughly 2 h, practically 100% of the cells are bound. At various times during the cell attachment process, the fraction of Triton-insoluble residue of the adherent fibroblast subpopulation was determined. The results depicted in Fig. 2B show that as early as 10—20 min after inoculation, almost 30% of the total proteins of the attached cells are rendered unextractable by

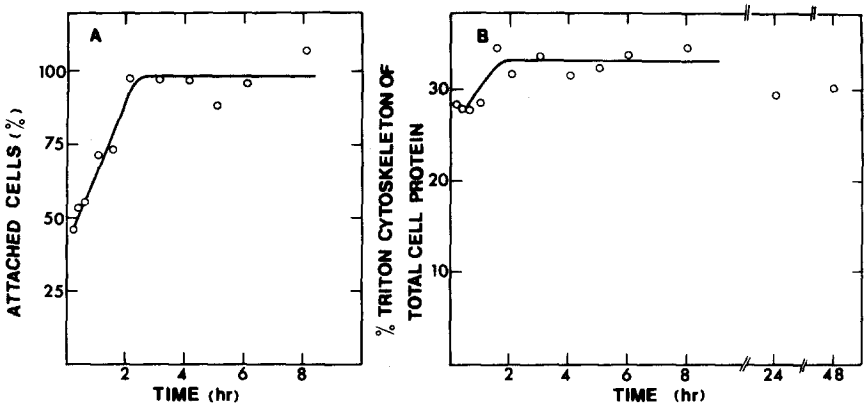

Fig. 2. Kinetics of cell adhesion and acquisition of cytoskeletal insolubility in Triton. Trypsin-detached chick fibroblasts, suspended in growth medium ($5 \cdot 10^5$ cells/ml) were seeded in a series of 60-mm polystyrene culture dishes ($7 \cdot 10^4$ cells/cm^2). Rates of cell adhesion and acquirement of Triton resistance by the adhered cell population were measured as described under Materials and Methods. (A) Cell adhesion, (B) Triton insolubility of the adhered cell population.

0.5% Triton. At 2 h, when complete cell attachment is attained, the Triton-insoluble fraction reaches a maximum value of 32% of the total cell protein. It seems therefore, that very early after cells adhere to the substratum or concurrently with their binding, about 30—32% of their proteins become resistant to solubilization by Triton. The mechanism of the rapid anchoring of the cytoskeletal system to the substratum is not clear. It could be that a transmembrane element links the plasma membrane-associated actin cables [17], and perhaps other cytoskeletal elements to the dish surface [1]. Another possibility is that the detergent insolubility of the cytoskeleton is artifactual and that actin, myosin and the 52K protein adhere nonspecifically to the substratum after being exposed during Triton-induced lysis of the cells. This last possibility was shown to be not valid. Confluent fibroblasts were detached from the substratum by either trypsin digestion or mechanical scarping and suspended in Buffer I. Triton X-100 was added to the cell suspension to a final concentration of 0.5% and the mixture was immediately put into contact with the culture dish at a density of $7 \cdot 10^4$ cells/cm^2. After incubation for 10 min at room temperature, the culture dish was rinsed several times with Buffer I and examined for the presence of an insoluble cytoskeletal residue. It was found that all cellular proteins were completely removed by the rinsing and no detergent-insoluble material remained adherent to the dish. Hence, it seems that rapid attachment of cytoskeletal elements to the substratum requires prior or concomitant adherence of the intact cell and that the exposed cytoskeletal elements by themselves are not capable of direct attachment to the surface in the presence of Triton.

Cell adhesion and acquirement of Triton insolubility by the cytoskeleton do not require protein synthesis

We have inquired whether cell adhesion as well as the rapid acquirement of detergent resistance by the cytoskeletal complex require de novo synthesis of

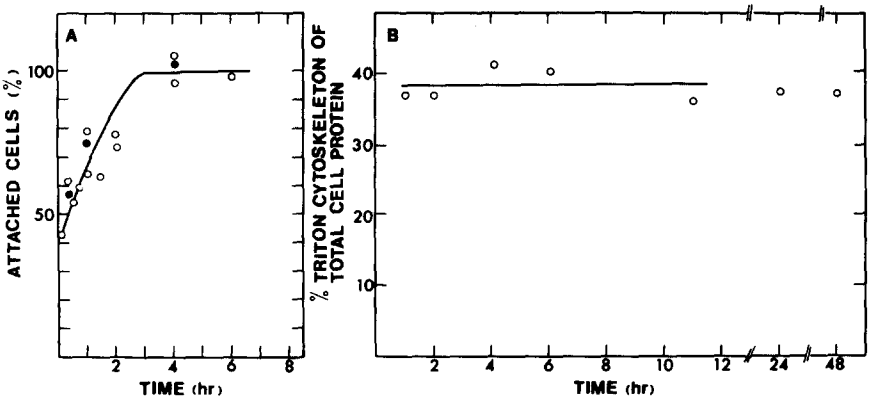

Fig. 3. Kinetics of cell adhesion and acquirement of cytoskeletal insolubility in Triton in the absence of protein synthesis. Trypsin-detached chick fibroblasts, suspended in growth medium which contained 0.2 mM cycloheximide ($5 \cdot 10^5$ cells/ml), were seeded in a series of 60-mm polystyrene culture dishes ($7 \cdot 10^4$ cells/cm^2). Control cultures were inoculated without cycloheximide. Rates of cell adhesion and acquirement of Triton resistance by the adhered cell population were measured as described under Materials and Methods. (A) Cell adhesion; ○———○, 0.2 mM cycloheximide; ●———●, control (no cycloheximide), (B) Triton insolubility of the adhered cell poulation in the presence of cycloheximide.

protein. In addition, we have studied the effect of prolonged inhibition of protein synthesis upon cell attachment and cytoskeleton binding to the substratum. Confluent chick fibroblasts were detached by trypsin and cycloheximide (final concentration 0.2 mM), together with [^{3}H]valine (1 μCi/ml) were added to the cell suspension. The fibroblasts were incubated with the drug for 30 min at room temperature and then seeded in its presence in a series of polystyrene culture dishes at a density of $7 \cdot 10^4$ cells/cm^2. Control cultures were similarly treated except that cycloheximide was omitted. Comparison of the incoration of [^{3}H]valine into acid-insoluble material in cycloheximide-treated and control cultures has indicated that protein synthesis was 97—99% inhibited in the presence of the drug. At various times, attachment of cells and Triton insolubility of the cytoskeleton were measured. The results of this experiment are shown in Fig. 3. It is evident that the kinetics of cell adherence in the presence of cycloheximide is not significantly different from that of untreated cultures (Fig. 3A, compare also with Fig. 2A). In addition, although protein synthesis was arrested, the culture acquired rapidly a Triton-insoluble residue which constituted about 38% of the total cell protein (Fig. 3B). The fraction of Triton-insoluble proteins was therefore, slightly larger in the cycloheximide-treated cultures than in untreated cells (Fig. 2B). For 8 h after seeding, cycloheximide-treated cells did not detach but exposure of the cultures to cycloheximide for prolonged periods of time (24—72 h), led to massive detachment of cells (not shown). However, the proportion of Triton-insoluble residue in cells which remained adherent was unaltered throughout the experiment (Fig. 3B). Electrophoretic analysis of the detergent-extractable and Triton-insoluble fractions of cells which were exposed to cycloheximide for 1—6 h revealed that the protein composition of these two fractions was the same as in untreated fibroblasts (not shown). These results indicate therefore, that protein synthesis is not required for the initial adhesion of cells and for cyto-

skeletal attachment. In addition, normal adhesion of cells and attachment of Triton cytoskeleton are maintained for 8 h in the absence of protein synthesis. Hence, it is conceivable that any protein element which links the intracellular cytoskeletal system to the substratum preexists in the binding cells and this putative component is not turned over at a very rapid rate.

Kinetics of attachment of cytoskeletal proteins

Of the major cytoskeletal components, LETS protein is an obvious candidate to serve as a link between the intracellular cytoskeletal system and the substratum. This surface glycoprotein might be a transmembrane protein [7], and seems to be involved in cell to cell and cell to substratum adherence [8,9,18]. The majority of LETS molecules are digested by trypsin treatment of

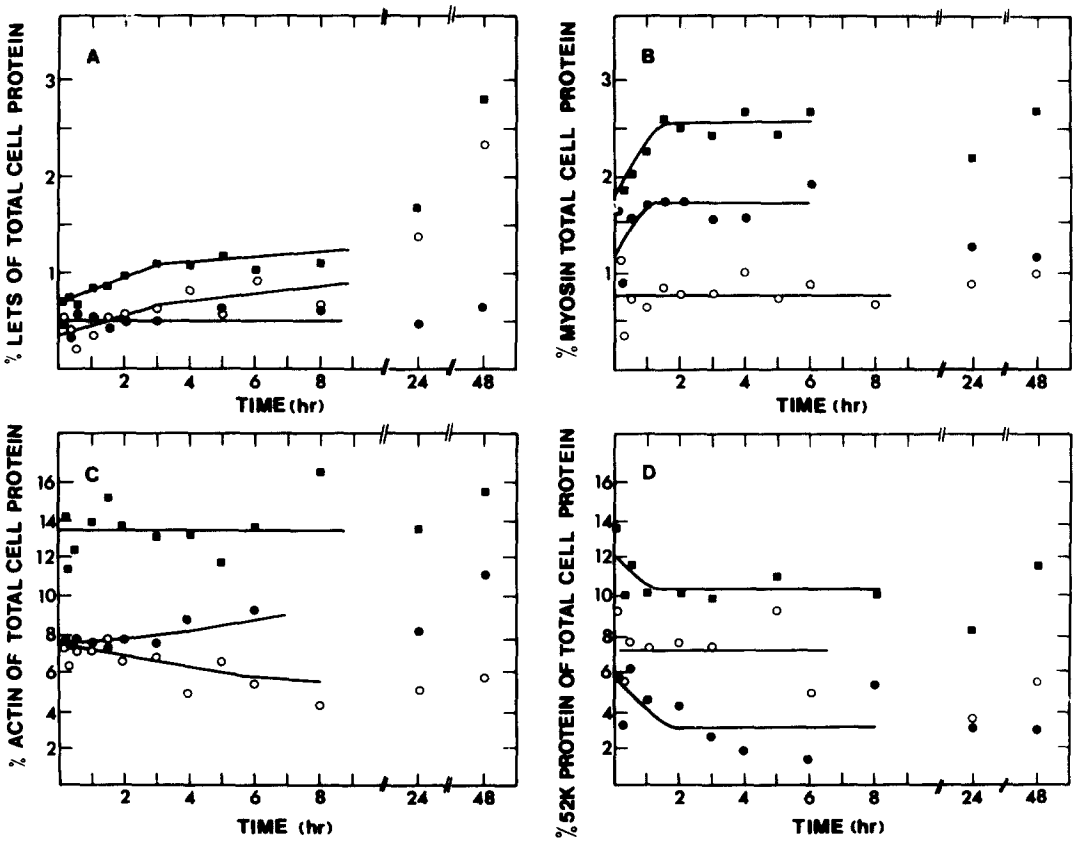

Fig. 4. Proportion of cytoskeletal proteins in cells inoculated in culture for various periods of time. Trypsin-detached chick fibroblasts were seeded in a series of culture dishes as in Fig. 2. At various times after inoculation, non-adherent cells were removed by rinsing the culture with Buffer I and the attached cells were either solubilized with 1% SDS to yield total cell protein or treated with 0.5% Triton X-100. Total proteins and the detergent-soluble fraction and the insoluble residue were collected and prepared for electrophoresis as described under Materials and Methods. The various protein preparations were electrophoresed in 0.1% SDS, 8.4% polyacrylamide gels. Relative amounts of LETS protein, heavy chain of myosin, 52K protein and actin were estimated by densitometry of the stained gels. (A) LETS protein, (B) Myosin, (C) 52K protein, (D) Actin. ■———■, total cell protein; ●———●, Triton extract; ○———○, Triton-insoluble residue.

intact cells [6,15]. In an attempt to assess the possible role of LETS protein in cytoskeletal attachment, we have followed the kinetics of LETS protein reappearance after its elimination by trypsinization and compared it with the kinetics of acquirement of Triton insolubility by LETS protein, myosin, actin and the 52K protein. Cultured chick fibroblasts were detached with trypsin and reinoculated in a series of 60-mm culture dishes at a density of $7 \cdot 10^4$ cells/cm^2. At various times after seeding, unbound fibroblasts were removed by three washes with Buffer I and the adherent cells were either solubilized directly with 1% SDS to yield total cell protein or treated with 0.5% Triton for 10 min to yield detergent-extractable and Triton-insoluble fractions. Total cell proteins, as well as proteins of the Triton-soluble and -insoluble fractions, were separated electrophoretically in 0.1% SDS, 8.4% polyacrylamide gels. The relative amounts of LETS protein, myosin, actin and 52K protein were determined by densitometry of the stained gels. Fig. 4 depicts the results of this experiment. As seen in Fig. 4A, the content of LETS protein at the time of inoculation is rather low: whereas this component constitutes about 2.7% of the total proteins of confluent cultures grown for 48 h (Fig. 4A and similar results in ref. 19) its initial content is only 0.7% of the total proteins of the newly seeded cells. This trypsin-insensitive species might contain non-LETS proteins and probably also represents the intracellular protease-resistant fraction of LETS protein which has been identified recently [19]. About 40% of the low initial amount of LETS protein are Triton insoluble whereas 60% are extracted by the detergent. During the first 2 h of incubaion, the amount of LETS protein increases linearly and when cell adhesion is completed, further increase in LETS protein is observed. All the newly synthesized LETS protein is exclusively located in the Triton-insoluble residue whereas the detergent-soluble fraction of this protein remains constantly at about 0.5% of the total cell protein. Thus, at 48 h, the insoluble fraction contains about 80% of the total LETS protein. These results indicate that the rapid initial adhesion of the cells (Fig. 2A) as well as acquirement of cytoskeletal detergent insolubility (Fig. 2B) occur while LETS protein is present at only 15—20% of its maximum total and cytoskeletal content. Complete adhesion and quantitative retention of the culture's cytoskeleton are achieved 2 h after seeding (Fig. 2) and at that time LETS protein is present at only 30—35% of its maximum amount. The levels of the other major components of the cytoskeleton change much less drastically in the course of cell binding and growth. As shown in Fig. 4B, the heavy chain of myosin initially constitutes about 2% of the total cell protein. Of this amount, roughly 25% are Triton insoluble whereas 75% are extracted by the detergent. Similar Triton solubility has been reported for the heavy chain of myosin of cultured 3T3 cells [3]. During the first 2 h of incubation, the cellular content of myosin increases to 2.6% and remains constant thereof. This relatively rapid increase in the level of myosin is solely due to a respective increment in the Triton-soluble fraction of this protein. The content of actin is about 13.5% of the total cell protein at the time of seeding (Fig. 4C, for comparable values see refs. 20 and 21). Of this amount, about 50% are located in the detergent-resistant cytoskeleton. Similar Triton solubility of actin was reported for confluent non-muscle cells grown in culture for prolonged periods of time [1,3]. As our results demonstrate, despite fluctuations in the amount

of actin in the Triton-soluble and -insoluble fractions, its total content after 48 h as well as its cytoskeletal fraction, remain subtantially the same as in newly seeded cultures. Fig. 4D shows that the cytoskeletal level of the 52K protein also remains constant throughout the experiment. The total amount of this protein, which ranges between 14 and 10% of the total cell protein, diminishes in the course of the first 8 h of growth. This decrease is due to a pronounced decline in the amount of the Triton-soluble fraction of this protein. However, the total amount of 52K protein, as well as its cytoskeletal and detergent-extractable fractions, are at similar levels in long-term and in newly seeded cultures.

Majority of LETS molecules is not required for cell adhesion and acquirement of Triton insolubility by the cytoskeleton

The results presented in the preceding section indicate that maximum levels of cytoskeletal actin and 52K protein and about 80% of the maximum amount of the heavy chain of myosin, are present in the Triton cytoskeleton very early after cell inoculation. By clear contrast, only a minor fraction of LETS protein is present in the cytoskeleton during adhesion of trypsinized cells and acquirement of detergent insolubility by their cytoskeleton. To examine whether a similar relationship may prevail between LETS protein, cell adhesion and cytoskeleton anchorage in adherent cultures, LETS protein of attached cells was digested by trypsin or extracted with urea under conditions which do not cause detachment of the firboblasts. After most LETS molecules were removed, the extent of solubilization of the cytoskeleton by Triton was examined, and the protein composition of the insoluble residue analyzed. Confluent cultures of chick fibroblasts were exposed to 1 M urea for 2 h under conditions that were described in detail by Yamada and Weston [15]. Parallel cultures were treated with 7.5 μg/ml trypsin (EC 3.4.21.4, crystallized, Worthington Biochem. Corp.) at 37° C for 5 min. The proteolytic reaction was stopped by washing the cells with growth medium which contained 2 mM of the protease inhibitor phenylmethylsulfonyl fluoride (PMSF). Neither urea extraction nor the mild trypsin digestion led to detachment of fibroblasts from the dish, although the treated cells rounded considerably. The trypsin- and urea-treated cultures, as well as untreated control cells were either solubilized with 1% SDS to yield total cell protein or extracted with 0.5% Triton X-100 to yield detergent-soluble fraction and -insoluble residue. The Triton-insoluble cytoskeleton was found to consist of roughly 30% of the total cell protein in both treated cultures and untreated cells. Fig. 5 shows electropherograms of the total proteins and the Triton-soluble and -insoluble fractions of the various cultures. It is evident that whereas cytoskeleton of untreated cells contains a considerable amount of LETS protein, the amount of this protein is greatly reduced in urea and trypsin-treated cells and in their Triton cytoskeleton. Densitometry of the gels revealed that the relative amount of cytoskeletal LETS protein is reduced by 62 and 83% of the control value in urea- and trypsin-treated cells, respectively (average of three experiments). By contrast, the amount of actin, 52K protein and the heavy chain of myosin as well as their distribution between the Triton-soluble and -insoluble fractions, were not affected by either the proteolytic digestion or urea extraction. Hence, a reduction of more than 80% in the

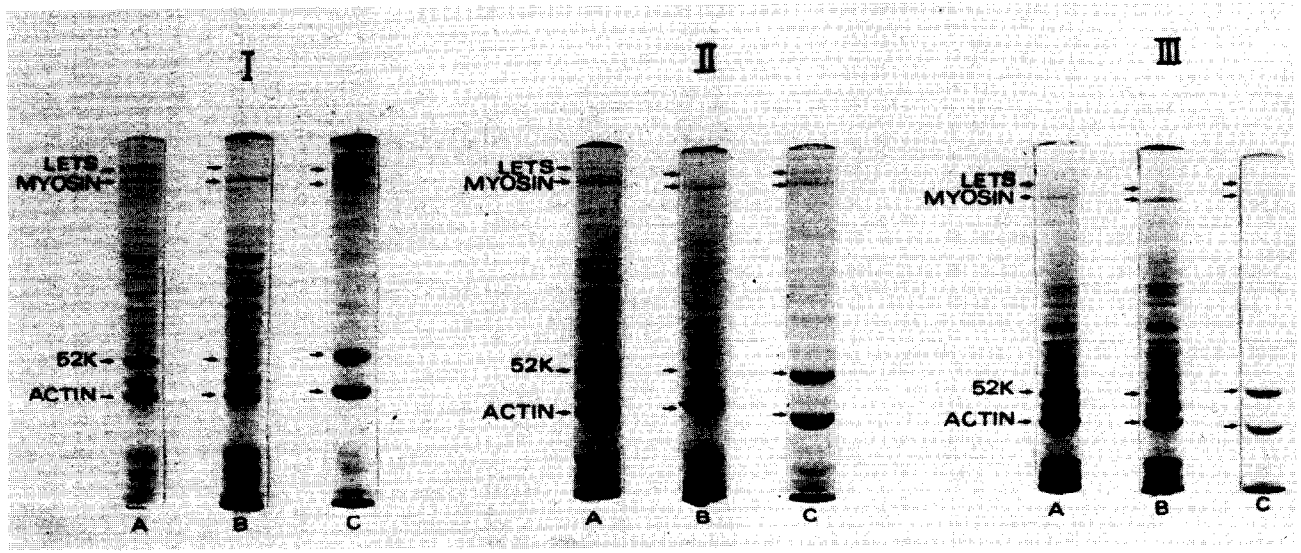

Fig. 5. Effect of urea extraction and trypsin digestion on the total protein composition and on protein elements of Triton extract and Triton cytoskeleton. Confluent cultures of chick fibroblasts were treated with urea and trypsin as described in the text. Total cell protein, Triton extract and Triton cytoskeleton were obtained as described under Materials and Methods. Gel electrophoresis was conducted as in Fig. 1. (I) Untreated control cells, (II) Urea-extracted cells, (III) Trypsin-digested cells. A, total cell protein; B, Triton extract; C, Triton cytoskeleton.

TABLE I

EFFECT OF LONG-TERM INHIBITON OF PROTEIN SYNTHESIS ON THE DISTRIBUTION AND AMOUNT OF CYTOSKELETAL PROTEINS

Protein	Relative amount of cytoskeletal proteins (percent of total cell protein) [a]					
	Control cells [b]		Trypsin treated cells [c]		Trypsin-treated cells, [d] cycloheximide, 72 h	
	Triton soluble	Triton cyto-skeleton	Triton soluble	Triton cyto-skeleton	Triton soluble	Triton cyto-skeleton
LETS protein	0.4	2.5	0.5	0.4	0.06	0.2
Myosin	1.8	0.6	1.5	0.5	0.7	1.0
52K	3.5	11.7	5.6	9.4	2.5	6.5
Actin	7.2	8.1	7.8	7.4	6.4	6.9

[a] Relative amounts of the four major cytoskeletal proteins were estimated by densitometry of stained electropherograms of the Triton fractions.

[b] Normal chick fibroblasts, grown for 48 h under normal conditions and extracted with 0.5% Triton X-100. Triton-insoluble residue constituted 31% of total cell proteins.

[c] Cells as in b, detached from the substratum with 0.25% trypsin, reinoculated and the adherent cells extracted with 0.5% Triton X-100, 60 min after inoculation. Triton-insoluble residue constituted 30% of the cell protein.

[d] Cells detached form the substratum as in b, reinoculated and incubated in the presence of 0.2 mM cycloheximide for 72 h. After the long-term incubation with the drug, only 35% of the originally seeded cells remained attached. Adherent cells were extracted with 0.5% Triton X-100. Triton-insoluble residue constituted 37% of total cell protein.

amount of cytoskeletal LETS protein does not affect cell adhesion and Triton insolubility of actin, 52K protein and myosin. In an attempt to further reduce the amount of LETS protein, the majority of LETS molecules was removed by trypsin and the culture was exposed to cycloheximide in order to inhibit de novo synthesis of LETS protein. During the long-term incubation of the culture with the drug, the trypsin-resistant fraction of LETS protein was degraded. Although inhibition of protein synthesis for extended periods of time (24—72 h) induces massive detachment of cells, it does not affect significantly the relative amount of Triton-insoluble residue in cells which remain adherent. Results of this experiment are summarized in Table I. Comparison of the total amount of LETS protein in trypsinized cells and in cells exposed to cycloheximide, indicate that in 72 h, about 70% of this protein were degraded. This result is in line with a recent estimation of the half-life of LETS protein of 36 h [19]. Clearly, the amount of both total and cytoskeletal LETS protein is reduced in cycloheximide-treated cultures to less than 10% of its value in control cells. By contrast, levels of total and cytoskeletal actin are only slightly reduced in the drug-inhibited cultures. Also, the amount of the 52K protein decreases by only 30% as compared to control Triton cytoskeleton. Although the level of total myosin is somewhat reduced in the presence of cycloheximide, the Triton-insoluble fraction of this protein even increases in comparison with control cells. We conclude therefore, that more than 90% of LETS protein can be removed without greatly affecting the composition of the cytoskeleton and its insolubility in Triton.

Discussion

The first objective of this work was to examine the relationship between cell adhesion and acquirement of Triton resistance by the cytoskeleton of the adherent cells. The assumptions inherent in this study were first, that the cytoskeleton might be involved in cell-substratum adhesion and second, that cytoskeleton insolubility in Triton reflects the in vivo attachment to the substratum of its cytoplasmic elements. The first assumption is well supported by numerous lines of evidence [9,22—24]. The second supposition is sutained by our observation that solubilized cytoskeletal proteins were incapable of attachment to the substratum. In addition, Brown et al. [1] have reported that adherent HeLa cells, transformed chick fibroblasts and rat tumour cells, which presumably possess altered cytoskeleton, came off the dish in the presence of Triton without leaving behind an insoluble residue. Hence, the cytoskeletal complex of various adherent cells is not necessarily insoluble in Triton under all conditions. It seems therefore, that the formation of Triton cytoskeleton is not artifactual and that it requires adhesion of intact cells which maintain connection between the cytoskeleton network and the substratum. Firstly, we followed the kinetics of both cell adhesion and acquirement of Triton-insoluble cytoskeleton by the adherent cell population. We have discriminated between adherent and non-adherent fibroblasts on the basis of resistance of the attached cells to mild rinsing procedure. A similar assay was recently described by Juliano and Galagang [25], who measured the rate of adhesion of CHO cells to protein-coated surface. As pointed out by these authors, although assay of this type determines the fraction of adherent cells at various times after inoculation, it does not provide information as to the firmness of cell attachment as other assays do [22,23]. We have found that under our experimental conditions, adhesion of the cells was non-dissociable from acquirement of complete Triton insolubility of their cytoskeleton (Fig. 1). In addition, the content of actin and 52K protein in cytoskeleton of freshly seeded cells was the same as in cytoskeleton of long-term cultures (Fig. 4). Furthermore, we showed that cell adhesion did not require synthesis of protein (Fig. 3A). Analogous findings by Weber et al. [22] showed that restoration of adhesiveness by conditional transformed chick cells was not inhibited by cycloheximide. Our experiments also provided evidence to show that acquirement of Triton insolubility of the cytoskeleton progressed normally in the presence of cycloheximide (Fig. 3B). Hence, neither cell adhesion nor cytoskeleton attachment depend on de novo synthesis of protein and these two processes could not be dissociated on the basis of their different requirement for synthesis of protein. It should be remarked, however, that all our kinetic experiments were of limited resolutions. Therefore, we could not distinguish between the possibilities that cell adhesion and cytoskeleton binding are interdependent and concurrent, or that these two events are dissociable but occur very closely in time. Moreover, our analysis of the extent of cytoskeleton insolubility and its composition was limited to the adherent fraction of the cell population. Study of the composition of the cytoskeleton of the non-adherent fraction of newly seeded cultures should be conductive for the understanding of the relationship between cell attachment and cytoskeleton anchoring. Lastly, it should be stressed that our

experiments do not indicate that a causative relationship exists between cytoskeletal attachment and adhesion of cells to the substratum. Future investigation should determine whether cell adhesion is dependent upon anchoring of the cytoskeleton or if these are two independent processes.

A second objective of the present work was to examine the possibility that LETS protein is involved in the binding of the cytoskeleton to the substratum. LETS protein is one of the four major protein components of the Triton cytoskeleton (refs. 1 and 4 and see Results). This protein, which might be a transmembrane component [7], has been implicated in cell-cell and cell-substratum adhesiveness [8,9]. Transformation which leads to loss or marked reduction in the amount of LETS protein [6], also alters the organization of the cytoskeleton [9,26,27]. It is conceivable therefore, that LETS protein might be associated with the cytoskeleton. Linking the substratum-bound LETS protein to membrane-associated elements of the cytoskeleton, such as actin [17], could provide mechanistic explanation to the anchoring of the cytoskeletal filaments to the substratum. In order to examine this model, we have studied the effect of removal of LETS protein upon cell adhesion and cytoskeleton attachment. It was found that trypsin-detached cells, which contained roughly 15—20% of the maximum amount of total and cytoskeletal LETS protein, adhered rapidly to polystyrene surface and their cytoskeleton became resistant to Triton solubilization (Figs. 1 and 4A). The great majority of LETS molecules was synthesized only after cell adhesion and cytoskeleton attachment have already been completed (Fig. 4A). Parallel experiments have similarly shown that removal of 62 and 83% of LETS protein of adherent cultures by urea extraction or trypsin digestion, respectively, did not affect either the attachment or the composition of the Triton cytoskeleton (Fig. 5). Furthermore, reduction of the amount of LETS protein to 10% of its maximum level by inhibition of its resynthesis after trypsin digestion, did not affect the proportion of the Triton-insoluble residue of adherent fibroblasts and caused only a limited decrease in the amount of other elements of the cytoskeleton (Table I). It seems therefore, that the majority of LETS molecules is not required for cell adhesion and cytoskeleton attachment to the substratum. Weber et al. [22] have shown that trypsin digestion of LETS protein from chick cells which would be iodinated did not affect the adhesiveness of these cells. Recent extensive studies by Iqbal et al. [9] and Mautner and Hynes [4] argued against a simple one-to-one association between LETS protein- and actin-containing filaments. However, these investigators could not exclude the possibility that more subtle interaction between LETS protein and actin does exist [4]. A particularly attractive proposition is that only a minor fraction of LETS protein is involved in cell adhesion and is associated with cytoskeletal elements. Hence, it could be that only 10—15% of the LETS protein is sufficient to initiate cell attachment to the substratum. This minor portion of LETS protein could perhaps be involved only in the initial phases of cell and cytoskeleton adhesion whereas at later stages, presence of LETS protein is no more required for these processes. Studies by Culp [18,28] have shown that a small fraction of LETS protein is located in the substratum-bound cell surface material, which also contains myosin, actin and a 56 000 molecular weight protein. It could be that only this minor fraction of LETS protein is involved in

cell-substrate adhesion and in cytoskeleton attachment. Such supposition requires therfore, that at least three different subpopulations of LETS protein should now be distinguished. First, intracellular, trypsin-resistant LETS protein [19]. Second, the greater part of exposed, cell surface LETS protein which is not involved in cell-substratum interaction and third, a minor part of the exposed portion of LETS molecules which might still serve in cell adhesion.

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