

# Two Pathways Through Cdc42 Couple the *N*-formyl Receptor to Actin Nucleation in Permeabilized Human Neutrophils

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**Abstract.** We developed a permeabilization method that retains coupling between *N*-formyl-methionyl-leucyl-phenylalanine tripeptide (FMLP) receptor stimulation, shape changes, and barbed-end actin nucleation in human neutrophils. Using GTP analogues, phosphoinositides, a phosphoinositide-binding peptide, constitutively active or inactive Rho GTPase mutants, and activating or inhibitory peptides derived from neural Wiskott-Aldrich syndrome family proteins (N-WASP), we identified signaling pathways leading from the FMLP receptor to actin nucleation that require Cdc42, but then diverge. One branch traverses the

actin nucleation pathway involving N-WASP and the Arp2/3 complex, whereas the other operates through active Rac to promote actin nucleation. Both pathways depend on phosphoinositide expression. Since maximal inhibition of the Arp2/3 pathway leaves an N17Rac inhibitable alternate pathway intact, we conclude that this alternate involves phosphoinositide-mediated uncapping of actin filament barbed ends.

**Key words:** Arp2/3 • actin assembly • signal transduction pathways • Rac • FMLP

## Introduction

Of human cells that translocate by crawling movements, neutrophilic polymorphonuclear leukocytes (neutrophils) are the fastest. They crawl toward diverse well-defined chemical stimuli (chemoattractants) that bind to surface receptors. Stimulation of their receptors by chemoattractants elicits extensively analyzed behavioral responses, reversible adhesion properties, and intracellular signal transduction cascades in these cells. The crawling behavior of neutrophils is medically important, because it enables these cells to contain and kill, by means of oxidants and microbicidal proteins, ubiquitous microorganisms that always infest humans. Impairments in this crawling activity predispose individuals to severe and often fatal infections (Devreotis and Zigmond, 1988; Gallin and Holland, 1999). Like other human cell crawling movements, the neutrophil's locomotion depends on the cycling of actin protein subunits between monomeric and polymeric pools, and the reversible cross-linking of the polymers into three-dimensional networks. Neutrophil signal transduction intermediates regulate (and are regulated by) the changes in actin assembly and architecture mediated by actin-binding proteins.

The most intensively studied chemoattractant, useful because of its chemical simplicity, is the *N*-formyl-methionyl-leucyl-phenylalanine tripeptide (FMLP)<sup>1</sup> (Schiffmann et al., 1975). By ligating a serpentine membrane receptor (Gerard and Gerard, 1994), it induces, among other responses important for host defense against infection (Gao et al., 1999), a several-fold increase in the proportion of polymerized neutrophil actin (Howard and Oresajo, 1985). Although this large burst of net actin assembly is not necessarily directly relevant to locomotion, it is a useful readout for linking the FMLP receptor to actin nucleation, a step that determines when and where new actin-based structures arise. Complicating the study of the linkage between signaling steps and actin turnover in neutrophils, however, is the fact that neutrophils are small, protease-rich, fragile end cells, not amenable to transfection or microinjection, techniques that have abetted investigations of this relationship in tissue culture cells. Indeed, focusing on one important actin remodeling reaction, the nucleation of actin assembly, reveals inconsistencies in the published literature.

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<sup>1</sup>Abbreviations used in this paper: F-actin, filamentous actin; FMLP, *N*-formyl methionyl leucyl phenylalanine tripeptide; GST, glutathione S-transferase; N-WASP, neural Wiskott-Aldrich syndrome family proteins; OG, *n*-octyl- $\beta$ -glucopyranoside; PIP<sub>2</sub>, phosphatidylinositol bisphosphate.

Some of the first evidence for involvement of GTPases in the regulation of cellular actin polymerization arose from research with neutrophils (Särndal et al., 1989; Downey et al., 1990). Rho family GTPases are presently the most prominent mediators of particular types of actin assembly in tissue culture cells, and of these GTPases the one most implicated in large increases in cellular actin assembly and locomotion is the Rac subfamily (Hall, 1998). The principal Rac species in neutrophils is Rac2, also clearly involved in activation of the neutrophil oxidase enzyme complex that generates microbicidal reactive oxygen radicals (Bokoch, 1995). FMLP stimulation activates Rac2 in intact neutrophils (Akasaki et al., 1999; Benard et al., 1999), and bone marrow neutrophils of Rac2 knockout mice have an almost negligible chemotactic responsiveness to FMLP, interleukin 8, and leukotriene B<sub>4</sub>. Furthermore, the Rac2 null neutrophils have marked impairment in the onset and extent of actin polymerization responses to FMLP, as well as to interleukin 8, although the comparatively delayed and less robust actin polymerization induced by G-CSF or TNF $\alpha$  is normal in these cells (Roberts et al., 1999).

Despite the abundant data supporting an intermediacy of Rac in intact neutrophil actin assembly, finding a consistent role for Rac in neutrophil actin turnover using biochemical approaches has been elusive. Arcaro (1998) reported that Rac caused complexes of actin with gelsolin to dissociate in soluble neutrophil extracts, a response that could promote actin nucleation by exposing the fast-growing (barbed) ends of actin protofilaments. Kaetanaev and Wymann (1998), however, while able to show that GTP $\gamma$ S induces actin assembly in neutrophil extracts, were unable to find any effect of Rac or Cdc42 on this reaction. In extensive investigations of actin assembly in neutrophils, Zigmond and coworkers have shown that GTP has an important role in actin nucleation activity (Zigmond et al., 1997, 1998). This nucleation activity promotes barbed orientation actin polymerization (Redmond et al., 1994). They have observed that the Rho GTPase Cdc42, which FMLP also activates in intact neutrophils (Benard et al., 1999), is a potent inducer of actin polymerization in soluble neutrophil extracts. But they have not detected any effect of Rac on this actin nucleation, although it stimulates phosphoinositide synthesis in the extracts (Zigmond et al., 1997, 1998). The linkage of Cdc42 to neutrophil actin polymerization resonates with an avalanche of recent data pointing to a pathway in which Cdc42, Wiskott-Aldrich Syndrome family proteins (WASP), and the actin-related protein (Arp2/3) complex interact to promote de novo actin polymerization in vitro and in vivo (Higgs and Pollard, 1999; Machesky and Insall, 1999; Welch, 1999). While some data indirectly links Rac to this reaction sequence, the connection is presently not very clear (Miki et al., 1998).

To try and resolve these discrepancies, we adapted an approach to neutrophils previously taken with another hematopoietic cell, the blood platelet, to analyzing the steps linking receptor perturbation to actin assembly. We previously studied the pathways between the PAR1 thrombin receptor of platelets and actin polymerization, finding that Rac1 was an important intermediary (Hartwig et al., 1995). The principal experimental technique involves con-

trolled partial permeabilization of the plasma membrane so as to preserve essential intermediates required to sustain the pathways, but to permit introduction of activators and inhibitors of signal transduction cascades. We describe our results here with neutrophils and conclude that complementary pathways encompassing components studied by different methodologies link FMLP receptor occupancy with actin assembly in the human neutrophil.

## Materials and Methods

### Materials

We purchased chemical reagents from Sigma-Aldrich unless otherwise noted. We prepared rabbit skeletal muscle actin and labeled it with as previously described (Hartwig et al., 1995).

A vector containing the sequence for N17Rac2 (pcDNA3m) was kindly provided by G. Bokoch (Scripps Research Institute, La Jolla, CA), and the N17Rac2 gene was isolated using a BamHI/EcoRI digest and subsequently subcloned into the BamHI/EcoRI site of pGEX2T (Amersham Pharmacia Biotech). We produced recombinant bacterial GST-Rho GTPase fusion proteins as described (Hartwig et al., 1995) using vectors encoding GST-V12Rac1 or GST-N17Rac1 (provided by Dr. A. Hall, University College, London, UK) or GST-N17Cdc42, GST-Rac2Q61L, or GST-V12Cdc42 (provided by Dr. G. Bokoch, Scripps Research Institute, La Jolla, CA) or GST-N17Rac2.

We used a 10-mer polyphosphoinositide-binding peptide based on the phosphoinositide-binding site of gelsolin (residues 160–169 QRLFQV-KGRR; Janmey et al., 1992). A control gelsolin peptide that included the same residues (in a random order) as the gelsolin 10-mer was used (FRVCLKQGQR). Rajat Rohatgi and Marc Kirschner (Harvard Medical School, Boston, MA) provided recombinant GST fusion proteins derived from human neural (N)-WASP (GST-V, aa 392–449; GST-CA, aa 450–505; and VCA, aa 392–505; Rohatgi et al., 1999). We dispersed PS, PI, PIP, PI(4,5)P<sub>2</sub>, and PI(3,4,5)P<sub>3</sub> into uniform micelles by sonication (Janmey and Stossel, 1989).

### Preparation of Neutrophils

We obtained blood from consenting healthy adult volunteers by venipuncture into 0.1 vol of sodium citrate anticoagulant (Sigma-Aldrich). We isolated neutrophils using neutrophil isolation media (NIM; Cardinal Associates) according to instructions provided by its manufacturer. The resulting cell preparation was >95% neutrophils as assessed by hematoxylin and eosin staining. We suspended neutrophils at  $5 \times 10^6$  cells/ml in HBSS. We performed all procedures with endotoxin-free solutions and completed all experiments within 3 h of blood collection.

### Permeabilization and Measurement of Actin Nuclei

To analyze actin nucleation activity, we determined the ability of permeabilized neutrophils to accelerate spontaneous actin assembly measured as enhancement of pyrene actin fluorescence with polymerization (Hartwig, 1992; Hartwig et al., 1995). We permeabilized resting neutrophils ( $5 \times 10^6$ /ml) for 10 s using 0.1 vol of OG buffer (PHEM buffer containing 4% octyl glucoside, 10  $\mu$ M phalloidin, 42 nM leupeptin, 10 mM benzamidine, and 0.123 mM aprotinin) or NP-40 (final concentration of 1%). We stopped the permeabilization process by diluting the detergent with 3 vol of buffer B (1 mM Tris, 1 mM EGTA, 2 mM MgCl<sub>2</sub>, 10 mM KCl, 5 mM  $\beta$ -mercaptoethanol, 5 mM ATP; pH 7.4). We then added activators or inhibitors of actin nucleation as described in Results. We then assayed for nuclei by adding pyrene-labeled rabbit skeletal muscle actin (Hartwig, 1992) to a final concentration of 1  $\mu$ M, and followed the fluorescence increase with a Perkin-Elmer spectrophotometer at excitation and emission wavelengths of 366 and 386 nm, respectively (Hartwig et al., 1995). We determined the contribution of actin filament barbed ends to the rate of fluorescence increase by the addition of 2  $\mu$ M cytochalasin B, and converted the cytochalasin B-sensitive actin assembly rate into the number of nucleation sites as described previously (Hartwig, 1992). We normalized the results to baseline control levels for each set of experiments. The range of absolute values for controls was 4,000–9,000 barbed ends per cell with a mean of  $7,500 \pm 667$  (mean  $\pm$  SEM; 14 experiments).

## Shape Change Experiments

To observe potential shape changes induced by FMLP in n-octyl-β-glucopyranoside (OG) permeabilized neutrophils, isolated cells were placed onto coverslips for 60 s. Attached cells were then permeabilized as outlined above, exposed to FMLP or vehicle alone, and were then fixed for 3 min with 3.7% formaldehyde. Cells were incubated with 0.5 U/ml Alexa 488 phalloidin for 5 min and observed. Under these conditions, Alexa phalloidin fluorescence was undetectable in nonpermeabilized cells. DIC and fluorescent images were acquired using a Princeton instruments Interline 1300 × 1030 12-bit digital camera and Isee software (Inovision). Images were prepared using Adobe Photoshop™.

## Confocal Fluorescence Microscopy

We exposed permeabilized cells to FMLP or vehicle alone for 150 s. We then added Alexa 488 phalloidin (0.5 U/300 μl sample; Molecular Probes) and 0.3 μM actin (1 rhodamine actin to 45 unlabeled actins; Cytoskeleton) for 30 s. We fixed the cells in 3.7% formaldehyde for 3 min and then gently centrifuged them onto polylysine-coated coverslips using a Cytospin (300 rpm for 3 min). We examined coverslips mounted in Moviol (Calbiochem) using a BioRad Confocal Microscope (MRC 1024 MP). We determined the cell dimensions in image sections of 0.5 μm with the final image of the middle section of the cell being stored for later analysis. Digital images were prepared using Adobe Photoshop™. Quantification of the rhodamine actin assembly was done using NIH image. Mean pixel fluorescence values were determined from the confocal images of the middle section of permeabilized neutrophils.

## Electron Microscopy

We attached permeabilized neutrophils to the surface of polylysine-coated glass coverslips by centrifugation at 280 *g* for 5 min and then fixed the cells with 1% glutaraldehyde in the PHEM buffer (Schliwa and Van Blerkom, 1981) for 10 min. We washed the fixed cells extensively with distilled water, subjected them to rapid freezing, freeze drying at -90°C, and coating with 1.4 nm of platinum with rotation and 2.5 nm of carbon without rotation (Cressington CFE-50 apparatus). We separated the replicas from the coverslips in 25% hydrofluoric acid and picked them up on carbon-formvar copper grids. We photographed the specimens at 100 kV in a JEOL-1200 EX electron microscope.

## Results

### Permeabilized Neutrophils Retain FMLP-mediated Signaling to Actin Nucleation

As expected from previous research (Howard and Ore-sajo, 1985), stimulation of intact neutrophils with 30 nM FMLP, followed by permeabilization with OG, increases the number of exposed barbed-end nucleation sites detectable in the permeabilized cells (Fig. 1 A). We designate these nucleation sites as free barbed ends. Free barbed ends increase on average from 7,000 per unstimulated cell to a maximum of 21,000 per FMLP-treated cell. A new finding, however, is that FMLP can also induce actin nucleation sites in PMN first permeabilized with OG. These sites are primarily barbed-end nucleation loci as evidenced by the inhibition of the FMLP-mediated pyrene actin polymerization rate by 2 μM cytochalasin B (Fig. 1 B). A small, but statistically significant ( $P < 0.03$ ; *t* test), increase in pointed ends also follows FMLP stimulation, as demonstrated by a fourfold change in the rate of actin assembly in permeabilized FMLP-stimulated neutrophils compared with unstimulated cells in the presence of cytochalasin B (Fig. 1 B).

The production and retention of FMLP-induced nucleation sites depends on the detergent type, concentration, and the detergent exposure time. FMLP-induced nucleat-

ing activity is optimal after exposure of neutrophils to 0.4% OG for 10 s. Higher detergent concentrations or increased exposure times greatly reduce the number of nuclei detectable after FMLP stimulation (Fig. 1, C and D). Omission of the protease inhibitors from the medium during the OG permeabilization step causes no significant difference in the FMLP-mediated increase in subsequent actin nucleation activity ( $242 \pm 50\%$  with protease inhibitors;  $230 \pm 35\%$  without protease inhibitors;  $P > 0.25$ ). These findings suggest that the optimal OG treatment does not unleash proteolytic enzymes.

We determined the optimal FMLP exposure time by determining free barbed ends after various FMLP exposure times. Cells were permeabilized, incubated with 30 nM FMLP for the indicated time, and then assayed for free barbed ends. Fig. 1 E demonstrates that the maximal detectable number of free barbed ends occurs at three minutes.

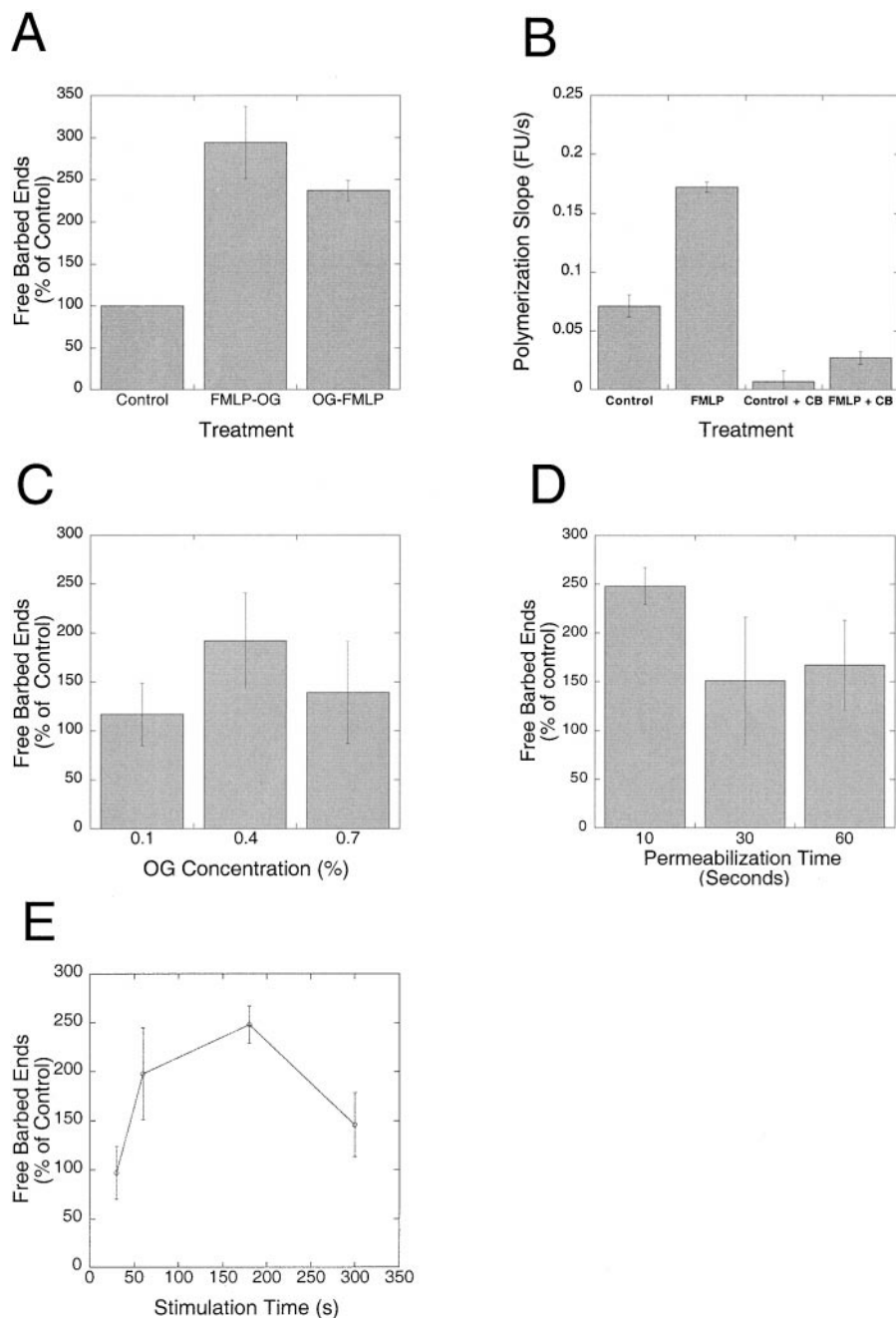
Weiner et al. (1999) noted that 1% NP-40 increased actin nucleation assessed qualitatively by light microscopy in neutrophils, and inferred that this detergent released proteases that degraded actin filament barbed-end capping proteins. Consistent with those findings, neutrophils treated with 1% NP-40 for ten seconds have much higher basal actin nucleation activity than neutrophils permeabilized with OG. The number of end equivalents in the unstimulated NP-40-treated cells ( $17,005 \pm 3,415$ ) is comparable to that of OG-permeabilized FMLP-stimulated neutrophils ( $17,444 \pm 4,340$ ). The addition of the protease inhibitors we use in the OG permeabilization solution does not alter the actin nucleation activity of the NP-40-treated cells. NP-40 permeabilized neutrophils fail to increase actin nucleation activity in response to FMLP either in the presence or absence of the protease inhibitors employed. Although the protease inhibitors do not affect actin nucleation activity in OG-permeabilized cells, we retained them as part of our standard assay system.

### Actin Nuclei Are Within Permeabilized Neutrophils

Centrifugation (14,000 *g* for 3 min) of OG permeabilized neutrophils exposed to 30 nM FMLP removes  $\geq 95\%$  of the actin nucleation activity from the resulting supernatant, demonstrating that the pyrene-actin in the assembly readout polymerized onto the neutrophil cytoskeleton.

70% of OG permeabilized cells picked up trypan blue and rhodamine phalloidin. We saw no difference in the percent of cells taking up trypan blue between 30 s and 300 s after permeabilization and dilution, confirming that the dilution of the OG with buffer B is effective in stopping further cell permeabilization.

Electron micrographs reveal perforations evenly distributed over the permeabilized neutrophil surface with large regions of intact membrane (Fig. 2 A). DIC images of neutrophils attached to poly-L-lysine-coated coverslips and then permeabilized as described demonstrate protrusive changes at their edges subsequent to FMLP treatment, which resemble early pseudopodia (Fig. 2 B). In three experiments,  $52 \pm 3\%$  of FMLP-treated cells demonstrated such shapes compared with  $18 \pm 8\%$  of control cells. Actin containing extensions visible in the FMLP exposed OG permeabilized neutrophils are absent in the control cells.

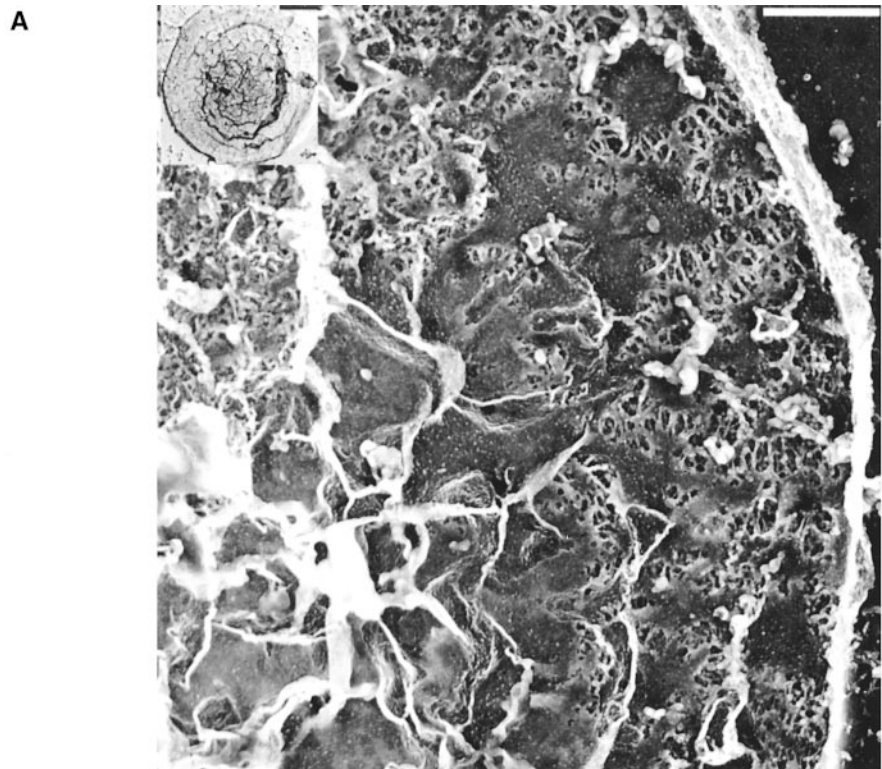


**Figure 1.** A, FMLP leads to free barbed ends on actin filaments in neutrophils permeabilized with OG. The increase in free barbed ends was determined. The values represent cytochalasin B-sensitive actin assembly initiated in neutrophils treated with FMLP (30 nM) for 3 min and then OG permeabilized (FMLP-OG), or OG permeabilized and then treated with FMLP for 3 min (OG-FMLP). The results are means  $\pm$  SEM of ten experiments normalized by setting the control free barbed ends in permeabilized cells at 100%. B, Effect of 2  $\mu$ M cytochalasin B on rates of pyrene-actin assembly of resting and FMLP-treated OG-permeabilized neutrophils. Cytochalasin B inhibits the FMLP-mediated increase in actin polymerization. There is a small but significant increase in free actin filament pointed ends in FMLP-stimulated permeabilized neutrophils ( $P < 0.05$ ). The results are means  $\pm$  SEM of five experiments. C, Relationship of the Octyl glucoside concentration and the retention of FMLP-mediated barb end exposure. Neutrophils were exposed to the indicated amount of OG for 10 s and then treated with 30 nM FMLP for 3 min. The data is from triplicate samples from a single experiment, representative of two experiments; mean  $\pm$  SD. D, Relationship of the Octyl glucoside permeabilization time and the retention of FMLP-mediated barb end exposure. Neutrophils were exposed to 0.4% OG for the indicated time periods and then treated with 30 nM FMLP for 3 min. The data is from triplicate samples from a single experiment, representative of two experiments; mean  $\pm$  SD. E, Relationship of FMLP incubation time and detectable free barbed ends. Neutrophils were permeabilized for 10 s with 0.4% OG, were then incubated with 30 nM FMLP for the indicated stimulation time, and then assayed for free barbed ends. The data is from triplicate samples from a single experiment, representative of two experiments; mean  $\pm$  SD.

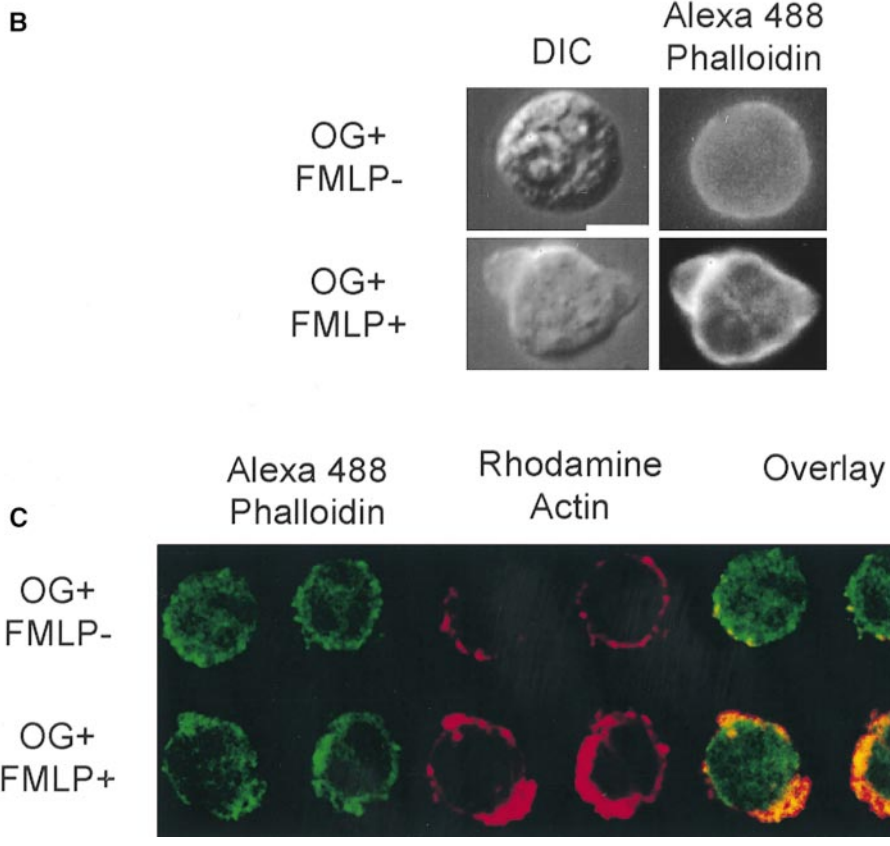
In confocal micrographs of permeabilized neutrophils, rhodamine actin assembles at the edge of the permeabilized neutrophils after treatment with FMLP (Fig. 2 C). Furthermore, the rhodamine actin assembles predominantly at one end of the permeabilized PMN, reminiscent of the polarized actin assembly caused by FMLP in intact neutrophils (Cassimeris et al., 1990; Weiner et al., 1999). As in the fluorimetric assays, cytochalasin B (2  $\mu$ M) inhibits detectable rhodamine actin association with permeabilized neutrophils. As described in Materials and Methods, quantification of the rhodamine actin

assembly by measurement of mean pixel intensity associated with individual neutrophils revealed that, as with the pyrene assay, there was an increase in rhodamine actin assembly associated with the FMLP-treated cells (FMLP,  $38.2 \pm 15.7$ ; control,  $14.4 \pm 9.2$ ; mean pixel intensity  $\pm$  SD).

Fig. 3 shows the relationship between the number of actin nuclei stimulated in optimally permeabilized cells and the FMLP concentration. FMLP  $>1$  nM induces detectable filament barbed ends, and FMLP concentrations  $>100$  nM effect maximal exposure.



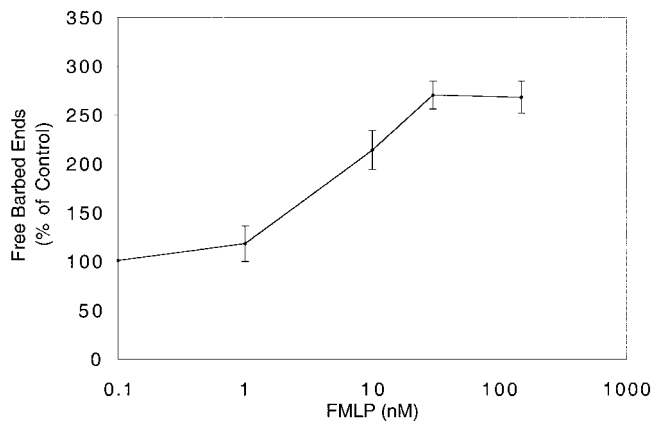
*Figure 2. A, Effect of OG on the integrity of the resting neutrophil plasma membrane. Electron micrograph showing perforation distribution on plasma membrane of neutrophils permeabilized with 0.4% OG. Neutrophils permeabilized with 0.4% OG for 10 s were placed onto polylysine-coated coverslips. Bar, 200 nM. Inset of whole neutrophil; bar, 2  $\mu$ m. B, Substrate-attached OG permeabilized neutrophils are able to undergo shape changes upon FMLP exposure. DIC and fluorescent images of attached neutrophils, which were OG permeabilized as described. Bar, 5  $\mu$ m. C, Incorporation of rhodamine actin in FMLP-stimulated permeabilized neutrophils. Images represent confocal micrographs from the middle third of permeabilized neutrophils. The top shows resting controls; the bottom shows FMLP-treated (30 nM) neutrophils. The phalloidin stain demonstrates both preexisting actin filaments and those filaments formed during the assay after permeabilization. The rhodamine actin represents exogenous actin associated with the cells after permeabilization. In the overlay, yellow represents the rhodamine actin in filamentous form that polymerized after permeabilization from free barbed nuclei. Bar, 5  $\mu$ m.*



***Polyphosphoinositides Alone Induce Actin Nucleation and Are Necessary for FMLP-induced Actin Nucleation in Permeabilized Neutrophils***

The appearance of nucleation of actin assembly in perme-

abilized neutrophils is detectable in the presence of 12  $\mu$ M PI(4,5)P<sub>2</sub>, and a maximal (fivefold) increase in nucleation activity follow the addition of 60  $\mu$ M PI(4,5)P<sub>2</sub> (Fig. 4 A). At the half-maximally effective concentration for



**Figure 3.** Effect of FMLP concentration on barbed-end exposure in OG-permeabilized neutrophils. Cells were exposed to 0.4% OG for 10 s and then exposed to the indicated FMLP concentration for 3 min. The results are means  $\pm$  SEM of five experiments.

PI(4,5)P<sub>2</sub> (30  $\mu$ M), PI(3,4,5)P<sub>3</sub> had equivalent activity for increasing free barbed ends in permeabilized neutrophils. PI and PI(4)P were much less effective and phosphatidyserine had no effect (Fig. 4 B).

A PI(4,5)P<sub>2</sub>-binding 10-mer peptide derived from the gelsolin phosphoinositide-binding site incubated with permeabilized neutrophils for 30 s before FMLP addition inhibits the nucleation response of the permeabilized neutrophils to FMLP (Fig. 4 C). Peptide concentrations  $\geq$ 45  $\mu$ M produce complete suppression of FMLP's effects. A random 10-mer peptide containing the same residues as the gelsolin 10-mer had no inhibitory activity on the FMLP-mediated increase in free barbed ends.

**Figure 4.** Effect of PIP<sub>2</sub> and phosphoinositide-binding peptide on barbed-end exposure in OG-permeabilized neutrophils. A, Effect of PI(4,5)P<sub>2</sub> concentration on barbed-end exposure in neutrophils. The appearance of nucleation of actin assembly in permeabilized neutrophils is detectable in the presence of 12  $\mu$ M PI(4,5)P<sub>2</sub>, and a maximal increase in nucleation activity follows the addition of 60  $\mu$ M PI(4,5)P<sub>2</sub>. Neutrophils are permeabilized as described and the lipids are added as micelles. The results are mean  $\pm$  SEM of three experiments. B, Effect of PS and PI, PIP, PI(4,5)P<sub>2</sub>, and PI(3,4,5)P<sub>3</sub> on barbed-end exposure in neutrophils. Both PI(4,5)P<sub>2</sub> and PI(3,4,5)P<sub>3</sub> induced a large increase in actin nucleation, whereas PI and PIP induced a small increase in free barbed ends. Phosphatidyl serine induced no change in actin nucleation. The lipids were added at 30  $\mu$ M. The results are mean  $\pm$  SEM of three experiments. C, Effect of a phosphoinositide-binding peptide on FMLP-induced barbed-end exposure. A PIP<sub>2</sub>-binding 10-mer peptide derived from the gelsolin phosphoinositide-binding site added to permeabilized neutrophils for 30 s before FMLP addition inhibits the nucleation response of the permeabilized neutrophils to FMLP. Peptide concentrations  $\geq$ 45  $\mu$ M produce complete suppression of FMLP's effects. A random 10-mer peptide (CP; 45  $\mu$ M) containing the same residues as the gelsolin 10-mer had no inhibitory activity on the FMLP-mediated increase in free barbed ends. The results are mean  $\pm$  SEM of three experiments.

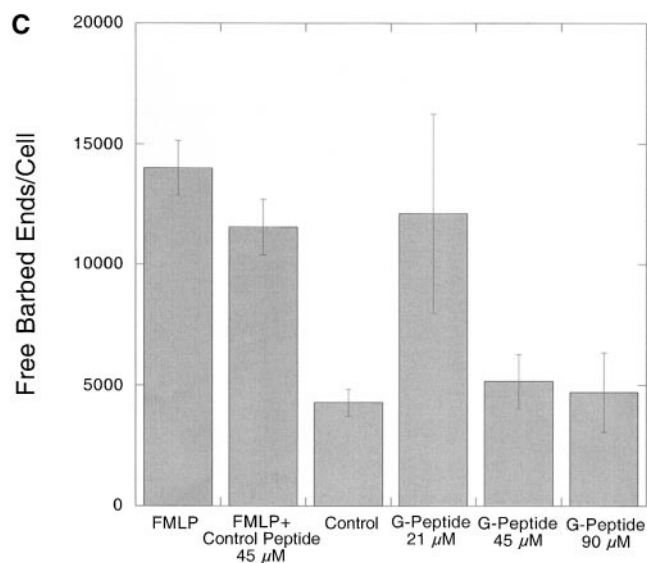
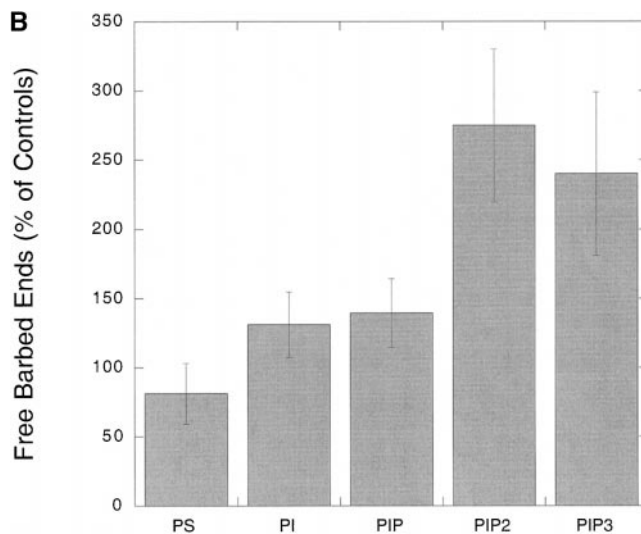
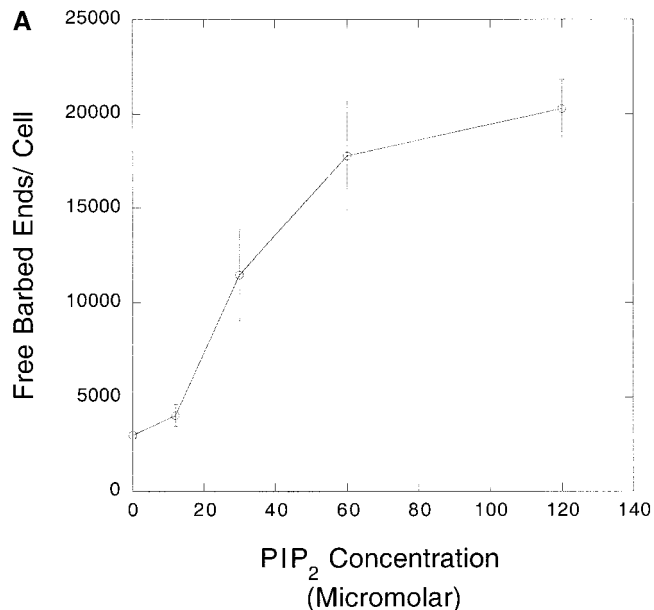


Table I. Effect of FMLP, Guanosine Nucleotides, PI(4,5)P<sub>2</sub>, 10-mer ppI-binding Peptide, V12Rac1, CDC42Q61L, N17Rac1, N17Rac2, and N17CDC42 GTPases on the Exposure of Barbed Filament Ends in Permeabilized Neutrophils

Treatment	Increase in barbed ends % of control
3 × 10 <sup>-8</sup> FMLP for 1 min and then permeabilize	294 ± 43
Permeabilize, and then 3 × 10 <sup>-8</sup> FMLP	237 ± 12
Permeabilize, and then 16 μM GTPγS	310 ± 67
Permeabilize, and then 3 × 10 <sup>-8</sup> FMLP, 100 μM GDPβS	110 ± 12
Permeabilize, 33 μM PI(4,5)P <sub>2</sub>	380 ± 74
Permeabilize, 33 μM PI(4,5)P <sub>2</sub> , 100 μM GDPβS	340 ± 60
Permeabilize, 10 <sup>-8</sup> FMLP, 45 μM 10-mer peptide	110 ± 5
Permeabilize, and then 300 nM V12Rac1	255 ± 26
Permeabilize, and then 300 nM V12Rac1, 45 μM 10-mer peptide	95 ± 14
Permeabilize, and then 3 × 10 <sup>-8</sup> FMLP, 2.7 μM N17Rac1	170 ± 6
Permeabilize, and then 3 × 10 <sup>-8</sup> FMLP, 2.7 μM N17Rac2	160 ± 23
Permeabilize, and then 300 nM V12CDC42	309 ± 29
Permeabilize, and then 300 nM V12CDC42, 45 μM 10-mer peptide	99 ± 39
Permeabilize, and then 3 × 10 <sup>-8</sup> FMLP, 1.2 μM N17CDC42	110 ± 5

Permeabilized cells were exposed to the various treatments for 3 min, after which 1 μM pyrene-labeled rabbit skeletal muscle G-actin was added to start the assay. Data are expressed as the mean ± SEM for at least three individual experiments. Controls were untreated permeabilized neutrophils.

### GTPases Induce and Are Intermediates in FMLP-mediated Actin Nucleation in OG-permeabilized Neutrophils

Addition of 16 μM GTPγS to OG-permeabilized neutrophils results in a greater than threefold increase in actin nuclei, whereas 100 μM GDPβS strongly inhibits all FMLP nucleation activity. PI(4,5)P<sub>2</sub> overcomes GDPβS inhibition, suggesting that the GDPβS effect is upstream from PI(4,5)P<sub>2</sub> in the signal transduction pathway from FMLP to actin nucleation (Table I).

The constitutively active Rho GTPases, V12Cdc42 and V12Rac1, both stimulate actin nucleation in permeabilized neutrophils (Fig. 5 A, Table I). At concentrations of 300 nM the effect of activated Cdc42 is slightly, but significantly, greater than that of Rac1 ( $P < 0.05$ ).

As shown in Fig. 5 B, the dominant-negative N17Cdc42 construct abolishes the FMLP-mediated increase in free barbed ends in permeabilized neutrophils. The dominant negative Rac GTPase, N17Rac1, at a maximal effective concentration, inhibits the FMLP-induced increase in actin nucleation by 60%. Although Rac2 is the major neutrophil isoform of Rac, previous work has not identified detectable differences between experimental effects of Rac1 and Rac2. As shown in Table I, N17Rac2 inhibits the FMLP-induced increase in actin nucleation to the same extent as N17Rac1. The maximal inhibitory concentration for both of these dominant negative GTPases in the permeabilized neutrophil is 2.7 μM. This concentration of small GTPase protein is similar to that used by others previously (Nobes and Hall, 1999). Addition of 5 μM of wild-type Rac1 had no inhibitory effect on the FMLP-induced increase in actin nucleation, ruling out the possibility that the inhibition caused by the dominant negative construct is simply a result of the relatively high protein concentration used.

N17Rac2 (3 μM) inhibited up to two-thirds of the barbed end increase mediated by 150 nM V12CDC42, whereas N17CDC42 (3 μM) had no significant inhibitory effect on the increase in free barbed ends caused by the

constitutively active Rac2 construct (Q61L; Fig. 5 C). These results place Rac downstream of CDC42.

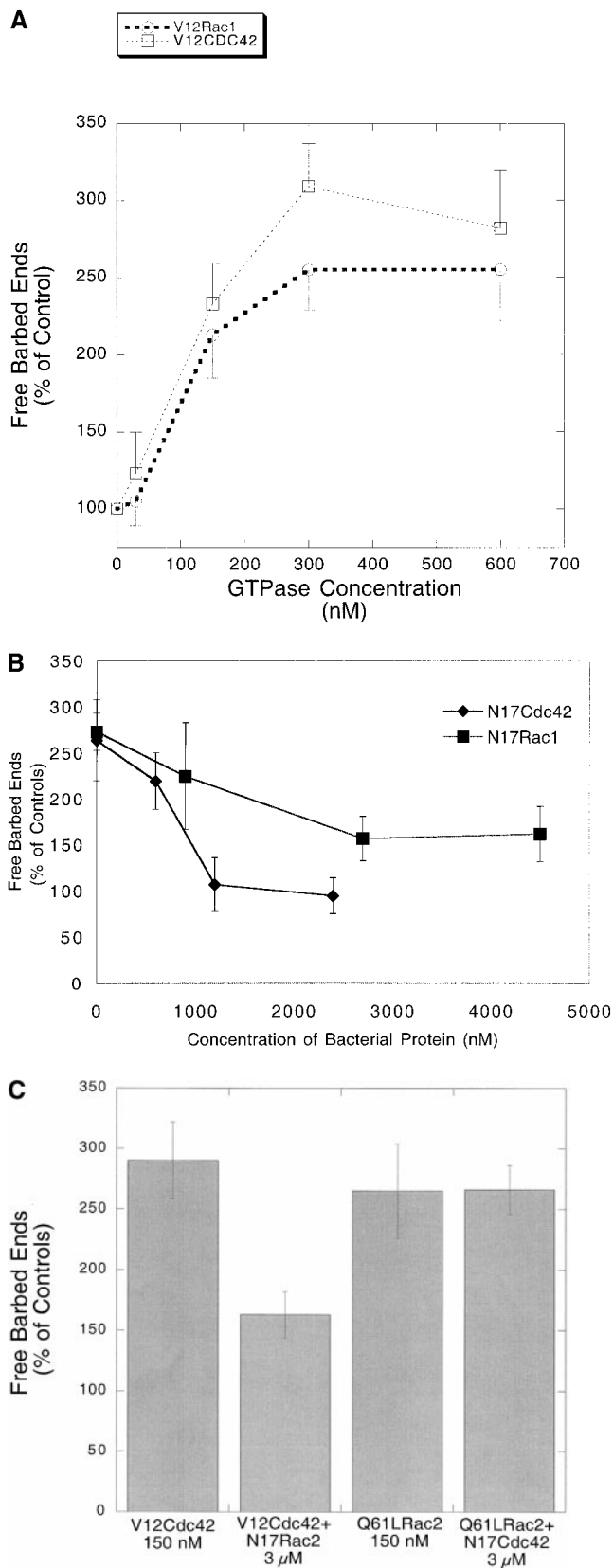
### Contribution of the Arp2/3 Nucleation Pathway in Response to FMLP Stimulation of Neutrophils

VCA (400 nM), a peptide derived from N-WASP that activates ARP2/3-mediated de novo actin assembly in vitro (Rohatgi et al., 1999), increases nucleation activity of OG-permeabilized neutrophils by 219 ± 28% (mean ± SEM of three separate experiments) over the control untreated level. Neither N17Rac1 nor N17CDC42 inhibited the VCA-mediated increase in actin nucleation (data not shown). Conversely, another N-WASP-derived peptide, CA, which inhibits ARP2/3-induced nucleation activity in *Xenopus laevis* egg extracts (Rohatgi et al., 1999), reduces by ~50% the ability of FMLP to nucleate actin in permeabilized neutrophils at saturating levels (Fig. 6 B). Combined with 2.7 μM N17Rac1, 3 μM CA peptide completely quenches the FMLP-mediated increase in nucleating activity (Fig. 6 A). A control peptide, GST-V (from the N-WASP V-domain), demonstrated no inhibitory effect on the FMLP-mediated increase in free barbed ends. The CA peptide (maximal inhibitory concentration of 3 μM) partially inhibits free barbed ends induced by GTPγS, V12CDC42, and PIP<sub>2</sub> (Fig. 6 C).

## Discussion

### Actin Nucleation in Neutrophils

Neutrophils, like other nonmuscle cells, have a large pool of unpolymerized actin that falls after FMLP stimulation. One way to account for this change is a capture of actin subunits to form nuclei that can then elongate in the barbed direction. In the last few years, many investigators have identified such a nucleation process and provided key reagents in the nucleation pathway. In this pathway, as proposed for mammalian cells including neutrophils, GTP-Cdc42 activates a WASP family protein, which in



**Figure 5.** A, Effect of V12Rac1 and V12Cdc42 concentration on barbed-end exposure in OG-permeabilized neutrophils. Cells were exposed to 0.4% OG for 10 s and then exposed to the indicated small GTPase concentration for 3 min. The results are mean  $\pm$  SEM of at least three experiments. B, Comparison of the

turn stimulates the Arp2/3 complex to nucleate actin assembly (Higgs and Pollard, 1999; Machesky and Insall, 1999; Welch, 1999).

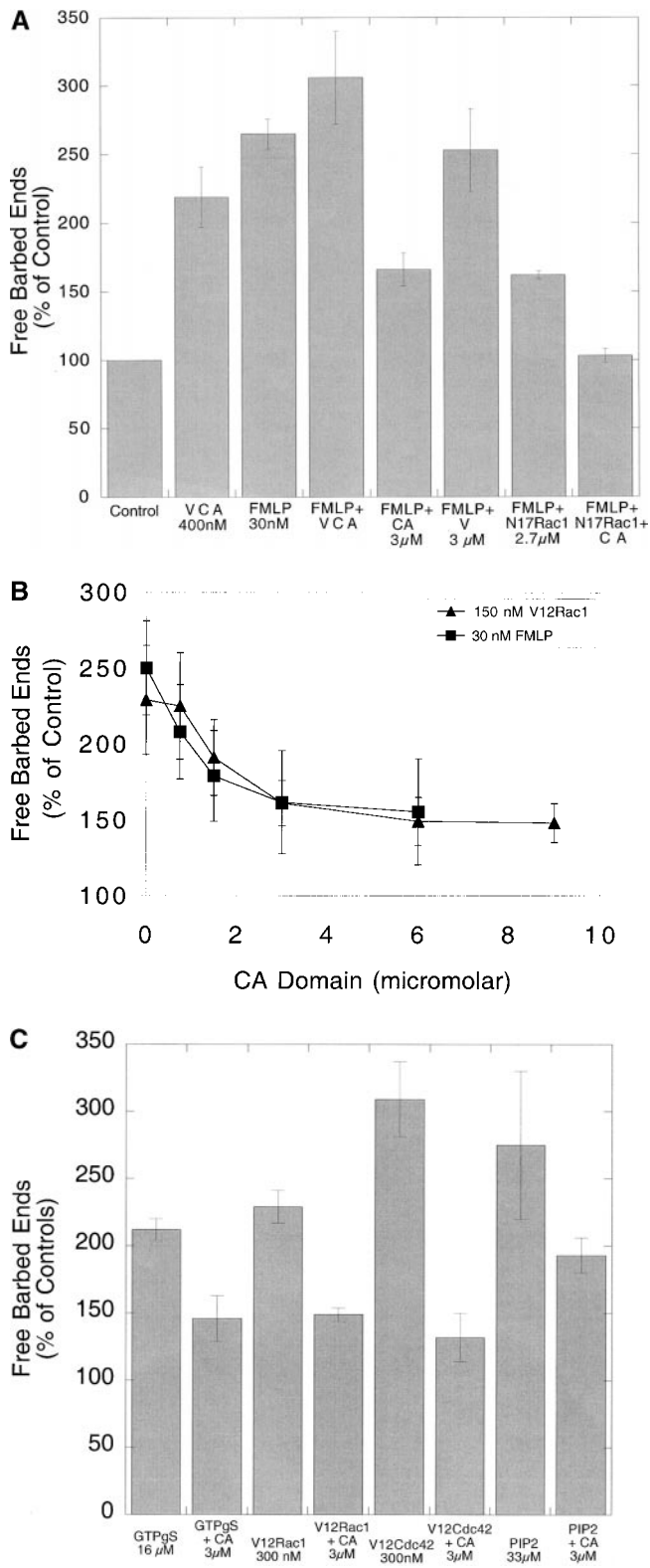
An alternative way to create nucleation sites in activated cells is to uncap the barbed ends of pre-existing actin filaments, and severing of actin filaments before uncapping can amplify the number of nuclei generated by this mechanism. Our morphological and biochemical investigations with activating platelets provided evidence favoring such a pathway with Rac as an important intermediary (Hartwig and Shevlin, 1991; Hartwig, 1992; Hartwig et al., 1995). A key support for this mechanism was the fact that inhibiting or ablating the actin severing and capping protein, gelsolin, markedly diminished nucleation activity in platelets or cultured fibroblasts after stimulation with agents that promote actin polymerization (Witke et al., 1995; Azuma et al., 1998). Experiments with gelsolin null neutrophils have revealed that upon FMLP activation, gelsolin null mouse neutrophils exhibit a 1.5-fold increase in free barbed ends compared with the almost threefold increase exhibited by wild-type mouse neutrophils (our unpublished results). In addition, actin filament barbed-end capping proteins release from permeabilized platelets after thrombin receptor stimulation (Barkalow et al., 1996; Meerschaert et al., 1998). We believe that the new information we obtained with permeabilized neutrophils supports both de novo nucleation and uncapping mechanisms for initiating actin assembly.

#### Comments Concerning Permeabilized Neutrophils

Permeabilized cells are useful for studying receptor-mediated cytoskeletal changes in hematopoietic cells (Redmond et al., 1994; Hartwig et al., 1995; Sullivan et al., 1999; Weiner et al., 1999). Redmond et al. (1994) demonstrated FMLP-mediated actin assembly in streptolysin-O-permeabilized rabbit neutrophils in the presence of exogenous GTP. In the study, GTP alone produced quantitatively similar actin assembly as GTP and FMLP added together. We report here a coupling of FMLP receptor perturbation to actin assembly in OG-permeabilized neutrophils that does not absolutely require GTP supplementation. We documented actin assembly in response to FMLP using both biochemical and morphological assays. The OG permeabilization method requires precise conditions that, if

effect of dominant negative Rho GTPase mutants of Cdc42 (N17Cdc42) and Rac1 (N17Rac1) on FMLP-induced free barbed-end creation in permeabilized neutrophils. The dominant negative Rac1 inhibited almost two-thirds of the FMLP-mediated increase in free barbed ends, whereas the CDC42 inhibited all the FMLP-mediated increase. The results are mean  $\pm$  SEM of two experiments. C, The hierarchy of CDC42 and Rac upstream of actin assembly in the permeabilized neutrophil. The dominant negative CDC42 and Rac2 proteins (N17) were tested downstream of the constitutively active version of the opposite partner. N17Rac2 (3  $\mu$ M) inhibited >50% of the V12CDC42- (150 nM) mediated barbed-end increase, whereas N17CDC42 (3  $\mu$ M) had no significant inhibitory effect on the Rac2Q61L- (150 nM) mediated increase in free barbed ends. Greater concentrations of either dominant negative did not lead to further inhibition. The results are mean  $\pm$  SEM of at least three experiments.





**Figure 6.** A, Effect of inhibitory peptides on FMLP-induced actin nucleation in permeabilized neutrophils. VCA (400 nM), a peptide derived from WASP, and 30 nM FMLP (F) increase nucleation activity in OG permeabilized when compared with control untreated cells. Addition of the WASP-derived peptide, CA, reduces by ~50% the ability of FMLP to nucleate actin in permeabilized neutrophils at saturating levels. N17Rac1 (2.7 μM) and CA peptide (3 μM) completely inhibit the FMLP-mediated in-

creased, deliver reproducible results. The technique creates holes in the plasma membrane without extracting receptors or key intermediates on the pathway to actin assembly. It does not liberate the potent elastase of neutrophils, which only treatment of intact cells with diisopropylfluorophosphate can otherwise prevent from damaging cell constituents under investigation (Amrein and Stossel, 1980; Weiner et al., 1999). Although we have concentrated on actin nucleation activity in this study, the procedure preserves some neutrophil shape change induced by FMLP stimulation. This approach adds a dimension of biochemical quantitation to the widely used morphological assessment of actin assembly not possible in studies with intact cells probed by microinjection or forced expression of signaling intermediates.

Our findings with OG-permeabilized neutrophils can reconcile some of the contradictions posed by previous studies addressing the regulation of neutrophil in actin assembly. The most likely reason for the inability of added GTPases to induce actin nucleation in some experiments (Kaetanaev and Wymann, 1998) was their inactivation by elastase. Arcaro (1998) documented Rac-dependent dissociation of gelsolin-actin complexes, but was unable to inhibit this effect with gelsolin-derived phosphinositide-binding peptide. This discrepancy is also explicable if elastase simply degraded the unprotected peptide. In cell extracts cleared of most membranes and cytoskeletal polymers, Cdc42, but not Rac, led to actin polymerization (Zigmond et al., 1997, 1998). We find that permeabilized neutrophils, like centrifuged neutrophil extracts, express GTPγS-stimulated cytochalasin B-sensitive actin nucleation activity, and GDPβS inhibits FMLP-induced actin nucleation activity, implicating intermediacy of GTPases. Like the extracts, permeabilized neutrophils develop actin nucleation activity in response to activated Cdc42. Contrasting the findings with cell-free extracts, however, activated Rac also promotes nucleation activity in the permeabilized neutrophils. This outcome is not surprising if one considers that membranes and cytoskeletal scaffoldings are important participants in the reactions coupling receptors to actin remodeling in intact cells. The permeabilized neutrophils presumably have the Cdc42-reactive components expressed in centrifuged extracts, but also have machinery, including FMLP receptors and the apparatus linking them to downstream components. The centrifugation step used to prepare neutrophil extracts probably removes this machinery.

crease in nucleating activity. The peptide GST-V (3 μM) demonstrated no inhibitory effect on the FMLP-mediated increase in free barbed ends. Data are the mean ± SEM from three separate experiments. B, Dose effect of the WASP-derived CA peptide on FMLP- or V12Rac1-induced actin nucleation. Each data point is from triplicate samples from a single experiment, representative of two experiments; mean ± SD. C, Effect of the WASP-derived CA peptide on actin nucleation induced by V12Rac1, V12CDC42, PIP<sub>2</sub>, and GTPγS. CA (3 μM), a peptide derived from WASP, inhibits the actin nucleation activity of V12Rac1 (300 nM), V12CDC42 (300 nM), PIP<sub>2</sub> (33 μM), and GTPγS (16 μM) in OG permeabilized by as much as two-thirds compared with control untreated levels.

Previously, we noted that stimulation of platelets with thrombin receptor activating peptide exposed far more barbed than pointed ends (Hartwig, 1992), and we note the same discrepancy in this study of neutrophils. We now know that a reasonable explanation is that some of the barbed ends arise from actin nucleation by Arp2/3, which yields free barbed, but not free pointed, ends.

### ***Piecing Together the Signaling Pathway from FMLP Receptor to Actin Assembly in Permeabilized Neutrophils***

***Essential Role of Phosphoinositides in FMLP-inhibited Actin Assembly.*** D3 and D4 phosphoinositides promote actin nucleation activity in permeabilized neutrophils, and a gelsolin-derived phosphoinositide-binding peptide fully inhibits FMLP-stimulated actin nucleation. These results mirror the findings with permeabilized platelets that these phosphoinositides also stimulate actin nucleation, and that the gelsolin peptide inhibits thrombin receptor-mediated nucleation (Hartwig et al., 1995). Phosphoinositides can operate at multiple steps in signal transduction (Martin, 1998; Hinchcliffe, 2000). As in permeabilized platelets, phosphoinositides overcome the inhibition by GDP $\beta$ S of FMLP receptor-mediated actin nucleation, indicating that the lipids act downstream of GTPase activation (although not excluding a role in upstream regulation as well). Phosphoinositides promote actin polymerization by monomer desequestration from profilin, by dissociating protein caps from actin filament barbed ends (Janmey, 1998), by aggregating actin filament barbed ends at the membrane (Machesky et al., 1997), and by activating WASP family proteins (Rohatgi et al., 1999). Therefore, phosphoinositides could be essential intermediates in all actin nucleation pathways.

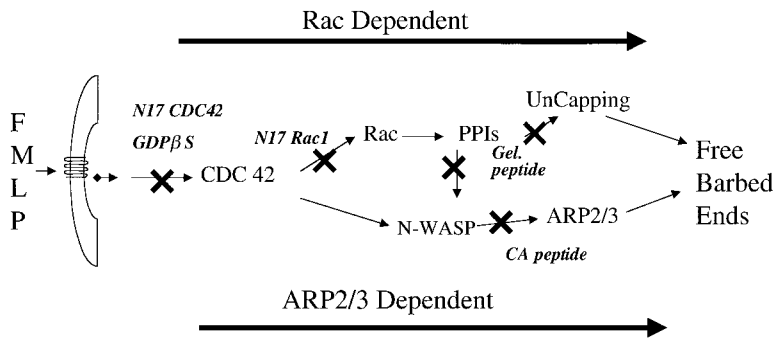
***The Relative Roles of Rac and Cdc42.*** Activated Cdc42 strongly induces nucleation activity in permeabilized neutrophils, and a dominant negative Cdc42 construct completely inhibits the effect of FMLP in promoting actin nucleation. Activated Rac's effect on actin nucleation in permeabilized neutrophils is slightly smaller than that of activated Cdc42, and a maximally effective concentration of a dominant negative Rac construct inhibits only about two-thirds of FMLP-stimulated actin nucleation. These results are consistent with Cdc42's being upstream of, and an obligatory intermediate for, Rac in the pathways leading from the FMLP receptor to actin nucleation. The demonstration that dominant negative Rac2 can dampen the activated CDC42-mediated increase in free barbed ends, whereas dominant negative CDC42 does not affect the Rac2-mediated increase in free barbed ends, supports this interpretation. Previous work has shown that Cdc42 activation can secondarily activate Rac in cells, although Rac activation independent of Cdc42 is also possible (Kjøller and Hall, 1999). Our findings indicate that, in neutrophils, Cdc42 and Rac represent distinct steps in a branching pathway, bifurcating off of activated Cdc42. The retention of Cdc42 effects in soluble neutrophil extracts with loss of the Rac responsiveness is consistent with these signaling branches residing on different subcellular structures. The effective concentrations of the GTPases (N17Cdc42, N17Rac1, V12Cdc42, and V12Rac1) added to the perme-

abilized neutrophils are similar to those used in permeabilized platelets previously (Hartwig et al., 1995; Hoffmeister and Hartwig, unpublished results), and by those microinjected into fibroblasts (Nobes and Hall, 1999).

The known downstream targets of Cdc42 leading to actin nucleation are the WASP family proteins and the Arp2/3 complex. Peptides derived from the Arp2/3 complex-binding domain of WASP family proteins are useful probes for the WASP-Arp2/3 interaction and are widely believed to inhibit actin nucleation through this nucleation pathway *in vitro* (Machesky and Insall, 1998; Rohatgi et al., 1999). A maximally effective concentration of the N-WASP peptide CA (3  $\mu$ M) inhibits about two-thirds of the FMLP-induced nucleation activity in permeabilized neutrophils. This amount of peptide is in the same order of magnitude as the ARP2/3 complex in human neutrophils (9.7  $\mu$ M; Higgs et al., 1999). This result attests to the importance of the Arp2/3 pathway in neutrophils and also points to the existence of a pathway that does not operate through Arp2/3. The data does not exclude a pathway that some have proposed leading from Rac to WASP family proteins (Miki et al., 1998; Machesky and Insall, 1999), but implies that this pathway is not the only way that activated Rac leads to actin nucleation.

Rac directly or indirectly promotes the synthesis of polyphosphoinositides (Honda et al., 1999; Toliás et al., 2000). Therefore, the effect of Rac activation on actin nucleation could theoretically be a result of the activation of WASP proteins by polyphosphoinositides. Such a role for Rac in neutrophil actin nucleation is possible. But the retention of substantial actin nucleation activity in the presence of the CA peptide is also consistent with phosphoinositides uncapping actin filament barbed ends in a pathway independent of the Arp2/3 complex. Furthermore, Zigmond et al. (1997) observed stimulation of PI(4)P and PI(4,5)P<sub>2</sub> synthesis in neutrophil extracts by Rac, but this phosphoinositide increase did not promote actin nucleation. Conversely, we interpret the substantial, but incomplete, inhibition of FMLP-induced actin nucleation by a dominant negative Rac construct as evidence for an independent limb of the signaling pathway leading directly from Cdc42 to actin nucleation, bypassing Rac. The greater inhibitory effectiveness of the gelsolin-derived phosphoinositide-binding peptide compared with inhibition of Rac activity may be a result of the peptide's ability to block preexisting, as well as newly synthesized, phosphoinositides or because Rac-independent pathways generate phosphoinositides. A recent paper proposed that the Arp2/3 nucleation pathway works by promoting branching actin elongation off the barbed ends of actin filaments (Pantaloni et al., 2000). If so, actin filament barbed end uncapping could contribute importantly to this nucleation mechanism. The complete inhibition of actin nucleation by phosphoinositide binding gelsolin peptide and the blocking of half of the actin nucleation by Arp2/3 inhibition in permeabilized FMLP-stimulated neutrophils are consistent with this hypothesis.

Neutrophils from Rac 2 knockout mice do not respond to FMLP with either chemotaxis or new actin assembly (Roberts et al., 1999), implicating an absolute requirement for both signaling arms defined by our results in mouse neutrophils. Possibly, Rac's main contribution is to pro-



**Figure 7.** Summary scheme of the pathway from the FMLP receptor to actin filament ends in OG-permeabilized neutrophils. The FMLP receptor leads to the small G protein Cdc42. The pathway then branches into a Rac-dependent path and a Rac-independent path that includes the ARP2/3 complex.

duce phosphoinositides, obligatorily required for expression of both pathways. Actin polymerization induced by G-CSF or TNF- $\alpha$  is normal in Rac2-null neutrophils, suggesting alternative signaling pathways from these ligands to actin nucleation. On the other hand, Ambruso et al. (2000) have observed that human neutrophils expressing a naturally occurring mutant Rac2 that completely inhibits FMLP-induced superoxide production have impaired, but not absent, actin assembly responses to FMLP. This finding is consistent with our results implicating dual pathways: a Rac-dependent pathway and a Rac-independent pathway in human neutrophils.

In conclusion, all of the evidence we have obtained with permeabilized human neutrophils is summarizable in the scheme shown in Fig. 7. We propose that FMLP receptor ligation activates Cdc42. Activated Cdc42 sets in motion signaling pathways leading through Rac, and presumably phosphoinositide synthesis to actin filament barbed-end uncapping and maximal catalytic activity of WASP family proteins activated by GTP-Cdc42. Active WASP proteins in turn cause the Arp2/3 complex to promote actin nucleation. Compared with the thrombin receptor pathway leading to actin assembly in permeabilized platelets, where Rac and actin filament barbed-end uncapping dominate quantitatively, Cdc42 and Arp2/3 nucleation account for a greater proportion of FMLP-induced actin assembly. Based on experience with the permeabilized platelet system, we anticipate that applying this approach to neutrophils will reveal many variations in the way that signaling from different receptors mobilizes actin assembly.

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