

Role of Gelsolin in Actin Depolymerization of Adherent Human Neutrophils

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Human neutrophils generally function adherent to an extracellular matrix. We have previously reported that upon adhesion to laminin- or fibronectin-coated, but not uncoated, plastic there is a depolymerization of actin in neutrophils. This phenomenon was not affected by inhibitors of the more well-studied components of the signal transduction pathway, specifically, pertussis toxin, an inhibitor of G-proteins, H-7 or staurosporine, inhibitors of protein kinase C, or herbimycin A, an inhibitor of nonreceptor tyrosine kinase. We therefore focused our attention on actin-binding proteins and measured the changes in the partitioning of gelsolin between the Triton X-100- soluble and -insoluble cellular fractions which occur upon neutrophil adhesion by means of quantitating anti-gelsolin antibody binding to aliquots of these fractions. It was found that approximately 90% of the total cellular gelsolin was found in the Triton X-100-soluble fraction in suspended cells, but that upon adherence to either fibronectin- or laminin-coated plastic about 40% of the soluble gelsolin could be detected in the insoluble fraction. This effect was not observed in cells adherent to uncoated plastic, wherein more than 90% of the gelsolin was found in the soluble fraction. Results of immunofluorescence microscopy of these cell preparations was consistent with this data. A gelsolin translocation to the insoluble cellular actin network may account for a part of the observed actin depolymerization.

INTRODUCTION

The dynamic changes of F-actin critically function in cell locomotion (Theriot and Mitchison, 1991; Zigmond, 1993), since the ability of monomeric actin (G-actin) to polymerize into filamentous actin (F-actin) is fundamental to its biological activity in all eukaryotic cells (Alberts *et al.*, 1994). A prominent feature of cell locomotion is that actin polymerization and depolymerization occur simultaneously at different locations in the cytoplasm of the moving cell (Wang, 1985; Fechheimer and Zigmond, 1993). The dynamic actin change is highly regulated by extracellular signals (Stossel, 1989) and by a large and diverse group of actin-binding proteins (Stossel *et al.*, 1985; Pollard and Cooper, 1986; Kabsch and Vandekerckhove, 1992).

The neutrophil is one of the most motile cells in the human body and serves as an important model for actin research. A variety of soluble agonists have been shown to induce the polymerization of actin in suspended cells (Cooper, 1991; Snyderman and Uhing, 1992), and the signal transduction pathways involved have been extensively studied (Omann *et al.*, 1987; Painter *et al.*, 1987; Dillon *et al.*, 1988; Sarndahl *et al.*, 1989; Therrien and Naccache, 1989; Downey *et al.*, 1990a,b; Niggli and Keller, 1991). However, the neutrophil *in vivo* usually functions adherent to either endothelial or interstitial tissues or cell target surfaces (Ginis and Tauber, 1990). Therefore, the study of adherent neutrophils on extracellular matrix (ECM) protein surfaces would more closely approximate *in vivo* conditions. Previous work by ourselves (Ginis *et al.*, 1992) and others (Southwick *et al.*, 1989) has shown that adherence of neutrophils to plastic surfaces induces actin polymerization by a pertussis toxin-insen-

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sitive signal pathway. In contrast, adherence to surfaces coated with ECM proteins, such as laminin (LN) and fibronectin (FN), initiates the opposite effect, i.e., transient actin depolymerization (Ginis *et al.*, 1992; Wang *et al.*, 1993), which occurs upon adherence of the suspended cell to the ECM-coated surface. Actin depolymerization under some conditions would be required for the cell to maintain a constant F-actin level in the presence of stimulants which tend to cause actin polymerization. The mechanisms controlling this effect and the consequent metabolic influence on other cellular constituents are still unknown, although we have noted that the process was accompanied by a rise in intracellular Ca^{2+} (Ginis *et al.*, 1992; Wang *et al.*, 1993).

The fraction of total cellular actin present in the soluble actin pool of the neutrophil is regulated by a number of proteins. Much of the actin monomer is sequestered by thymosin β 4 and to a lesser extent by profilin (Southwick and Young, 1990; Cassimeris *et al.*, 1992), whereas the F-actin is stabilized by cross-linking with α -actinin (Cano *et al.*, 1992). Gelsolin is an important actin-regulating protein present in the neutrophil which, in the presence of micromolar calcium, can cut and cap actin filaments (Yin and Stossel, 1980; Yin *et al.*, 1980). It has also been postulated to be an important nucleating factor responsible for the F-actin increase found after formylmethionylleucylphenylalanine stimulation (Howard *et al.*, 1990). In the present report, we have attempted to further define the underlying control mechanisms for actin depolymerization and have focused on the distribution of gelsolin between the soluble and insoluble phases of the cell.

MATERIALS AND METHODS

Materials

Human plasma FN was obtained from Calbiochem (La Jolla, CA). Murine LN, monoclonal antigelsolin, anti-mouse IgG peroxidase conjugate, double-stranded calf thymus DNA, bovine pancreas DNase I, phalloidin, proteinase inhibitors antipain, aprotonin, chymostatin, diisopropyl fluorophosphate, leupeptin, pepstatin A, phenylmethylsulfonyl fluoride, and Dulbecco's phosphate-buffered saline (PBS; 10 \times) were purchased from Sigma Chemical Co. (St. Louis, MO). Monoclonal antiactin, high-performance luminescence detection film (Hyperfilm-ECL), and enhanced chemiluminescence (ECL) Western blotting detection reagents were purchased from Amersham Life Sciences (Arlington Heights, IL). Bicinchoninic acid (BCA) protein assay reagents were obtained from Pierce (Rockford, IL). Microconcentrators were obtained from Amicon Inc. (Beverly, MA). Polyvinylidene difluoride (PVDF) transfer membrane (Plyscreen) was obtained from DuPont New England Nuclear Research Products (Boston, MA). Nitrocellulose (0.22 μm) was obtained from MSI (Westboro, MA). SDS-polyacrylamide high and low molecular range standards were purchased from Bio-Rad (Richmond, CA). Protogel (concentrated polyacrylamide/Bis) was purchased from National Diagnostics (Manville, NJ). Triton X-100 was purchased from EM Science (Gibbstown, NJ). A standard G-actin solution was made from rabbit skeletal muscle actin obtained from an acetone powder using the method of Spudich and Watt (1971). Gelsolin was

purified from human plasma according to the methods of Cooper *et al.* (1987). All other chemicals are molecular biology grade and commercially available.

Isolation of Neutrophils

Human neutrophils were isolated from normal donors by RBC sedimentation in dextran-citrate, followed by hypotonic lysis and Ficoll-Paque density gradient centrifugation as described previously (Higson *et al.*, 1985). The isolated cells (>95% pure) were then suspended in PBS containing 140 mM NaCl, 3 mM KCl, 1 mM K_2HPO_4 , 1 mM CaCl_2 , and 1 mM MgCl_2 (pH 7.4) and were maintained at 4°C until studied.

Adhesion and Estimation of the Number of Adherent Cells

In 1 ml of PBS $0.5\text{--}1 \times 10^7$ cells were prewarmed to 37°C for 5 to 10 min, plated into the 35 \times 10-mm plastic culture dishes (Falcon 3001) coated with LN (30 μg) or FN (30 μg). The cells were incubated for indicated times at 37°C. At the end of the incubation, nonadherent cells were washed out by successive rinsing the dishes three times with PBS. To estimate the number of adherent cells, culture dishes with adherent cells were incubated with Pierce BCA protein assay reagent for 60 min at 37°C. Each sample was then read at 562 nm and compared with a standard curve of known cell numbers.

Fractionation of Adherent and Suspended Neutrophils

The BCA protein assay was used for measuring total protein from monolayer cell culture (Goldschmidt and Kimelberg, 1989). LN- and FN-adherent and suspended neutrophils were fractionated into two fractions: the Triton X-100 soluble and the Triton X-100 insoluble, by extraction with 0.5% Triton X-100 in PBS including 10 mM EGTA, 50 μM phalloidin, 1 $\mu\text{g}/\text{ml}$ leupeptin, chymostatin, antipain, pepstatin A, 10 $\mu\text{g}/\text{ml}$ aprotonin, and 0.1 mM phenylmethylsulfonyl fluoride (pH 7.4). The lysates were centrifuged at 14,000 rpm for 5 min, and supernatants (Triton X-100-soluble fraction) were transferred to an Amicon microconcentrator for concentration as directed by the manufacturer's guide. We found that testing of samples from a batch of filtrator membranes with a known concentration of protein was important. Recovery of 95–100% was obtained with different concentrations of bovine serum albumin concentrated in the filtrator unit. The pellet was combined with the scraped unextractable part to constitute the Triton X-100-insoluble fraction. Aliquots of these fractions were incubated with Pierce BCA protein assay reagent for 60 min at 37°C. Each sample was then read at 562 nm, and protein concentrations were obtained by comparison to a standard solution of bovine serum albumin supplied by the manufacturer and compared with a standard curve of known cell numbers (Smith *et al.*, 1985). The absorbance at 562 nm was linear for up to 1×10^5 cells/dish under the experimental conditions.

Analysis of SDS-PAGE

Aliquots with an equal amount of proteins (usually 10 μg) from each fraction were combined with Laemmli sample buffer (Laemmli, 1970) containing 62.5 mM Tris, 2% SDS, 10% glycerol, and 5% 2-mercaptoethanol and solubilized for 2 to 5 min at 100°C. The gradient (5–15%) SDS-polyacrylamide gels were prepared by a gradient maker in SE 215 multiple casting chamber (Hoefer Scientific Instruments,* San Francisco, CA). The samples with high and low SDS-PAGE molecular standard and solubilized standard solutions of actin or gelsolin, which were varied, were analyzed in a Hoffer SE 250 Mighty Small II slab gel electrophoresis unit powered by a PS 500X DC power source. The gels were also used for Western blotting detection.

Western Blotting and ECL Detection

After the completion of SDS-PAGE, gels were equilibrated in 10 mM 3-(cyclohexylamino)-1-propanesulfonic acid buffer (pH 11.0) containing 10% methanol for 15 min and subsequently transferred in this buffer onto PVDF transfer membrane (Polyscreen) by Semi-Phor TE-70 semidry transfer units (Hoefler). The membrane was washed in Tris-buffered saline-Tween 20 (TBS-T; 0.2–0.3%), pH 7.4, for twice for 10 min and was either stained with India ink (0.1%) or "blocked" in TBS-T containing 5% powdered milk (Carnation) for 1 h. The blocked membrane was washed three times in TBS-T and then incubated overnight with primary monoclonal antibody in a 1:1000 in TBS-T containing 5% powdered milk at 4°C. Following the incubation, the membrane was washed three times in TBS-T and incubated in a 1:2000 dilution of a peroxidase-conjugated goat anti-mouse IgG for 1 h at room temperature. After thoroughly washing several times with TBS-T, the membrane was incubated with ECL reagents, covered with plastic wrap, exposed to high-performance luminescence detection film (Hyperfilm-ECL), and developed in a dark room. The protein bands were further quantified by densitometry in comparison to a standard band.

Dot-Blot Method for Quantitation of Gelsolin in Fractions of Adherent and Suspended Neutrophils

To characterize the localization of gelsolin in adherent and suspended cells, the Triton X-100-soluble and -insoluble fractions were prepared as described previously. Protein concentrations were determined by a Pierce BCA protein assay. Equal volumes (2–5 μ l) of samples from the fractions and standard solutions of gelsolin were carefully hand pipetted onto PVDF transfer membrane (Polyscreen). We found that we could obtain more reproducible results if the protein solutions were pipetted onto the membrane by hand rather than using a suction apparatus, despite the fact the dots obtained were irregular in outline. The membrane was kept in a 37°C chamber for 15 min, washed in TBS-T twice for 10 min, blocked with 5% powdered milk, washed in TBS-T three times, incubated with monoclonal antigelsolin in a 1:1000 dilution overnight at 4°C, washed three times in TBS-T, and further incubated in a 1:2000 dilution of a peroxidase-conjugated goat anti-mouse IgG for 1 h at room temperature. After thoroughly washing several times with TBS-T, the membrane was incubated with ECL reagents, covered with plastic wrap, exposed to the high-performance luminescence detection film (Hyperfilm-ECL), and developed in a dark room. The amount of gelsolin was determined by scanning the dots of the samples in the film with a Shimadzu CS-930 dual wavelength scanner (Kyoto, Japan) and comparing with the dots of a standard solution of purified gelsolin. The dots were scanned using an integrated area program so that the total intensity of the dot was obtained independent of its shape or size. Between 1 and 60 ng of gelsolin, purified from human serum, were always applied to the PVDF membrane on which cell samples had been applied to quantitate the gelsolin present in these fractions. The film was exposed over a range of times so that the dots of the cell fractions were in the linear absorbance range. A representative dot-blot and standard curve for purified plasma gelsolin is shown in Figure 1. The data analyzed by a linear regression had excellent correlation ($r^2 = 0.995$) for all of the values as well as the range 1–10 ng ($r^2 = 0.948$).

Statistical Analysis

The amount of gelsolin in different fractions was analyzed by analysis of variance and multiple comparisons. The data of adhesion ratio and number of cells were analyzed using χ^2 analysis. $p < 0.05$ was considered significant.

Gelsolin Staining of Neutrophils

Neutrophils were allowed to adhere to 20 \times 20-mm squares cut from small 35 \times 10-mm plastic tissue culture dishes coated with 30

μ g of LN (Sigma), 30 μ g of FN (Sigma), or left uncoated and permeabilized for 5 min using 1% Triton X-100 in PBS with the protease inhibitors in a similar manner to that described above. The samples were fixed, stained with monoclonal antibody to gelsolin (Sigma), and counterstained with a rhodamine-labeled affinity-purified mouse IgG (H + L, Kirkegaard and Perry, Gaithersburg, MD) by standard methods (Harlow and Lane, 1988). Three microliters of PBS were placed on the plastic squares followed by 18 \times 18-mm glass coverslips which were sealed in place using colorless fingernail polish. Sealed slides were stored at 4°C in the dark.

RESULTS

Actin Changes in the Fractions of Adherent Human Neutrophils

The actin present in the Triton X-100-extractable and -unextractable fractions of neutrophils adherent to either LN- or FN-coated plastic were evaluated with a Western blot of SDS-PAGE of these samples using an anti-actin antibody. The fractions from adherent neutrophils were concentrated by a Microcon concentrator (Amicon) and compared with the fractions of the suspended cells. The same amount of total protein was present in each sample. As shown in Figure 2, A and B, there is an increase in actin in the Triton X-100-soluble fraction, as compared with suspended cells, after 1 min of adherence to either FN or LN, followed by a decrease over the next 10 min. As shown in Figure 2C, there is a corresponding decrease in actin in the Triton X-100-insoluble fraction of adherent as op-

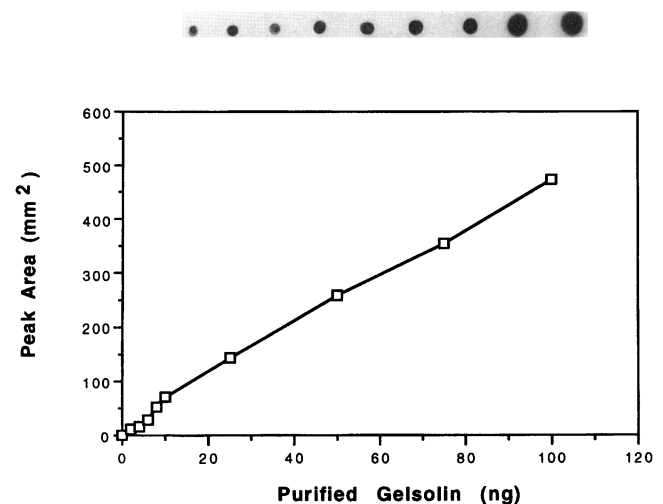


Figure 1. Standard curve of plasma gelsolin. Different concentrations of gelsolin in 3–5 μ l of PBS (dot-blot of gelsolin from human plasma in the range of 1–60 ng/3 μ l of PBS shown above graph) were freshly prepared by a series of dilutions from concentrated gelsolin (0.2 mg/ml), which was made from human plasma, and spotted onto nitrocellulose membranes, dried, washed, blocked, incubated with a primary monoclonal antigelsolin antibody and secondary goat anti-mouse IgG horseradish peroxidase conjugate followed by ECL detection. Each point represents the mean \pm SD of three independent experiments.

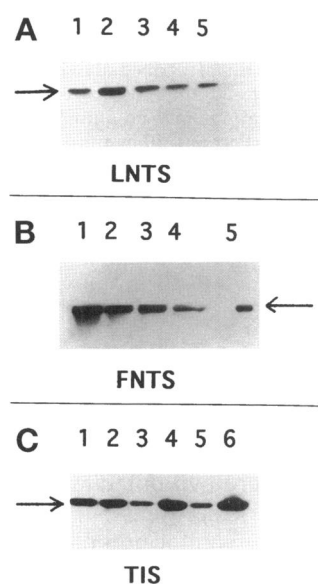


Figure 2. Actin changes in Triton X-100-soluble and -insoluble fractions of adherent and suspended neutrophils. The samples (10 μ g) from each fraction were analyzed by SDS-PAGE and Western detection as detailed in MATERIALS AND METHODS. Arrow, 42-kDa molecular weight marker. (A) Lane 1 is the purified actin band from rabbit skeletal muscle; lanes 2-4, Triton X-100-soluble fractions of 1-, 5-, and 10-min LN-adherent cells; and lane 5, Triton X-100-soluble fraction of suspended cells. (B) Lanes 1-3 represent Triton X-100-soluble fractions of 1-, 5-, and 10-min FN-adherent cells; lane 4, Triton X-100-soluble fraction of suspended cells; and lane 5, purified actin from rabbit skeletal muscle. (C) Lane 1 is the purified rabbit skeletal muscle actin

band; lane 2, Triton X-100-insoluble fraction of suspended cells; lanes 3 and 4, Triton X-100-insoluble fractions of 1- and 10-min LN-adherent cells; and lanes 5 and 6, Triton X-100-insoluble fractions of 1- and 10-min FN-adherent cells. FNTS, Triton X-100-soluble fractions of FN-adherent cells; LNTS, Triton X-100-soluble fractions of LN-adherent cells; TIS, Triton X-100-insoluble fractions of LN- and FN-adherent cells.

posed to suspended cells at 1 min, which increases to approximately control by 10 min. These results are consistent with our previous observations (Ginis *et al.*, 1992; Wang *et al.*, 1993) that adhesion of neutrophils to extracellular proteins initiates transient mobilization of F-actin into a G-actin pool which then serves as a source for repolymerization of actin.

Regulation of Actin Depolymerization

We have previously shown that the actin depolymerization event was not affected by the G-protein-inhibiting agent, pertussis toxin, or the protein kinase C inhibitors, H-7 or staurosporine (Wang *et al.*, 1993). These studies were extended by examining the behavior of LN- and FN-adherent neutrophils that had been treated with the potent nonreceptor tyrosine kinase inhibitor herbimycin A, at a concentration of 5 μ g/ml, and again found no statistically significant difference in actin depolymerization (our unpublished results). Thus, actin depolymerization in the adherent neutrophil does not appear to be controlled by the more well-studied components of the signal transduction pathway. In an attempt to gain insight into the control of this phenomena, it seemed reasonable to determine whether the quantitative changes in actin were a result of alterations of the distribution of actin-associated proteins between the Triton X-100-soluble and -insoluble fractions of the neutrophil. Since α -actinin may be

responsible for stabilizing the actin network in the neutrophil (Cano *et al.*, 1992) and gelsolin can cut and shorten actin filaments, we examined these proteins by Western blotting of SDS-PAGE. Although no α -actinin was found in the soluble fraction of either suspended or adherent neutrophils, we did find gelsolin in the Triton X-100-insoluble fraction of adherent but not suspended neutrophils (our unpublished results). To determine whether the latter observation could be relevant to adherence-induced actin depolymerization, we quantitated the translocation.

Quantitation of Gelsolin Translocation into F-Actin Fractions in the Process of Adhesion to LN- and FN-Coated Surfaces

We quantitated the amount of gelsolin present in the different fractions by pipetting samples directly onto Immobilon-P (dot-blot method), followed by incubation with anti-gelsolin antibody, and then detecting the antigelsolin antibody by means of the ECL system. The dots were scanned with a Shimadzu CS-930 dual wavelength scanner. The dot-blot method was used rather than Western blotting of SDS-PAGE because of the ease of controlling the amount of the total protein and the volume in each fraction; avoidance of artifacts which can arise from incomplete solubilization of the samples, especially the insoluble cytoskeletal (F-actin) pool; the elimination of variable transfer of the protein from the SDS gel to the membrane; and finally dot-blotting allows a direct comparison to be made between the cellular fractions and the purified standard on the same membrane. The values obtained were quantitated by means of a calibration curve obtained from pure gelsolin (Figure 1). As can be seen in the representative dot-blot shown in Figure 3, there is little gelsolin present in the Triton X-100-insoluble fraction of neutrophils, but upon adherence to either a LN- or FN-coated surface there is a significant translocation of gelsolin from the soluble to the insoluble fraction. The blot shown was exposed for a longer time than those used for quantitation to show the lack of gelsolin in the Triton X-100-insoluble fraction of

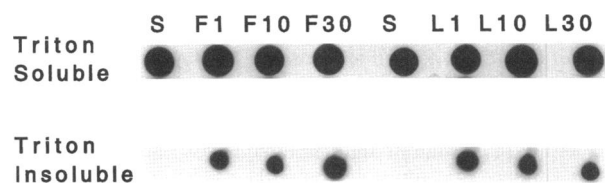


Figure 3. Dot-blot analysis gelsolin from fractions of adherent and suspended neutrophils. Lanes 1 and 5 are the fractions from suspended cells; lanes 2-4 are the fractions from 1-, 10-, and 30-min LN-adherent cells; and lanes 6-8, the fractions of 1-, 10-, and 30-min FN-adherent cells. The top row is the Triton X-100-soluble fraction and the bottom row is the Triton X-100-insoluble cytoskeleton.

Table 1. Gelsolin distribution in fractions of adherent and suspended human neutrophils

Fractions	% Total protein	% Gelsolin
Suspended		
Triton X-100 soluble	0.61 ± 0.09	89.1 ± 13.3
Triton X-100 insoluble	0.07 ± 0.02	10.9 ± 2.7
Adherent		
LN, 1 min		
Triton X-100 soluble	0.83 ± 0.14	63.7 ± 11.2
Triton X-100 insoluble	0.47 ± 0.12	36.3 ± 7.1 ^a
LN, 10 min		
Triton X-100 soluble	0.73 ± 0.18	60.3 ± 12.1
Triton X-100 insoluble	0.48 ± 0.13	40.7 ± 2.7 ^a
LN, 30 min		
Triton X-100 soluble	0.50 ± 0.09	43.2 ± 7.9 ^a
Triton X-100 insoluble	0.66 ± 0.15	56.8 ± 13.3 ^a
FN, 1 min		
Triton X-100 soluble	0.60 ± 0.08	59.7 ± 7.6
Triton X-100 insoluble	0.40 ± 0.12	40.3 ± 10.6 ^a
FN, 10 min		
Triton X-100 soluble	0.71 ± 0.14	60.0 ± 11.2
Triton X-100 insoluble	0.48 ± 0.12	40.0 ± 8.4 ^a
FN, 30 min		
Triton X-100 soluble	0.67 ± 0.12	59.0 ± 10.1
Triton X-100 insoluble	0.47 ± 0.09	41.0 ± 7.8 ^a

Note: Data are the means ± SD of six experiments.

^a $p < 0.05$.

suspended cells. The gelsolin in the two neutrophil fractions was quantitated as described in MATERIALS AND METHODS from dot-blot and is presented in Table 1. In suspended neutrophils only 10% of the total gelsolin could be detected in the insoluble cytoskeleton. However, upon adherence, nearly 40% of the gelsolin was found in the insoluble fraction. These distributions were paralleled by a concomitant fall of the Triton X-100-soluble gelsolin from 90% of the total to 60%. The translocation occurred within the first minute of adherence and there was little change over the next 30 min. Similar results were obtained for cells adherent to both LN- and FN-coated surfaces. Although one- to twofold variations were found in the absolute amount of gelsolin detected in neutrophils from different donors, ranging from 0.6% to 1.4% of total cellular protein, the distribution of gelsolin was uniformly different between suspended and adherent cells.

Comparison of Gelsolin Distribution and G-Actin Changes in LN- and Plastic-adherent Human Neutrophils

We have previously shown that there is no depolymerization of F-actin in neutrophils adherent to plastic in contrast to cells adherent to FN- or LN-coated surfaces. It was therefore of interest to compare the gel-

solin translocation observed for neutrophils adherent to FN and LN to plastic. The results obtained from dot-blot similar to that shown in Figure 3 are presented in Table 2. Almost all of the gelsolin (> 95%) was found in the Triton X-100-soluble fraction of cells adherent to plastic, and little was detected in the F-actin fractions. However, in matched experiments, significant amounts of gelsolin were found in the F-actin fractions of cells adherent to LN. These data imply that the change in gelsolin partitioning which occurs upon adhesion to either LN-coated or FN-coated surfaces is a specific response to adhesion to these substrates and not a result of adhesion per se.

Immunofluorescence Microscopy of Adherent Neutrophils

The differences between cells adherent to LN-coated plastic and uncoated plastic suggested that it would be of interest to determine whether there was a difference in morphology and/or gelsolin staining between these cell preparations when examined by microscopy. Photomicrographs of neutrophils that had been extracted with Triton X-100 containing PBS after adhering to either LN-coated or uncoated plastic for 10 min, but prior to fixation with Formalin, then stained for gelsolin using an antigelsolin antibody as described in MATERIALS AND METHODS, are shown in Figure 4. It can be seen under phase contrast that the cells adherent to plastic have a better defined nucleus and a faint lamellipodia surrounding them. The cells adherent to LN are more rounded and the nucleus is difficult to discern. This representation may reflect the fact that there is less F-actin in the latter

Table 2. Gelsolin distribution in fractions of plastic and LN-adherent human neutrophils

Fractions	% Total protein	% Gelsolin
Plastic adherence		
1 min		
Triton X-100 soluble	1.02 ± 0.09	98.7 ± 0.5
Triton X-100 insoluble	0.02 ± 0.003	1.3 ± 0.7
10 min		
Triton X-100 soluble	1.11 ± 0.18	98.5 ± 1.4
Triton X-100 insoluble	0.15 ± 0.01	1.5 ± 0.7
Laminin adherence		
1 min		
Triton X-100 soluble	0.44 ± 0.08	58.4 ± 5.2 ^a
Triton X-100 insoluble	0.32 ± 0.05	41.6 ± 5.8 ^b
10 min		
Triton X-100 soluble	0.47 ± 0.07	61.7 ± 4.7 ^a
Triton X-100 insoluble	0.30 ± 0.04	38.3 ± 5.02 ^b

Note: Data are the means ± SD of four experiments.

^a $p < 0.05$.

^b $p < 0.01$ (analysis of variance) compared with the amount of gelsolin in the fractions of suspended cells.

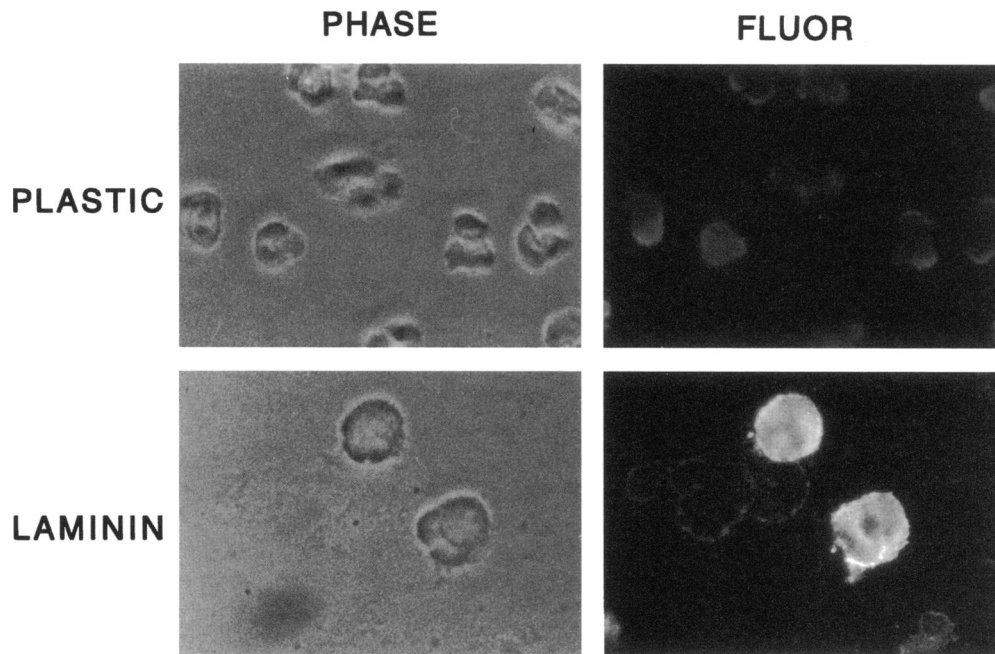


Figure 4. Phase contrast and immunofluorescence of adherent neutrophils. Neutrophils were allowed to adhere to either plastic or LN-coated plastic. Cells were stained for gelsolin with antigelsolin antibody as described in MATERIALS AND METHODS.

cells, and when the membrane is permeabilized with detergent the nucleus is less well anchored and is washed away, since permeabilization is performed prior to fixation. The cells adherent to LN show intense staining for gelsolin as compared with those adherent to plastic, consistent with the biochemical data. The stained material probably represents gelsolin bound to a layer of F-actin which remains adherent to the surface after washing. The faint circles of staining for the cells adherent to LN, which are not seen in the corresponding phase-contrast frames, probably represent cells where all that is remaining is a tightly adherent rim of the F-actin cytoskeleton with bound gelsolin.

DISCUSSION

A dynamically structured actin-containing cytoskeleton is considered to be important to the mechanism by which cells move. We have previously observed that there is a net depolymerization of actin when neutrophils adhere to LN-coated or FN-coated surfaces. In the present study, we have confirmed the previous results and have begun to explore the biochemical mechanism by which this occurs. We have found that this phenomenon was not inhibited by pertussis toxin and is thus likely not linked to adhesion by a G-protein pathway as is stimulation of actin polymerization by formyl-methionylleucylphenylalanine (Bengtsson *et al.*, 1990). In addition, we found no inhibition of this phenomenon by the presence of known inhibitors of protein kinase C, which has been shown to be activated upon cell adhesion (Vuori and Ruoslahti, 1993), nor by an inhibitor of

tyrosine kinase, which is activated by integrin receptor binding in the platelets (Haimovich *et al.*, 1993). We therefore decided to pursue a different approach and began to explore the cytoskeletal distribution of actin-binding proteins, which are potent regulators of F-actin in vitro, and found that although adhesion had no effect on α -actinin, which is thought to be associated with integrins (Oteg *et al.*, 1990), there was a significant translocation of gelsolin.

We quantitated the distribution of gelsolin between the Triton X-100- soluble and -insoluble fraction and found that nearly one-half of the cellular gelsolin is found in the actin cytoskeleton after the neutrophil adheres to a LN-coated or FN-coated surface, and, moreover, this event occurs within the first minute of adherence. This effect appears to be a specific consequence of adherence to a LN-coated or FN-coated surface since the translocation is not observed when neutrophils adhere to uncoated plastic surfaces.

It is important to attempt to quantitate the amount of actin depolymerization that occurs concomitant to the gelsolin translocation. From the data presented in Table 1 and the fact that there is about 50 pg of protein per cell in the neutrophil (White *et al.*, 1983; Ginis *et al.*, 1992; Zaner *et al.*, 1992), it can be calculated that about 1.3×10^6 molecules of gelsolin translocate to the insoluble cytoskeleton upon adherence. We have previously shown that the G-actin pool increases by about 6 pg/100 pg protein (Wang *et al.*, 1993) or 4.2×10^7 molecules per cell in the adherent neutrophil, from which it can be calculated that about 32 actin monomers depolymerize for each gelsolin that binds to the insoluble actin net-

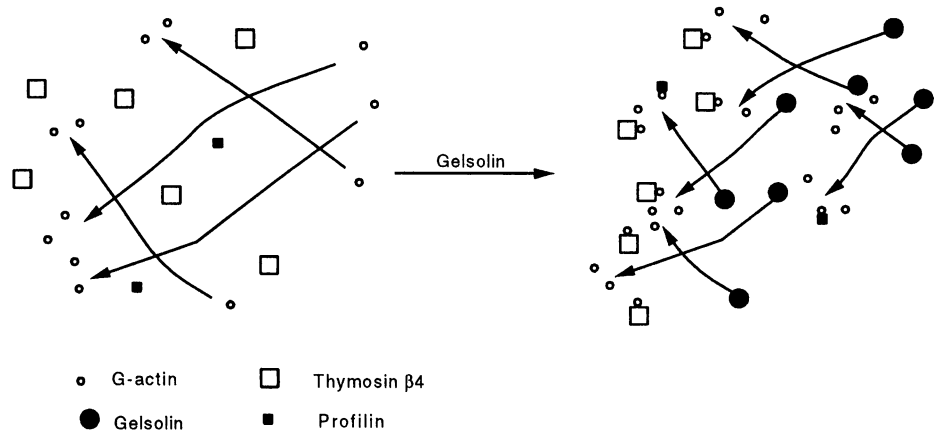


Figure 5. Scheme for the depolymerization of actin by gelsolin binding to the cytoskeleton.

work within 1 min of adherence. This could occur if a gelsolin molecule cut an actin filament that was part of the actin network, then bound to the newly exposed barbed end and created a free pointed end. It has been shown that the Triton X-100-insoluble network of the neutrophil is difficult to dissociate, probably because of α -actinin stabilization (Cano *et al.*, 1992), so that a gelsolin molecule could cut and cap an actin filament which is part of the network, remaining bound to the insoluble cytoskeleton, thereby generating a new pointed end, as shown schematically in Figure 5. Since, from *in vitro* data, the dissociation rate of actin from the pointed end is 0.8 s^{-1} (Pollard, 1986), 48 monomers can dissociate from a single pointed end in 1 min if the free monomer concentration in the cytoplasm is zero. However, since the free monomer concentration is higher than zero, it is necessary to include the fact that the addition of monomers will decrease the amount of actin liberated in 1 min. By using the rate constant for addition of monomers to the pointed end, k_{+p} , of $1.3 \mu\text{M s}^{-1}$ (Pollard, 1986), it can be calculated that 35, 25, or 9 monomers could dissociate in 1 min with free monomer concentrations of 0.17 (the monomer concentration for both ends free), 0.3, or $0.5 \mu\text{M}$. Therefore, the translocation of gelsolin can explain some or most of the observed actin depolymerization, although further independent measurement of the concentration of free actin would be required to definitively establish this result. Another consideration is whether there is enough thymosin β_4 to bind all of the liberated actin. The increase in actin of 4.2×10^7 molecules per cell is within the range of concentrations of thymosin β_4 , although at the high end (Cassimeris *et al.*, 1992; Safer *et al.*, 1992), implying that there may be enough thymosin β_4 to bind to the liberated actin, but there may be other factors involved.

We have observed in this report that the depolymerization of F-actin is associated with a translocation of gelsolin to the cytoskeleton. Whether the translocation is responsible for depolymerization and the interplay with other proteins such as thymosin β_4 or capping

protein- β_2 , recently reported to be an important barbed end capping protein in neutrophils (DiNubile *et al.*, 1995), is yet to be determined. In contrast to actin, where there is a time-dependent repolymerization, the gelsolin remains in the Triton X-100-insoluble fraction of the cell. This is consistent with *in vitro* data which have shown that actin-gelsolin binding is generally irreversible (Kurth and Bryan, 1984) and can only be dissociated by phosphatidylinositol 4,5-bisphosphate (Janmey and Stossel, 1987). It is conceivable that other cell stimulants can induce a dissociation of gelsolin from the cytoskeleton and also physiologically plausible, since the adherence of the neutrophil to an ECM is only the first phase of its journey to the sites of infection. The mechanism by which gelsolin is induced to dissociate from the actin cytoskeleton is of interest for further study.

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