Role of Gelsolin Interaction with Actin in Regulation and Creation of Actin Nuclei in Chemotactic Peptide Activated Polymorphonuclear Neutrophils

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In vitro Ca^{++} activates gelsolin to sever F-actin and form a gelsolin-actin (GA) complex at the + end of F-actin that is not dissociated by ethylene glycol-bis(β -aminoethyl ether)- N,N,N',N' -tetraacetic acid (EGTA) but is separated by EGTA + PIP/PIP_2 . The gelsolin blocks the + end on the actin filament, but the - end of the filament can still initiate actin polymerization. In thrombin activated platelets, evidence suggests that severing of F-actin by gelsolin increases GA complex, creates one - end actin nucleus and one cryptic + end actin nucleus per cut, and then dissociates to yield free + ends to nucleate rapid actin assembly. We examined the role of F-actin severing in creation and regulation of nuclei and polymerization in polymorphonuclear neutrophils (PMNs). At 2-s intervals after formyl peptide (FMLP) activation of endotoxin free (ETF) PMNs, change in GA complex was correlated with change in + end actin nuclei, - end actin nuclei, and F-actin content. GA complex was quantitated by electrophoretograms of proteins absorbed by antigelsolin from cells lysed in 10 mM EGTA, + end actin nuclei as cytochalasin (CD) sensitive and - end actin nuclei as CD insensitive increases in G-pyrenyl actin polymerization rates induced by the same PMNs, and F-actin content by NBDphalloidin binding to fixed cells. Thirty three percent of gelsolin was in GA complex in basal ETF PMNs; from 2-6 s, GA complexes dissociate (low = 15% at 10 s) and sequentially + end nuclei and F-actin content and then - end nuclei increase to a maximum at 10 s. At >40 s GA complex increase toward basal and + end nuclei and F-actin content returned toward basal. These kinetic data show gelsolin regulates availability of + end nuclei and actin polymerization in FMLP. However, absence of an initial increase in GA complex or - end nucleating activity shows FMLP activation does not cause gelsolin to sever F- or to bind G-actin to create cryptic + end nuclei in PMNs; the results suggest the + nucleus formation is gelsolin independent.

INTRODUCTION

Gelsolin is a 90 kilodalton actin binding/regulatory protein. Interactions of gelsolin with actin in vitro are regulated by Ca^{++} and PIP_2 (Yin and Stossel, 1979; Yin *et al.*, 1981b; Janmey *et al.*, 1985, 1987; Janmey and Stossel, 1987). In vitro micromolar Ca^{++} activates gelsolin's ability to sever filamentous (F) actin, cap the barbed (+) end of F-actin, and nucleate globular (G) actin polymerization at the pointed end of the filament. All three actions create actin filaments or actin nuclei that are polymerization competent at the pointed end but not the barbed end (Bryan and Coluccio, 1985) and create stable one mole gelsolin: one mole actin com-

plexes [gelsolin-actin (GA) complexes] that are resistant to dissociation by Ca^{++} chelation with ethylene glycol-bis(β -aminoethyl ether)- N,N,N',N' -tetraacetic acid (EGTA) (Chaponnier *et al.*, 1987b) but are sensitive to dissociation by PIP_2 micelles (Janmey and Stossel, 1987; Janmey *et al.*, 1987). Furthermore, gelsolin is present in blood cells, including platelets (Lind *et al.*, 1982) and polymorphonuclear neutrophils (PMNs) (Yin *et al.*, 1981). In these cells increase in $[\text{Ca}^{++}]_i$ and phosphoinositide turnover (Pozzan *et al.*, 1983; Cockcroft *et al.*, 1985; Lapetina, 1990; Stephens *et al.*, 1991) accompanies ligand activated actin polymerization at the barbed end of actin filaments (Rao and Varani, 1982; Fechtner and Zigmond, 1983; Fox and Phillips, 1983; How-

ard and Meyer, 1984; Howard and Oresajo, 1985a,b; Wallace *et al.*, 1985), and GA complex dissociation temporally correlates with F-actin growth in these cells (Lind *et al.*, 1987; Howard *et al.*, 1990a). The in vitro effects of gelsolin on creation and regulation of actin nuclei paired with gelsolin's behavior in blood cells suggest that gelsolin is an excellent candidate to regulate actin polymerization in platelets and PMNs.

Surprisingly, however, reported studies of GA interactions in platelets and PMNs suggest the actions of gelsolin in the two cells are not identical (Lind *et al.*, 1987; Howard *et al.*, 1990a). First, the low levels of EGTA resistant 1:1 GA complex in basal platelets contrast to the high levels of complex in basal Ficoll-Hypaque purified PMNs (FH-PMNs). Second, the kinetic changes in GA complexes in ligand activated platelets and PMNs differ. In thrombin activated platelets that experience a Ca^{++} transient GA complexes form rapidly and then dissociate concurrent with an increase in cytoskeleton associated actin (Lind *et al.*, 1987). This observation suggests Ca^{++} dependent severing of F-actin by gelsolin simultaneously creates new GA complexes and cryptic barbed-end nuclei that are unmasked as GA complexes dissociate (Lind *et al.*, 1987); however, no direct measures of barbed- or pointed-end nucleating activity to support the suggestion were reported.

In contrast, after chemotactic peptide activation of FH-PMN, which compared to platelets have an elevated basal level of GA complex, there is no further increase in GA complex levels above basal and rapid dissociation of GA complex is associated with increase in barbed-end nucleating activity and growth of F-actin (Howard *et al.*, 1990a). This suggests that in FH-PMNs gelsolin severing of F-actin does not create barbed-end nuclei. The distinctions between GA interactions in platelets and PMNs are consistent with either of two possibilities: 1) gelsolin's role in creation and regulation of actin nuclei differs in PMNs and platelets, i.e., gelsolin creates and regulates barbed-end nuclei in platelets but only regulates the barbed ends of filaments in PMNs; or 2) as previously suggested (Lind *et al.*, 1987; Howard *et al.*, 1990a), gelsolin creates barbed-end nuclei similarly in both cells, and the elevated basal GA complex in FH-PMN simply reflects a prior Ca^{++} transient that caused gelsolin dependent nucleus creation during FH-PMN preparation.

To directly determine whether GA interactions in PMNs differ significantly from those in platelets and whether Ca^{++} dependent gelsolin severing of F-actin or nucleation with G-actin creates as well as regulates barbed-end nuclei in PMNs, a minimally activated PMN, the endotoxin free PMN (ETF-PMN) was prepared (Howard *et al.*, 1990b). Because, unlike the FH-PMN, the ETF-PMN has low basal levels of GA complex, this cell can clearly show evidence of increases as well as decreases in GA complex and could demonstrate sequential formation and dissociation of GA complex

as reported in platelets (Fox and Phillips, 1983), if such occurs. Therefore, in formyl peptide (FMLP) activated ETF-PMN, the time course for changes in F-actin content, GA complex, barbed-end and pointed-end nucleating activity was measured to determine whether gelsolin creates or simply regulates the barbed-end nuclei in PMNs. The effect of gelsolin on G- or F-actin was documented by measurement of EGTA-resistant GA complex and by the amount of barbed-end and pointed-end nucleating activity in PMNs. An increase in GA complex would reflect Ca^{++} dependent severing of F-actin by gelsolin or Ca^{++} activated binding of gelsolin to G-actin to create pointed-end nuclei. Based on the in vitro behavior of gelsolin, each cut of F-actin by gelsolin or each gelsolin bound to an actin monomer creates one pointed-end nucleus and one cryptic barbed-end nucleus (Yin *et al.*, 1981; Bryan and Coluccio, 1985); similarly, each dissociation of one GA complex would uncap one cryptic barbed-end nucleus (Yin *et al.*, 1981; Bryan and Coluccio, 1985). Results of these studies demonstrate three points. First, the time course for formation/dissociation of GA complex in ETF-PMN differs from that in platelets and is similar to that in FH-PMNs. Specifically, dissociation of GA complex occurs concurrent with increase in barbed-end nucleating activity and actin polymerization. Second, unlike in platelets, in ETF-PMNs there is no initial net increase in GA complex after PMN activation. Third, in ETF-PMNs there is no increase in pointed-end nucleating activity before GA complex dissociation. These results show that 1) the dynamics of GA interactions differ in platelets and PMNs, 2) gelsolin capping of actin filaments regulates the availability of barbed-ends for growth in PMNs, and 3) because neither increase in GA complex or pointed-end nucleating activity occurs, Ca^{++} activated gelsolin-mediated severing of F-actin or binding to G-actin does not create cryptic barbed-end nuclei in ETF-PMNs. These results do not exclude a role for gelsolin-actin interactions in regenerating barbed-end nucleating activity in PMNs; however, they exclude a role for gelsolin-actin interactions in the initial creation of nuclei in this dynamic nonmuscle cell.

MATERIALS AND METHODS

Human PMNs were prepared ETF as previously described by Haslett (Haslett *et al.*, 1985), Chaponnier (Chaponnier *et al.*, 1987), and Howard (Howard *et al.*, 1990b) over Percoll with exposure to <12 pg/ml of lipopolysaccharides by *Limulus* amoebocyte assay. Cells were 97–99% neutrophils and were utilized within 1.5 h of preparation. Cells were studied in Hanks N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid balanced salt solution (H/HBSS) as previously described (Howard *et al.*, 1990b). The preparation was made at room temperature. The cells contain $<1\%$ polarized cells and 98% absolutely round cells as described (Howard *et al.*, 1990b).

Preparation of Triton-insoluble Cytoskeletons and Triton-soluble Supernatants

These experiments were done as previously described (Chaponnier *et al.*, 1987b; Howard *et al.*, 1990a). Briefly, cells in H/HBSS were

incubated for 10 min at 37°C and then exposed either to FMLP (Sigma Chemical, St. Louis, MO) or to dimethyl sulfoxide (DMSO) ≤ 0.0001 vol% (the vehicle used to dissolve FMLP) for various times by adding an equal volume of HBSSG containing DMSO or FMLP. Reactions were terminated by addition of an equal volume of a lysing buffer (2.0% Triton X-100, 80 mM KCl, 20 mM EGTA, 20 mM imidazole HCl, 7 mM diisopropyl fluorophosphate, pH 7.15). Cells were vortexed and immediately centrifuged at $16\,700 \times g$ for 1 min to separate Triton-soluble supernatants and Triton-insoluble cytoskeletons. Cells were incubated for 10 min with lysis buffer, centrifuged, and the supernatants were immediately used to quantify gelsolin-actin complexes.

To eliminate variations between cell preparations, determinations of F-actin content, nucleating activity, gelsolin-actin complex, and cytoskeleton-associated actin were measured concurrently on the same cell preparation. For F-actin content and nucleating activity, a 0.5-ml aliquot of cells was activated by brisk addition of $2\times$ final FMLP or DMSO in HBSSG buffer and fixed with formalin at indicated time intervals. The zero time point represents simultaneous addition of formalin and FMLP. The order of timed samples was varied to assure reproducibility. Each timed sample was then split into two aliquots, one for F-actin determination and one for nucleating activity from fixed cells. For GA complex and F-actin analysis after 10-min incubation at 37°C, a separate 0.125-ml aliquot of cells was activated by addition of 0.125 ml of $2\times$ FMLP or DMSO in HBSSG and, at 2-s intervals, 0.250 ml of lysis buffer containing diisopropyl fluorophosphate (DFP) was briskly added and then separated into Triton-soluble supernatants and Triton-insoluble cytoskeletons as noted above. Order of samples with respect to duration of exposure to FMLP was varied to assure reproducibility.

Quantification of F-Actin Content by NBDPhalloidin Binding

The F-actin content was determined as previously described by Howard *et al.* (Howard *et al.*, 1990a). Briefly, 2×10^6 neutrophils were exposed to FMLP or its solvent DMSO for the indicated times at 37°C, fixed with 3.7% formalin for 15 min at 37°C, and then permeabilized with lysophosphatidylcholine and stained with NBDphalloidin (3.3×10^{-7} M; Molecular Probes, Junction City, OR) at 25°C for 10 min. Cells were analyzed on FACStar flow cytometer (Becton Dickinson, Mountain View, CA) as previously described (Howard and Oresajo, 1985b). The results are expressed as the relative F-actin content indicated by mean fluorescence channel number of test sample/mean fluorescence channel number of cells simultaneously exposed to FMLP and fixative at 0 s. The values presented are the means $\pm$ SD from multiple trials.

Measurement of the Quantity of Gelsolin Bound to Actin in EGTA-resistant 1:1 Complex

These assays were done as previously described (Chaponnier *et al.*, 1987; Howard *et al.*, 1990a). Gelsolin and GA complexes in supernatants were immunoabsorbed onto Sepharose beads conjugated with a monoclonal antigelsolin antibody (Chaponnier *et al.*, 1987a) by mixing in Eppendorf tubes (Brinkmann Instruments, Westbury, NY) at 4°C on a rotary shaker (Scientific Industries, State College, PA). After 2 h, the beads were centrifuged at $15,000 \times g$ for 2 min washed successively with 1 ml of $1\times$ lysing buffer, 1 ml of 0.3 M $MgCl_2$, 1 mM EGTA in PTBS (10 mM tris(hydroxymethyl)aminomethane, 100 mM NaCl, pH 7.4) and 1 ml of TBS, pH 7.4. The washed beads were boiled in solubilizing buffer and analyzed on 5–15% sodium dodecyl sulfate-polyacrylamide gels. The intensities of the gelsolin and actin bands were measured by laser scanning densitometer (Ultrascan, LKB Instruments, Piscataway, NJ), and their relative molar ratios were calculated as previously described (Chaponnier *et al.*, 1987). The results are expressed as moles of actin per mole of gelsolin at each indicated time, such that a value of 1.0 means all gelsolin is bound to actin in

an EGTA-resistant 1:1 gelsolin complex/GA complex; a value of 0.5 means one half of cellular gelsolin is bound to actin in GA complex.

Repeated control experiments revealed that the duration (2–10 min) of cell exposure to lysis buffer did not alter the amount of GA complex observed and that, during 30 min of incubation of resting cells at 37°C, the amount of GA complex was constant. The presence or absence of Ca^{++} or Mg^{++} in the original cell buffer did not alter the amount of GA complex, and the quantity of GA complex in cells is not altered by addition of DMSO (<0.0001 vol%). Repeated trials also showed that immunoabsorption of Triton soluble supernatants with antigelsolin beads removed $\geq 95\%$ of the gelsolin, as evidenced by the absence of gelsolin in the postabsorption Triton-soluble supernatant fluids and by the inability to absorb additional gelsolin onto monoclonal antigelsolin-coated beads from pre-absorbed Triton soluble supernatants.

Quantification of Barbed- and Pointed-End Nucleating Activity in Neutrophils

Actin nucleating activity in PMNs was quantified with a rabbit muscle pyrenyl actin (Kouiyama and Mihashi, 1981) indicator system similar to that described by Carson *et al.* (1986) but modified by cell fixation to allow 2-s time resolution of nucleating activity. Rabbit muscle actin was purified by polymerization/depolymerization cycles (Spudich and Watt, 1971) and column purified over Sephadex G150 (Pharmacia, Piscataway, NJ) to remove endogenous nucleating activity (Cassella and Maack, 1987). Cells were exposed to FMLP or DMSO and fixed at various times with 3.7% formaldehyde for 10 min as described. One half of the fixed cells were used for F-actin determination. To measure barbed-end nucleating activity, the remainder of fixed cells were gently washed free of formaldehyde, resuspended in HBSSG, and added to a pyrenyl actin indicator solution at 30°C that contained 1.0–1.5 μ M G-pyrenyl actin, 0.1 vol% Triton X-100 in polymerization buffer (138 mM KCl, 2 mM $MgCl_2$, 1 mM ATP, 10 mM imidazole, pH 7.4). The 407-nm fluorescence emission of the actin solution excited at 370 nm was monitored. The G-pyrenyl actin concentration was determined from extinction coefficients of Selden and Estes (1983). The G-pyrenyl actin concentration was between the experimentally determined critical concentration for polymerization at the pointed end (2.2 μ M) and at the barbed end (0.2 μ M) in this polymerization buffer. No actin polymerization was observed for >20 min unless exogenous nuclei from neutrophils or sonicated F-actin were added. Control experiments showed that the rate of pyrenyl-actin polymerization was proportional to the number of exogenous nuclei added. For each assay, 1.2×10^6 fixed cells were added in a 2.78-ml cuvette containing G-pyrenyl actin, and the rate of actin polymerization was monitored with spectrofluorometer (SLM 8000; SLM-Aminco, Urbana, IL) at 15-s intervals. The polymerization initiated by lysed PMNs, fixed PMNs, or sonicated exogenous F-actin was completely inhibited by 10^{-6} M cytochalasin D, confirming that the pyrenyl-actin polymerization reflects growth at the barbed end of nuclei. The barbed-end nucleating activity was expressed as arbitrary fluorescence units -407 nm EM (370 nm EX)/min/ 1.25×10^6 cells. Further control experiments showed that the fixation of basal or FMLP-activated PMNs did not alter the nucleating activity in PMNs. Specifically, the nucleating activity of formaldehyde-fixed cells in the basal state or activated with 10^{-7} FMLP for 10–15 s, i.e., times when F-actin content and nucleating activity was constant (low in the basal and increased in the 10^{-7} FMLP activated cell), is the same as that of nonfixed cells. Fixation allows 2-s time resolution of nucleating activity.

Pointed-end nucleating activity in PMNs was quantified using the same pyrene actin polymerization indicator system in a manner similar to that previously described (Hartwig and Janmey, 1989). An aliquot of the fixed PMNs was added to the same pyrenyl actin indicator system except the cuvette contained 2.4 μ M G-pyrenyl actin and 10^{-6} M cytochalasin (CD). The experimentally determined critical concentration for actin polymerization at the pointed end, i.e., that in the presence of CD in this polymerization buffer, is 2.2 μ M. A range of G-actin concentrations was tested (2.2–4.8 μ M), and the lowest con-

Figure 1. (A) Time course of GA complex formation/dissociation in FMLP-activated ETF-PMNs. ETF-PMNs exposed to 10^{-7} FMLP at 37°C were assayed as previously described (Howard *et al.*, 1990a) for 1:1 EGTA-resistant GA complex at times after activation. Results are expressed as moles actin:moles gelsolin such that a value of 1.0 means 100% SD from $n = 7$ trials. Control (DMSO only) (○); FMLP-activated (●). (B) Time course of F-actin content in FMLP-activated ETF-PMNs. An aliquot of the same ETF-PMNs in A exposed to 10^{-7} M FMLP at 37°C was assayed for F-actin content by NBD phalloidin binding (Howard and Meyer, 1984) at indicated times after activation. Results are expressed as F-actin content of activated PMN (●) relative to basal cells (○). Values are means $\pm$ SD from seven trials. (C) Time course of barbed-end nucleating activity in FMLP-activated ETF-PMNs. An aliquot of the same ETF-PMNs in A exposed to 10^{-7} M FMLP at 37°C was assayed for barbed-end (+ end) nucleating activity

centration was selected because it assured detectable nucleating activity by exogenous pure actin nuclei exposed to CD. All barbed-end nucleating activity from PMN or exogenously added pure actin nuclei was inhibited by the 10^{-6} M CD in the presence of $1.5 \mu\text{M}$ G-pyrenyl actin. Results are expressed as arbitrary fluorescence units/min/ 1.25×10^6 PMN.

RESULTS

GA Interactions in Basal ETF-PMNs

The F-actin content of basal ETF-PMN is similar to that of PMN circulating in peripheral blood (Howard *et al.*, 1990a). In comparison to PMN purified with FH (Boyum, 1963) or by other standard methods, ETF-PMN are the least activated purified PMN available for study of cytoskeletal dynamics. FH-PMN are more motile, produce more superoxide, and exhibit greater FMLP-induced actin polymerization than ETF-PMN (Howard *et al.*, 1990b). Furthermore, GA interactions differ both qualitatively and quantitatively in the basal FH-PMN and ETF-PMN. As shown in Figure 1A, the amount of EGTA resistant 1:1 GA complex in basal ETF-PMN ($32 \pm 4\%$ of total cellular gelsolin is complexed to actin; means $\pm$ SD, $n = 5$) is lower than that reported in basal FH-PMN ($82 \pm 11\%$, means $\pm$ SD, $n = 5$) (Howard *et al.*, 1990a). The level of GA complex in ETF-PMN is similar to the level in basal platelets (Lind *et al.*, 1987). The actin:gelsolin ratio in Figure 1A is measured by the GA complex immunoabsorption assay (see MATERIALS AND METHODS) and is established such that a ratio of 1.0 means 100% of the gelsolin in the cell is bound to actin in a 1:1 EGTA resistant GA complex and a ratio of 0.5 means 50% of cellular gelsolin is complexed with actin. The amount of GA complex in ETF-PMN is not altered by exposure to DMSO, the carrier solvent for PMN activators, and, unlike basal platelets (Lind *et al.*, 1987), the level of GA complex remains constant in PMN incubated for up to 30 min at 37°C in vitro.

GA Interactions in FMLP Activated ETF-PMN

After FMLP activation, PMNs experience a $[\text{Ca}^{++}]_i$ transient (Pozzan *et al.*, 1983; Sullivan *et al.*, 1989) and modulate GA interactions (Howard, *et al.*, 1990a). Because ETF-PMNs have a low basal level of GA complex, they can exhibit either an increase or decrease in level of GA complex after activation. FMLP activated ETF-PMNs were studied to determine whether, as suggested in platelets, gelsolin severing of F-actin or binding to G-actin increases GA complex to create cryptic barbed-end nuclei or whether gelsolin simply regulates barbed-end nuclei and filament growth by uncapping and capping barbed-end nuclei. To describe the time course of

(Howard *et al.*, 1990a) at indicated times after activation with pyrene actin assay. Values shown are means $\pm$ SD of seven trials. Control (DMSO only) (○); FMLP-activated (●).

changes in GA complex in FMLP activated ETF-PMN, the fraction of total cellular gelsolin bound in 1:1 EGTA resistant GA complex (expressed as mole actin:mole gelsolin) (Chaponnier *et al.*, 1987b; Howard *et al.*, 1990a) was determined at 1- to 2-s intervals after 10^{-7} M FMLP activation at 37°C. The 10^{-7} M FMLP concentration is required for maximum actin polymerization in ETF-PMN (Howard *et al.*, 1990b).

As shown in Figure 1A, unlike reported changes in platelets (Lind *et al.*, 1987), the amount of GA complex in FMLP activated ETF-PMN never increases above basal during 5 min of observation. Even when GA complex is quantified at 1-s intervals during the first 4 s of activation, no increase in GA complex is observed. However, after 2–3 s of activation, the fraction of gelsolin bound in 1:1 GA complex steadily declines, reaches a minimum of (0.16%) 10 s after activation, and then returns to basal levels (0.29%) after 5 min. Figure 1, B and C shows concurrent changes in F-actin content and barbed-end nucleating activity determined simultaneously on the same preparations of ETF-PMNs. During an initial 3-s lag, as previously described (Kurth and Bryan, 1984; Howard *et al.*, 1990b), F-actin content remains constant as does the amount of GA complex. A subsequent decline in GA complex, which reflects dissociation of the complex from cryptic barbed-end nuclei, coincides with a more rapid increase in barbed-end nucleating activity and F-actin content. The maximum F-actin content, maximum barbed-end nucleating activity, and nadir for GA complex occurs at 10 s after FMLP activation.

Concurrent dissociation of GA complex, increase in barbed-end nucleating activity, and growth in F-actin suggest that gelsolin regulates the availability of barbed-end nuclei and filament growth in ETF-PMNs. In addition, after 10 s of FMLP activation, there is a direct inverse relationship between the quantity of GA complex dissociated and the quantity of barbed-end nucleating activity that appears in PMNs. Specifically, the amount of gelsolin bound in 1:1 GA complex decreases from $0.37 \pm 0.03\%$ to $0.16 \pm 0.03\%$, approximately a 50% reduction. If GA dissociation is a major regulator of barbed-end nuclei in PMNs and if GA dissociation is linked to the uncapping of cryptic barbed-end nuclei, then the observed 50% reduction in GA complex predicts approximately a 2.0-fold increase in barbed-end nucleating activity. The observed 1.6- to 1.7-fold increase in barbed-end nuclei approaches the value predicted from GA complex dissociation and suggests GA interactions play an important role in regulating barbed-end nucleating activity as suggested in platelets and FH-PMNs (Lind *et al.*, 1987; Howard *et al.*, 1990a). However, quantification of absolute numbers of nuclei are required to prove the events are quantitatively balanced.

Although the time course of GA interactions in FMLP activated ETF-PMN includes dissociation of GA complex to unmask barbed-end nuclei as suggested in

platelets, the GA interactions in ETF-PMN also differ significantly from the reported GA interactions in platelets. Specifically, unlike in platelets (Lind *et al.*, 1987), the level of GA complex never exceeds basal levels in the ETF-PMN. Because Ca^{++} activation of gelsolin in macrophages and platelets (Pollard and Cooper, 1986; Chaponnier *et al.*, 1987b) or in the presence of actin in vitro (Yin *et al.*, 1981b; Bryan and Coluccio, 1985) increases GA complex and forms pointed-end nuclei through gelsolin's ability to sever F-actin or bind to G-actin (Yin *et al.*, 1981b; Bryan and Coluccio, 1985), absence of increase in GA complex paired with subsequent GA complex dissociation are consistent with a role for gelsolin in regulating, but not in creating, the barbed-end nuclei in ETF-PMNs.

To assure that FMLP activation does not significantly increase GA complex during the first 2–4 s of activation, the effect of varied FMLP concentration (10^{-11} to 10^{-7} M) on the amount of GA complex in ETF-PMNs at 1, 2, 3, 4, and 10 s after activation was determined. As shown in Figure 2, the amount of GA complex present 2 s after FMLP (1, 3, and 4 s not shown but similar to 2 s) was not increased at any FMLP concentration, and significant decreases in GA complex after 10 s required FMLP concentrations in excess of 10^{-9} M FMLP. Therefore, absence of an observed increase in GA complex in FMLP activated cells is not unique to the 10^{-7} M FMLP activated cell but is similar regardless of FMLP concentration.

Time Course of Barbed-End and Pointed-End Nucleating Activity in FMLP Activated ETF PMNs

When in the presence of Ca^{++} , gelsolin binds to actin monomer or severs a previously existing actin filament

Figure 2. Effect of FMLP concentration on GA complex in ETF-PMNs. ETF-PMNs activated with no FMLP (□) or varied concentrations of FMLP at 37°C for 2 (●) or 10 s (○) were assayed for 1:1 EGTA-resistant gelsolin actin complex. The amount of GA complex is measured as moles actin:moles gelsolin such that a value of 1.0 means 100% of gelsolin is bound in 1:1 complex (Howard *et al.*, 1990a). Values shown are means $\pm$ SD from four trials.

and there are three consequences: 1) the amount of EGTA resistant GA complex increases (Chaponnier *et al.*, 1987), 2) the average filament length decreases (Yin *et al.*, 1981), and 3) one pointed-end nucleus and one cryptic or capped barbed-end nucleus is created for each gelsolin bound to G-actin and for each gelsolin cut of an F-actin filament. Therefore, if in PMNs or other non-muscle cells, Ca^{++} dependent severing of F-actin or nucleus formation via gelsolin and actin monomer interaction creates cryptic barbed-end nuclei, then barbed-end nucleating activity should remain constant and the quantity of pointed-end nucleating activity should increase. If cryptic barbed-end nuclei are subsequently uncapped, then barbed-end nucleating activity too should increase. Theoretically, if as suggested in platelets (Lind *et al.*, 1987), gelsolin severs F-actin and creates cryptic or gelsolin-capped barbed-end nuclei and GA complexes, then the amount of pointed-end nucleating activity should increase in FMLP activated PMNs without concurrent increase in barbed-end nucleating activity. Therefore, analysis of pointed-end nucleating activity as an additional indicator of the nature of GA interactions in FMLP activated PMNs was pursued.

To analyze barbed- and pointed-end nucleating activity, a pyrene actin polymerization assay (26) that depends on addition of exogenous nuclei was utilized (Howard *et al.*, 1990a) to simultaneously measure barbed-end (+) and pointed-end (-) nucleating activity. Barbed-end nucleating activity was assayed as the rate of $1.0 \mu\text{M}$ pyrenyl-G actin polymerization induced by PMNs in the absence of CD, and pointed-end nucleating activity was measured as the rate of CD-insensitive polymerization of $2.5 \mu\text{M}$ pyrenyl actin (Janmey and Stossel, 1987). The quantity of barbed ($1 \mu\text{M}$ actin, -CD) and pointed-end ($2.5 \mu\text{M}$ actin, +CD) nucleating activity were measured at 2-s intervals after exposure to 10^{-7} M FMLP was determined. The CD concentration utilized to measure pointed-end nucleating activity inhibits all of the barbed-end nucleating activity in 10^{-7} M FMLP activated PMNs from 0 to 10 s after activation. Two significant results are apparent in Figure 3 that plots the time course of barbed- (+) and pointed- (-) end nuclei. First, 2 s after FMLP activation, when $[\text{Ca}^{++}]_i$ increases (Wang *et al.*, 1990) and actin filaments would be severed or new nuclei formed if gelsolin creates cryptic nuclei, there is no evidence of an increase in pointed-end nucleating activity. However, at subsequent times when GA complexes dissociate, there is an increase in barbed-end, followed by a slightly slower increase in pointed-end nucleating activity that is maximal after 10 s. The changes in pointed-end nuclei occur during a period when GA complexes are dissociating. These results, like the measurements of GA complex, show that gelsolin severing of F-actin or binding to G-actin is not an initial FMLP-activated event in ETF-PMNs and that in those cells gelsolin does not initially create cryptic barbed-end nuclei that are subsequently unmasked. The results

Figure 3. Time course of changes in barbed-end and pointed-end nucleating activity in FMLP-activated PMNs. ETF-PMNs were activated with 10^{-7} M FMLP for indicated times at 37°C , and the amount of barbed-end (+) (O) and pointed-end (-) (●) nucleating activity in PMNs was determined simultaneously from a single batch of PMNs. Barbed-end (+) nuclei were measured in $1 \mu\text{M}$ pyrene actin without CD; pointed-end (-) nuclei were measured in $2.5 \mu\text{M}$ pyrene actin in the presence of 10^{-6} M CD. Results represent means $\pm$ SD on five trials of cells with nucleating activity reported as arbitrary fluorescence units/ 10^6 ETC-PMNs/min.

suggest the original gelsolin capped nuclei in basal PMNs are created either by gelsolin independent mechanism(s), by a previous net turnover of GA complexes between severing/capping and uncapping states during differentiation, or by a combination of these events. The results do not exclude the possibility that in FMLP activated PMNs net turnover of GA complexes between severing/capping and uncapping states in generation of nuclei plays a role in the regeneration of actin nuclei before subsequent ligand activations. However, they are not consistent with an initial net increase in gelsolin severing/capping to FMLP activated PMNs (Wallace *et al.*, 1985).

DISCUSSION

One critical step in regulating the state of actin polymerization in nonmuscle cells is the creation and regulation of actin nuclei (Stossel *et al.*, 1985; Pollard and Cooper, 1986). In vitro, an actin nucleus is formed by the condensation of at least two or three actin monomers and is the rate-limiting step in actin polymerization (Janmey and Matsudaira, 1988); a preformed actin filament of $>2-3$ monomers in length can also serve as a nucleus for polymerization. Like actin filaments, actin nuclei either long or short are polar. Each has a positive (+) or barbed end and a negative (-) or pointed end assigned by electron microscopic image of filaments decorated with heavy meromyosin (Stossel *et al.*, 1985; Pollard and Cooper, 1986; Korn *et al.*, 1987). The two ends differ functionally. The affinity constant of actin

monomer (G-actin) for the barbed-end is 10-fold greater than that for the pointed end (Korn *et al.*, 1987) and under identical buffer conditions the critical concentration for polymerization at the two ends differ dramatically. Because the barbed end is the preferred end for filament growth and the majority (~80%) of the ligand activated actin polymerization in nonmuscle cells like platelets and PMNs occurs on barbed-end nuclei (Rao and Varani, 1982; Fechtmeier and Zigmond, 1983; Fox and Phillips, 1983; Howard and Oresajo, 1985b; Wallace *et al.*, 1985; Lind *et al.*, 1987; Howard *et al.*, 1990a,b), the creation and regulation of barbed-end nuclei is a critical step in regulating actin polymerization in PMNs.

During the past decade, several actin binding/regulatory proteins capable of regulating monomer binding to the barbed-end of actin nuclei have been identified. These include villin, gelsolin, and gCap39 (Yin and Stossel, 1979; Southwick and DiNubile, 1986; Janmey and Matsudaira, 1988; Young *et al.*, 1990; Yu *et al.*, 1990). All three actin regulatory proteins are activated by micromolar Ca^{++} to bind actin at the barbed-end of actin filaments. However, each is otherwise unique. In vitro, villin functions as a bundling protein and does not sever actin filaments (Janmey and Matsudaira, 1988; Petrucci *et al.*, 1988); gCap39 binding to actin is reversible by Ca^{++} chelation and has limited ability to sever filaments (Southwick and DiNubile, 1986; Young *et al.*, 1990; Yu *et al.*, 1990). In contrast, gelsolin severs actin filaments, caps the barbed-end of filaments, and is not dissociated from actin by Ca^{++} chelation but only by exposure to PIP_2 micelles (Janmey *et al.*, 1985, 1987; Janmey and Stossel, 1987; Janmey and Matsudaira, 1988). Because villin is not found in platelets or PMNs, because $[\text{Ca}^{++}]_i$ is "tonically" elevated (for ≥ 5 min) (Sullivan *et al.*, 1989) in FMLP activated PMN, and because ligand activation causes both Ca^{++} transient and PIP_2 turnover (Pozzan *et al.*, 1983; Cockcroft *et al.*, 1985; Lapetina, 1990; Stephens *et al.*, 1991), we investigated the role of gelsolin as a plausible regulator and creator of barbed-end nuclei in FMLP activated neutrophils.

Several lines of evidence in platelets and PMNs clearly show that gelsolin can regulate the barbed ends of actin nuclei. Dissociation of GA complexes occurs concurrent with actin polymerization in both cells (Lind *et al.*, 1987; Howard *et al.*, 1990a) and the dissociation correlates temporally with appearance of barbed-end nucleating activity (Howard *et al.*, 1990a). These results suggest a role of nuclei regulation for gelsolin; however, the protein's role in creating barbed-end nuclei is less clearly defined. Studies in platelets suggest gelsolin severing or G-actin binding creates nuclei (Lind *et al.*, 1987). This notion is not supported by the observation of GA interactions in FH-PMN (Howard *et al.*, 1990a). However, interpretation of FH-PMN studies is confused by the high basal level of GA complex in basal FH-PMN (Wallace *et al.*, 1985).

In the studies reported here, the role gelsolin plays in both the creation and regulation of barbed-end nuclei in PMNs is examined by simultaneously assaying the amount of GA complex, barbed-end and pointed-end nucleating activity, and F-actin content in FMLP activated PMN. Specifically, experiments were designed to determine whether gelsolin binding to G-actin or severing of F-actin creates cryptic barbed-end nuclei by assaying for the quantitative changes in GA complex and seeking evidence of severing or nucleation by analysis of pointed-end nucleating activity. Increase in pointed-end nucleating activity should parallel gelsolin's effect because in vitro each filament cut or gelsolin-generated nucleus creates a new pointed end and a new cryptic barbed end. Our studies show 1) the time course of formation/dissociation of GA complexes of ETF-PMN differs from that in platelets and is similar to that of FH-PMN, 2) in ETF-PMNs there is no initial increase in GA complex after PMN activation, and 3) in ETF-PMN there is no increase in pointed-end nucleating activity before net GA complex dissociation. Therefore, the conclusions are that the dynamics of GA interaction differ in platelets and PMNs, gelsolin capping regulates barbed-end nuclei in PMNs, and gelsolin severing of F-actin or binding to G-actin does not create the cryptic barbed-end nuclei in PMNs.

Two important findings emphasize the importance of gelsolin as a regulator of the barbed ends of actin nuclei in PMNs. First, temporal correlations between dissociation of GA complex and the appearance of barbed-end nuclei link GA dissociation to barbed-end nucleus appearance. Second, quantitative agreement between the predicted increase in barbed-end nuclei (1.6- to 1.7-fold) expected from 50% reduction in GA complex in FMLP activated PMNs suggests dissociation and nucleus appearance are linked. Although PMNs have other barbed-end capping proteins, such as gCap39 (Southwick and DiNubile, 1986; Young *et al.*, 1990; Yu *et al.*, 1990), a barbed-end capping protein regulated primarily by Ca^{++} , an association of gCap39 with actin nuclei, not a dissociation like that of gelsolin, would be expected of gCap39 in the face of a persistent elevation of $[\text{Ca}^{++}]_i$ during the first 10 s (Sullivan *et al.*, 1989) after PMN activation. Therefore, complex association strongly suggests that gelsolin, if not the sole regulator of the barbed-end nuclei in PMNs, is certainly a significant regulator.

In contrast, gelsolin, though likely an important regulator of barbed-end nuclei, is not the producer of the barbed-end nuclei in FMLP activated ETF-PMNs. Our observations on GA complex in FMLP activated PMNs are inconsistent with the suggestion in platelets (Lind *et al.*, 1987) that gelsolin creates the barbed-end nuclei. Although the observations do not exclude a role for gelsolin in creating cryptic barbed-end nuclei during myeloid differentiation to mature PMNs, the observations show that in mature PMNs gelsolin does not create

the cryptic nuclei responsible for FMLP-induced actin polymerization and therefore suggest the importance of gelsolin independent mechanism for creating cryptic barbed-end nuclei.

In PMNs, gelsolin-independent mechanisms for creating nuclei could involve proteins like ponticulin, an F-actin side binding integral membrane protein of *Dictyostelium sp* described by Luna *et al.* (Luna *et al.*, 1990; Schwartz and Luna, 1988; Shariff and Luna, 1990). FMLP-dependent side binding of actin oligomers to membrane via a ponticulin-like protein could explain both the presence of cryptic barbed-end nuclei in basal cells and the increase in barbed- and pointed-end nuclei reported here (Figure 3) in the FMLP activated ETF-PMN. Other possible mechanisms could involve the de novo FMLP-dependent formation of nuclei from monomers without the involvement of gelsolin or a ponticulin-like protein. Further studies should consider a developmental role for gelsolin, the de novo creation of nuclei from monomers and the role of oligomer side-binding ponticulin-like proteins. Finally, despite the fact that gelsolin does not create the barbed nuclei in the FMLP-activated ETF-PMN, the rapid turnover of gelsolin between severing/nucleating and dissociated states could be involved in regenerating cryptic barbed-end nuclei for actin polymerization in response to subsequent ligand activations as described in C5a activated cells previously exposed to FMLP (Wallace *et al.*, 1985).

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