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Selective Inhibition of IgG-Mediated Phagocytosis in Gelsolin-Deficient Murine Neutrophils[1]

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Phagocytosis and the microbicidal functions of neutrophils require dynamic changes of the actin cytoskeleton. We have investigated the role of gelsolin, a calcium-dependent actin severing and capping protein, in peripheral blood neutrophils from gelsolin-null (*Gsn*$^-$) mice. The phagocytosis of complement opsonized yeast was only minimally affected. In contrast, phagocytosis of IgG-opsonized yeast was reduced close to background level in *Gsn*$^-$ neutrophils. Thus, gelsolin is essential for efficient IgG- but not complement-mediated phagocytosis. Furthermore, attachment of IgG-opsonized yeast to *Gsn*$^-$ neutrophils was reduced (~50%) but not to the same extent as ingestion (~73%). This was not due to reduced surface expression of the Fcγ-receptor or its lateral mobility. This suggests that attachment and ingestion of IgG-opsonized yeast by murine neutrophils are actin-dependent and gelsolin is important for both steps in phagocytosis. We also investigated granule exocytosis and several steps in phagosome processing, namely the formation of actin around the phagosome, translocation of granules, and activation of the NADPH-oxidase. All these functions were normal in *Gsn*$^-$ neutrophils. Thus, the role of gelsolin is specific for IgG-mediated phagocytosis. Our data suggest that gelsolin is part of the molecular machinery that distinguishes complement and IgG-mediated phagocytosis. The latter requires a more dynamic reorganization of the cytoskeleton. *The Journal of Immunology,* **2000, 165: 2451–2457.**

Polymorphonuclear leukocytes (neutrophils) constantly rearrange their actin cytoskeleton to perform chemotaxis and phagocytosis. Such rearrangements depend on actin severing and capping proteins of which gelsolin is the best-characterized example (1). Gelsolin is an 82-kDa cytoplasmic protein widely expressed in mammalian cells. Upon activation by Ca^{2+}, gelsolin severs actin filaments, binds, and caps barbed ends of actin filaments. Gelsolin-actin complexes dissociate in the presence of polyphosphoinositides (2, 3). Gelsolin-null (*Gsn*$^-$)[5] mice have normal embryonic development and longevity (4). *Gsn*$^-$ mice show a prolonged bleeding time due to decreased platelet shape changes. Migration of neutrophils into peritoneal exudates is delayed, and fibroblasts have excessive stress fibers and decreased motility. The GTP-binding protein rac is overexpressed in *Gsn*$^-$ tissues and regulates gelsolin in fibroblasts (5).

Neutrophil functions, such as adhesion, chemotaxis, phagocytosis and finally microbial killing by secretion of oxidative metabolites and granular enzymes into the phagosomal space, demand rapid and dynamic cytoskeletal rearrangements. Gelsolin constitutes about 1% of the total neutrophil protein and is a good candidate to mediate Ca^{2+}-dependent actin rearrangements (6). Phagocytosis in neutrophils is well studied, but its molecular mechanisms are still poorly understood. The two main pathways are complement-receptor (CR)-mediated and Fcγ-receptor (FcγR)-mediated phagocytosis (reviewed in Ref. 7). The important receptors are CR1, CR3, and CR4 for CR-mediated phagocytosis and FcγR II and FcγR III for FcγR-mediated phagocytosis in nonstimulated neutrophils.

Binding and clustering of CRs by complement-opsonized particles mediates serine phosphorylation of the receptor and the formation of punctuate structures containing F-actin, paxillin, α-actinin, and vinculin assembled to the membrane (8). This leads to a sinking of the opsonized particle into the membrane without protruding pseudopods (9). FcγR-mediated phagocytosis has been shown to have another morphology with protruding pseudopodia encircling the prey, thus demanding another kind of actin remodeling (9). FcγR-receptor stimulation mediates signaling via immune receptor tyrosine-based activation motifs (ITAM) with the initiation of a signaling cascade involving phosphorylation of syk, src-kinases activation of PI3 kinases, rac and phospholipase C, that stimulate protein kinase C-activation and calcium release from intracellular stores (7–9).

An actin ring that slowly dissolves after ingestion is completed encircles the phagosome (10). The actin ring may be a barrier, preventing granules from fusion with the phagosome. Gelsolin translocates to the actin ring and might be important for its removal (P.S. and O.S., unpublished observations). Intracellular Ca^{2+} stores and granules also translocate to the phagosome and phagolysosome fusion takes place (11). In neutrophils, this is a calcium-dependent process (12).

Extracellular secretion of granule proteins is another neutrophil activity, important for killing, but also causing tissue damage. The cortical actin network may form a physical barrier that prevents

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[5] Abbreviations used in this paper: *Gsn*$^-$, Gelsolin-null; E.S., external solution; FcγR, Fcγ receptor; CR, complement receptor; MPO, myeloperoxidase; NBT, nitroblue tetrazolium; FRAP, fluorescence recovery after photobleaching.

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granules from reaching the plasma/phagosomal membrane. The role of gelsolin could be to break up this barrier. It has indeed been shown that actin depolymerization in itself is sufficient to induce secretion in some cell types and that gelsolin can stimulate secretion (13–15).

We have investigated neutrophils from *Gsn*$^-$ mice to study the role of gelsolin in neutrophil functions. Our results show the feasibility of using transgenic mice to study the specific role of a protein in peripheral blood neutrophils. Gelsolin is the prototype of actin severing and capping proteins. It is thought to play a central role in the actin dynamics of phagocytes. We show here that gelsolin plays an important role in FcγR-mediated phagocytosis, but not in several other phagocyte functions.

Materials and Methods

Materials

Rabbit-anti-yeast Abs were produced by Dakopatts (Glostrup, Denmark). Percoll was purchased from Pharmacia (Uppsala, Sweden) and HBSS from Life Technologies (Grand Island, NY). FITC, FITC-Phalloidin, lyso-phosphatidyl choline, cytochalasin B, and polyclonal rabbit anti-lactoferrin Ab were bought from Sigma (St. Louis, MO). Polyclonal rabbit anti-myeloperoxidase (MPO) Ab was provided by Dr. Inge Olsson in Lund, Sweden and rabbit anti-gelsolin Ab by Dr. Christine Chaponnier, CMU, Geneva, Switzerland. Goat IgG, FITC-labeled, and nonlabeled goat anti-rat $F(ab')_2$, and dichlorotriazinylaminofluorescein-labeled goat anti rabbit $F(ab')_2$ was purchased from Jackson ImmunoResearch (West Grove, PA). Rat monoclonal anti mouse CD16/32 (clone 2.4G2) was bought from PharMingen (San Diego, CA) and anti-mouse CD18 (clone C71/16) from AMS, Biotechnology (Täby, Sweden).

Preparation of mouse neutrophils

Mouse blood (200–400 μl per animal) was obtained from either wild-type or *Gsn*$^-$ mice (C57LB/6) (4) in EDTA-tubes by tail bleeding. NaCl (0.9%) was added to a final volume of 4 ml. After centrifugation at 450 × *g* for 10 min, cells were resuspended in 1 ml HBSS supplemented with 0.2% EDTA. The cells were overlaid a 3-layer Percoll gradient 75%, 67%, and 52% Percoll, respectively, diluted in HBSS (100% Percoll = 9 parts Percoll and 1 part 10× HBSS) and centrifuged at 1500 × *g* for 30 min. The neutrophils were harvested from the 67%/75% interface after carefully removing the cells from the upper phases. After one wash, remaining red cells in the neutrophil fraction were eliminated by hypotonic lysis (0.5 ml water for 35 s and 0.5 ml 1.8% NaCl). After a final wash, 50,000–500,000 cells, >90% neutrophils, identified by staining the nuclei with Türks reagent, were obtained per mouse. These cells were kept in Medium 199 or external solution (E.S.: 140 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 2 mM $CaCl_2$, 10 mM HEPES, pH 7.2, 8.9 mM glucose) and used within 6 h.

Phagocytosis assay

Phagocytosis was assayed in suspension and not with adherent cells to minimize the contribution active cell movement, which is known to be influenced by gelsolin (4). Furthermore, additional receptors may be activated upon adherence to surfaces and influence the phagocytic uptake. Heat-killed bakers yeast (*Saccharomyces cervisiae*) (10^8/ml) were labeled with FITC (0.25 mg/ml) in 0.2 M carbonate buffer (pH 9.5) for 60 min at 37°C, washed several times in PBS, and resuspended in E.S. (16). The yeast particles (10^7/ml) were opsonized with either 25% mouse serum (complement C3b-opsonisation) or with a rabbit anti-yeast Ab (20 μg/ml, produced at the Department of Medicine and Microbiology, Linköping, Sweden) and 25% heat-inactivated (30 min, 56°C) mouse serum (IgG-opsonization). Nonopsonized control particles were prepared by incubation with heat-inactivated serum alone. After 30 min at 37°C, the yeast was washed twice and resuspended to 10^7/ml in E.S. Mouse neutrophils (10^6/ml) were incubated with opsonized yeast at a ratio 10 yeast particles/neutrophil for indicated times at 4°C (see Fig. 2) or 37°C. Phagocytosis was stopped by cooling the mixture tubes on ice. Fluorescence from extracellular yeast was quenched by trypan blue (17). Extracellular particles turned dark blue (16, 18). To distinguish bound particles from particles close to the cell, but not bound, the microscope slide was moved gently, and the particles that followed the cells were considered bound. The number of ingested and bound yeast particles per cell was counted immediately in 50 cells per condition with an inverted fluorescence microscope (Nikon, Melville, NY; Diaphot) with 40× oil immersion objective.

FACS analysis

Mouse neutrophils (20,000 cells/tube) were pelleted and resuspended in 0.1 ml cold 4% paraformaldehyde. After 30 min, the cells were washed twice and incubated for 1 h with 0.1 ml either rat monoclonal anti-mouse CD16/32 (clone 2.4G2) or anti-mouse CD18 (clone C71/16) diluted 1/100 in 0.1% BSA/PBS. After one wash, 0.1 ml of an FITC-labeled secondary Ab, goat anti-rat $F(ab')_2$, -fragments diluted 1/200 in 0.1% BSA/PBS supplemented with 5% goat IgG, was added. Cells were washed and resuspended in 0.2 ml PBS.

Cell labeling and fluorescence recovery after photobleaching (FRAP)

Neutrophils (10^6/ml) were incubated at 4°C for 1 h with 100 μl Krebs-Ringer's phosphate buffer supplemented with 10 mM glucose, 1.2 mM Mg^{2+}, and 1 mM Ca^{2+} (KRG) with FITC-labeled monoclonal rat-anti mouse CD16/32 (5 μg/ml) in KRG. Cells were plated on cool 3-well glass slides for 10 min, rinsed, and mounted by adding 50 μl ice-cold KRG and placing a coverslip over the well. The chambers were sealed with hot wax-vaseline mixture (1:1). The equipment for FRAP measurements has been described in detail elsewhere (19). Fluorescently labeled receptors in a small circular area (the bleach spot) on the cell surface were illuminated and photobleached (×100–1000 intensity, 500 ms) with an Argon laser (type 2020-03; Spectra Physics, Mountain View, CA) at 488 nm through a 160-μm circular aperture in a Zeiss Universal microscope (Zeiss, Jena, Germany). The 63× oil-immersion planachromatic objective used gave an estimated bleach spot radius of 0.89 μm at $1/e^2$ intensity. Fluorescence in the bleach spot was measured before bleaching and for 120 s after bleaching at increasing time intervals. The diffusion coefficient (D, × 10^{-10} cm^2/s) and the mobile fraction (R, %) was calculated according to Axelrod et al. (20) and Jacobson et al. (21). On small mouse neutrophils, up to 10% of the total cell surface might be bleached and thereby reduce R. However, bleaching would be identical in neutrophils from both *Gsn*$^-$ and wild-type mice and would not affect the comparison. Measurements were completed within 60 min after labeling and equilibration of the cells to microscope stage temperature. The preparation was kept at 15–17°C on a temperature-regulated microscope stage to minimize internalization of the receptors.

Capacitance measurements of secretion

For patch-clamp recordings, the neutrophils were plated on glass coverslips, in some cases coated with 1% BSA. Capacitance recordings with the time domain technique were performed as described for human neutrophils (22). Mouse neutrophils have an initial capacitance of 1.6 ± 0.1 pF on albumin and 2.3 ± 0.13 on glass in the presence of cytochalasin B (control cells), which probably reflects some spontaneous degranulation on glass. *Gsn*$^-$ neutrophils display similar initial capacitance values (1.7 ± 0.15 on albumin and 2.4 ± 0.14 on glass with 5 μM cytochalasin B). The capacitance values for mouse neutrophils on glass are around 25% smaller than human neutrophils (23). The cells were stimulated with either 20 μM GTPγS or 100 μM Ca^{2+} in the pipette to elicit a significant secretory response (22, 23).

Visualization of F-actin with FITC-labeled phalloidin during phagocytosis

Neutrophils were adhered on glass slides for 5 min at 37°C. Complement-opsonized yeast was added. After 3, 10, and 15 min, cells were fixed in cold 4% paraformaldehyde for 30 min. After thorough washes, cells were incubated for 20 min in lyso-phosphatidylcholine (100 μg/ml) and FITC phalloidin (0.6 μM). The preparations were washed in PBS and mounted. A Zeiss Axiovert S100 microscope equipped with epifluorescence and differential interference contrast and a 100× objective was used. Images were acquired with a cooled charge-coupled device camera (Vivicam, Visitron System, Puchheim, Germany) and the Metafluor software (Universal Imaging, West Chester, PA).

Immunofluorescence

Mouse neutrophils were adhered for 5 min to 3-well microscope slides. Complement-opsonized yeast particles were added. After 3, 10, and 15 min at 37°C, cells were fixed in ice-cold paraformaldehyde (4%), permeabilized with 0.1% Triton X-100 in 0.1% BSA/PBS and labeled with $F(ab')_2$ of polyclonal Abs to either MPO (primary granules), diluted 1/250, or lactoferrin (secondary granules), diluted 1/500 or gelsolin 1/500. After 30 min at room temperature, the cells were washed three times in 0.1% BSA/PBS. A secondary anti-rabbit Ab was added 1/100 in 0.1% BSA/PBS supplemented with 5% IgG from host species. After 30 min, the cells were washed carefully, mounted, and analyzed with fluorescence microscopy.

Nitro blue tetrazolium (NBT) test

Neutrophils were incubated in E.S. at 37°C with opsonized yeast particles in the presence of 1.7 mg/ml NBT (yellow) for 20 min at 37°C. In the presence of oxidative metabolites, NBT is reduced to formazan, which forms a blue precipitate. Cells were observed under the microscope for blue precipitates around the yeast particles.

Statistical analysis

The average number of ingested and bound yeast particles was determined from the pool of all cells observed under the same conditions (n = 150–450 from three to nine independent experiments). Mean and SE of mean were calculated and the probability that two pools were identical was determined by Student's t test on two independent populations (performed with Microsoft Excel). Differences between pools were considered statistically significant at $p < 0.05$.

Results

Gelsolin is necessary for FcγR-mediated phagocytosis, but not for CR-mediated phagocytosis

To analyze the role of gelsolin in the regulation of phagocytosis, we studied the uptake of yeast through the main phagocytic receptors of neutrophils, namely FcγRs and CRs. Fig. 1*A* shows the uptake of complement-opsonized yeast by wild-type and *Gsn*$^-$ neutrophils. The overall uptake of yeast (40 min) was the same under these conditions. However, there were kinetic differences: *Gsn*$^-$ neutrophils took up significantly less than wild-type controls during the first 5–20 min ($p < 0.001$). The situation was different for IgG-opsonized yeast (Fig. 1*B*): its uptake was markedly inhibited in *Gsn*$^-$ cells at all time points ($p < 0.001$). We also determined the number of yeast particles attached to the outside of cell. Similar to phagocytosis, the association of complement opsonized particles to *Gsn*$^-$ neutrophils was reduced up to 20 min (Fig. 1*C*). After 40 min of incubation, the differences were no longer significant. For IgG-opsonized particles, a marked decrease in the number of cell associated particles was found in *Gsn*$^-$ cells at all time points (Fig. 1*D*, $p < 0.03$).

Particle uptake requires pseudopod formation upon particle attachment, pseudopod extension, and closure of the phagosome. There is recent evidence that these steps are regulated differentially. For example, in macrophages, the tyrosine kinase syk and phosphoinositide 3-kinase appears to be required for closure of the phagosome, rather than initial pseudopod formation (24, 25). A decreased number of internalized particles, but an increased number of cell-associated particles characterize failure of phagosome closure. In contrast, the decreased number of cell-associated particles in *Gsn*$^-$ neutrophils suggest that gelsolin is needed for phagocytic steps preceding pseudopod extension and phagosome closure.

Phagocytosis and binding of IgG-opsonized particles are active processes

We compared complement and IgG-mediated phagocytosis with conditions that are known to inhibit phagocytosis (Fig. 2). Inhibition of actin polymerization by cytochalasin B reduced particle ingestion and attachment. IgG-mediated phagocytosis (Fig. 2*A*)

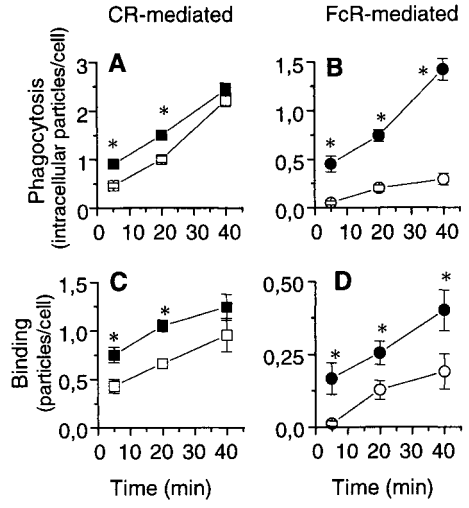

FIGURE 1. CR and FcγR-mediated phagocytosis in *Gsn*$^-$ and wild-type mouse neutrophils. Opsonized yeast particles were added to mouse neutrophils at a ratio 10:1 (yeast/neutrophil). The mixture tubes were incubated at 37°C for the indicated times, put on ice, and particle phagocytosis and attachment were determined by quenching extracellularly bound particles with trypan blue. Fluorescent and quenched particles were counted in 50 cells/sample at each time point. *A*, CR-mediated phagocytosis of *Gsn*$^-$ (□) and control neutrophils (■). *B*, FcγR-mediated phagocytosis of *Gsn*$^-$ (○) and control neutrophils (●). *C*, CR-mediated binding of *Gsn*$^-$ (□) and control neutrophils (■). *D*, FcγR-mediated binding of *Gsn*$^-$ (○) and control neutrophils (●). The numbers of particles per cell are mean values ± SEM from 150–450 cells per condition from three to nine experiments. ∗, Time points with statistically significant differences ($p < 0.05$) in ingestion or binding between *Gsn*$^-$ and controls. Several controls are shown in Fig. 2.

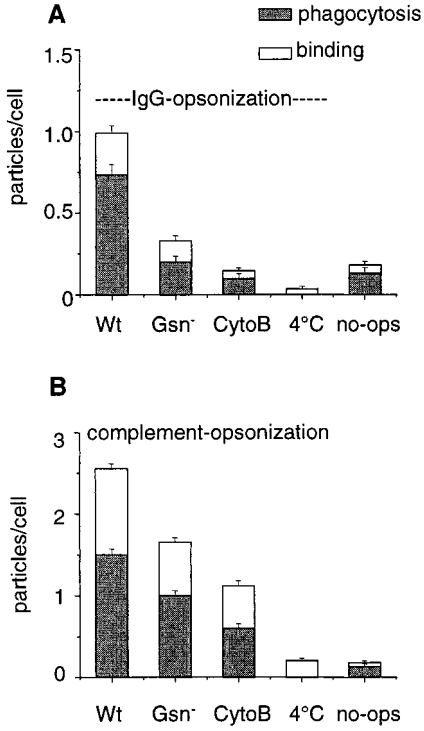

FIGURE 2. Inhibition of phagocytosis and binding by cooling, cytochalasin B, or lack of opsonization. Neutrophils were incubated with FITC-labeled IgG, complement, or nonopsonized yeast particles for 20 min. Total number of bound and ingested particles per cell are shown. *Wt*, Wild-type cells at 37°C; *Gsn*$^-$, gelsolin deficient cells at 37°C; *CytoB*, wild-type cells at 37°C in the presence of 5 μg/ml cytochalasin B; *4°C*, wild-type cells at 4°C; and *no-ops* , wild-type cells at 37°C with yeast particles opsonized with normal heat-inactivated serum. Average particle phagocytosis and average particle binding per cell was determined for IgG-opsonized (*A*) and complement-opsonized (*B*) particles. The last column in *A* and *B* concerns nonopsonized particles for comparison. Data are mean values ± SEM from 150 to 450 cells per condition. The differences in binding and ingestion between control cells at 37°C and all other conditions are statistically significant ($p < 0.02$).

was more sensitive to cytochalasin B and more affected by the absence of gelsolin than complement-mediated phagocytosis (Fig. 2*B*). Furthermore, particle ingestion was more sensitive than particle binding. At 4°C, almost no particles were attached to mouse neutrophils and none were internalized. Nonopsonized particles were poorly bound and internalized. In fact, IgG-mediated phagocytosis by *Gsn*$^-$ cells was not significantly different from phagocytosis of nonopsonized particles by control cells ($p = 0.14$). Thus, attachment and internalization of IgG-opsonized particles to mouse neutrophils are active, actin-dependent processes, and gelsolin is important for both.

Previous studies had shown that FcγR-, but not CR-mediated phagocytosis is a Ca^{2+}-dependent process (26). Our results suggest that gelsolin is the sensor for Ca^{2+}-dependent actin rearrangements occurring during FcγR-mediated phagocytosis. However, alternative explanations might involve effects of gelsolin on cell surface expression of FcγRs or on lateral receptor mobility.

Surface expression of phagocytic receptors is not reduced in Gsn$^-$ neutrophils

We compared the cell surface expression of Fc receptors and CRs on wild-type and *Gsn*$^-$ neutrophils in suspension by FACS-analysis with Abs against defined FcγRs and CR3. The FACS data showed no detectable difference in either CR3 (CD18) or FcγRII/III (CD16/32) surface expression on wild-type and *Gsn*$^-$ neutrophils, respectively (Fig. 3). Thus, the inhibition of FcγR-mediated phagocytosis in *Gsn*$^-$ neutrophils was not due to decreased cell surface density of receptors.

Lateral mobility of Fcγreceptors II/III is not altered in Gsn$^-$ *neutrophils*

Phagocytic receptors must move in the plane of the membrane to mediate phagocytosis (27). To investigate whether the difference in FcγR-mediated phagocytosis is due to altered receptor mobility in the neutrophil membrane, FRAP was used. We found no differences ($p = 0.94$) between the FcγR diffusion coefficients of *Gsn*$^-$ ($3.54 \times 10^{-10} \pm 0.30$ cm^2/s, $n = 39$) and wild-type ($3.57 \times 10^{-10} \pm 0.27$ cm^2/s, $n = 41$) neutrophils. The calculated diffusion coefficients of mouse neutrophil Fcγ-receptors are similar to what has been shown for CR1/CD35 and the fMLP-receptor in human neutrophils (2.7–4.5 and 5×10^{-10} cm^2/s, respectively) (28, 29). Note that the only available Ab against mouse FcγRs (2.4G2) binds both mFcγRII and mFcγRIII. Thus, our data represent the average mobility of FcγRIIs and FcγRIIIs on mouse neutrophils.

FRAP analysis also reveals the mobile fraction, R, of receptors that can move freely in the plane of the membrane without confinement by cytoskeletal structures or other interactions (30). Like the diffusion coefficients, the mobile fraction of receptors was unaltered in *Gsn*$^-$ neutrophils ($25.4 \pm 2.6\%$, $n = 41$ for wild-type vs $27.8 \pm 1.9\%$, $n = 39$ for *Gsn*$^-$, $p = 0.45$). Therefore, the cytoskeletal elements that may regulate FcγR movement in the plane of the plasma membrane are functional in the absence of gelsolin. We conclude that the gelsolin-sensitive step in FcγR-mediated phagocytosis is downstream of FcγR movement in the plasma membrane. However, we cannot exclude that adherence of the cells, which is needed for this technique, influences receptor mobility.

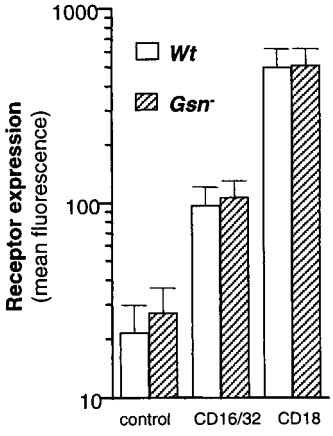

FIGURE 3. FACS analysis of phagocytic receptors. Neutrophils from *Gsn*$^-$ (▨) and wild-type (□) mice were stained with monoclonal rat Abs to CD16/32, CD18, or without primary Ab (*control*) and FITC-labeled goat anti-rat secondary Ab. At least 2000 cells per sample were measured by flow cytometry. The bars show mean fluorescence ± SEM from three experiments with no significant differences between *Gsn*$^-$ and wild-type cells ($0.6 < p < 0.94$).

Secretion induced by Ca^{2+} and GTPγS is not dependent on gelsolin

Gelsolin might be important not only for Ca^{2+} -dependent phagocytosis, but also for Ca^{2+}-dependent exocytosis. Indeed, it has been suggested from morphological studies that granules are prevented from fusing with the plasma membrane by the submembraneous actin network and that Ca^{2+}/gelsolin-dependent actin severing might therefore induce exocytosis (31). Several pieces of experimental evidence also argue in favor of this model, including the enhanced neutrophil secretion in the presence of cytochalasin B (32), and a direct effect of gelsolin on Ca^{2+}-dependent secretion in permeabilized mast cells (15). As gelsolin is regulated by the GTP-binding protein rac (5), and rac activates secretion in mast cells (33), the activation of secretion by GTPγS (23) might also involve a gelsolin-dependent pathway.

To test these hypotheses, we investigated secretion in *Gsn*$^-$ neutrophils using patch-clamp capacitance measurements. Perfusion of 100 μM Ca^{2+} and/or 20 μM GTPγS into single cells via the patch-pipette induced secretion in wild-type mouse neutrophils. The Ca^{2+} concentrations were chosen based on our previous studies in human neutrophils (22), where 100 μM Ca^{2+} was needed to elicit significant release of primary and secondary granules.

Neither amplitude nor kinetics of Ca^{2+} or GTPγS-induced capacitance increases were different in *Gsn*$^-$ vs wild-type neutrophils (Fig. 4, *A* and *B*). Normal capacitance increases occurred in *Gsn*$^-$ neutrophils both in the presence and the absence of cytochalasin B, suggesting that gelsolin does not enhance secretion like cytochalasin B and is not involved in this enhancement. Thus, we conclude that gelsolin is not required for Ca^{2+}- and GTPγS-induced secretion.

Phagosomal actin reorganization, granule translocation, and NBT reduction occur normally in Gsn$^-$ *neutrophils*

As shown above, CR-mediated phagocytosis is slowed down, but not substantially inhibited in the absence of gelsolin. However, gelsolin might not only be important for formation of the phagosome (as is the case for FcγR-mediated phagocytosis), but also for later events in phagocytosis, such as remodeling of the actin cytoskeleton after particle ingestion, phagolysosome fusion, and NADPH oxidase activation. We first investigated whether gelsolin was translocated to the phagosome. As shown in Fig. 5, *A* and *B*, in wild-type mouse neutrophils, gelsolin was enriched around the phagosome. Thus, the periphagosomal localization of gelsolin is

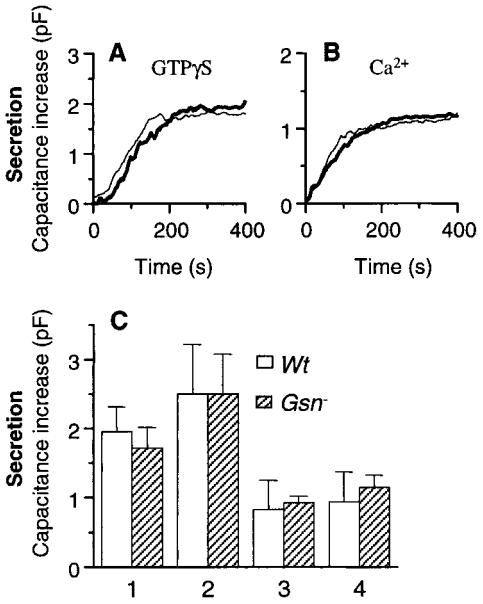

FIGURE 4. Patch-clamp capacitance recordings reveal gelsolin-independent granule secretion. Representative traces of capacitance changes from neutrophils stimulated with either 20 μM GTPγS (*A*) or 100 μM Ca^{2+} (*B*) via the patch pipette. The traces show *Gsn*$^-$ (thick line) compared with control (thin line) cells. Initial capacitance is subtracted for clarity and traces were smoothed by adjacent averaging. *A*, Capacitance traces from a *Gsn*$^-$ neutrophil and a control neutrophil plated on glass with 5 μg/ml cytochalasin B in the medium stimulated with GTPγS (20 μM). *B*, Capacitance traces from a *Gsn*$^-$ neutrophil and a control neutrophil plated on BSA-coated glass stimulated with 100 μM Ca^{2+}. *C*, Quantitative analysis of secretion induced by GTPγS and/or Ca^{2+} on glass or albumin ± cytochalasin B in *Gsn*$^-$ (▨) and control (□) neutrophils. Each bar represents the average maximal capacitance of three to seven cells ± SEM. The neutrophils were plated and stimulated under the following conditions: 1 + 2, plating on glass in the presence of cytochalasin B; 1, stimulation with 20 μM GTPγS; 2, stimulation with 100 μM Ca^{2+} and 20 μM GTPγS; 3 + 4, plating on albumin in the absence of cytochalasin B; 3, stimulation with 100 μM Ca^{2+}; 4, stimulation with 100 μM Ca^{2+} and 20 μM GTPγS. Differences between control and *Gsn*$^-$ cells are not statistically significant ($0.36 < p < 1$).

compatible with its potential involvement in phagosome maturation. As expected, no immunolabeling was detected in *Gsn*- neutrophils (Fig. 5, *C* and *D*). Because of the periphagosomal translocation of gelsolin, we analyzed late events in CR-mediated phagocytosis in *Gsn*$^-$ neutrophils. During particle ingestion, an actin ring forms around the phagosome (11). The actin ring is later dissolved in a Ca^{2+}-dependent fashion (10), possibly to facilitate the fusion of granules with the phagosome. Surprisingly, the F-actin distribution at different time points during phagocytosis in *Gsn*$^-$ neutrophils was similar to wild-type controls. F-actin rings around the phagosomes were indistinguishable in *Gsn*$^-$ and wild-type neutrophils (Fig. 5, *E–H*). Thus, although Ca^{2+}-dependent actin depolymerization is involved in limiting periphagosomal F-actin accumulation (10), gelsolin is not required in this process.

Despite the fact that gelsolin was not important for granule fusion with the plasma membrane, it might be important for the regulation of granule fusion with phagosomal membranes. Therefore, we investigated the translocation of two granule markers, namely MPO (primary granules) and lactoferrin (secondary granules), to the phagosome. Both granule markers translocated normally in *Gsn*$^-$ neutrophils (Fig. 5, *I–L* and *M–P*). Thus, gelsolin is not essential for granule translocation.

Another possible function of gelsolin might be in the regulation of the NADPH-oxidase activation during phagocytosis. Indeed, the small GTP-binding protein rac plays a role in both NADPH oxidase activation and regulation of gelsolin function. To study NADPH oxidase activation in the phagosome, we used the NBT test. In both, *Gsn*$^-$ and wild-type neutrophils, an intense periphagosomal NBT staining was observed in all phagocytosing cells (Fig. 5, *Q* and *R*). Thus, gelsolin is not required for the activation of the NADPH-oxidase in the phagosome.

Discussion

We have investigated the role of gelsolin in actin-dependent neutrophil functions. Our data clearly show that gelsolin plays a specific role in IgG-mediated phagocytosis. Phagocytosis of IgG-opsonized particles was strongly inhibited in *Gsn*$^-$ cells, whereas phagocytosis of complement-opsonized particles was merely delayed and several steps in phagosome maturation were unaffected.

Gelsolin exerts a dual role on actin filaments (reviewed in Ref. 34). First, upon activation by Ca^{2+}, gelsolin severs filaments and generates capped barbed ends. Second, binding of polyphosphoinositides dissociates gelsolin from these filaments providing free ends for polymerization. Both functions may be localized in subcellular domains. Localized accumulation of polyphosphoinositides could provide a mechanism to direct actin polymerization to specific areas near the plasma membrane like the pseudopods during phagocytosis. The selective inhibition of IgG-mediated phagocytosis in *Gsn*$^-$ cells correlates well with earlier reports, showing calcium-dependence of IgG-mediated phagocytosis in neutrophils and calcium-independence of CR-mediated phagocytosis (26). In macrophages, phagocytosis via FcR is calcium-independent (35). Thus, gelsolin might be the calcium sensor in IgG-mediated phagocytosis by neutrophils. The morphology of FcγR-mediated phagocytosis differs from CR-mediated phagocytosis. The pseudopods protrude from the cell body when an IgG-opsonized particle is ingested, whereas a complement-opsonized particle appears to sink down into the membrane (9). Different cytoskeletal structures are associated with complement- and IgG-mediated phagocytosis in macrophages (8), and different GTP-binding proteins seem to be involved (36). The formation of pseudopods in FcγR-mediated phagocytosis might require a more intense actin reorganization that depends on gelsolin.

Several intracellular mediators of phagocytosis have been identified recently and the following hypothetical signaling cascade of IgG-mediated phagocytosis emerges: The activation of rac in FcγR-mediated phagocytosis probably occurs via activation of src and syk tyrosine kinases, leading to activation of PI3-kinase (24, 37), which in turn activates the GTP-binding protein rac (38). Gelsolin has been identified as an effector of rac (5), providing a possible link to cytoskeletal rearrangements in phagocytosis, as we show here. The precise role of the proposed syk-PI3-kinase-rac-gelsolin cascade remains to be proven. However, syk, PI3-kinase, and rac are needed to complete phagocytosis (24, 25, 36, 39, 40). Rac appears to be specific for IgG-mediated phagocytosis and rho for complement-mediated phagocytosis (36), although a role for rho in FcγR-mediated phagocytosis has also been reported (41). Lamellopodia-like structures engulf IgG-opsonized particles in macrophages (8). In fibroblasts, lamellopodia formation is mediated by rac via gelsolin (5) and rac promotes the dissociation of gelsolin from actin filaments in neutrophils (42). Together, these data lead to the hypothesis that FcγRs activate rac, which interacts with gelsolin, leading to ingestion of the IgG-coated particle. Complement-opsonized particles may activate multiple signaling pathways. Gelsolin appears to be involved but is not essential, because in its absence, CR-mediated phagocytosis is merely delayed.

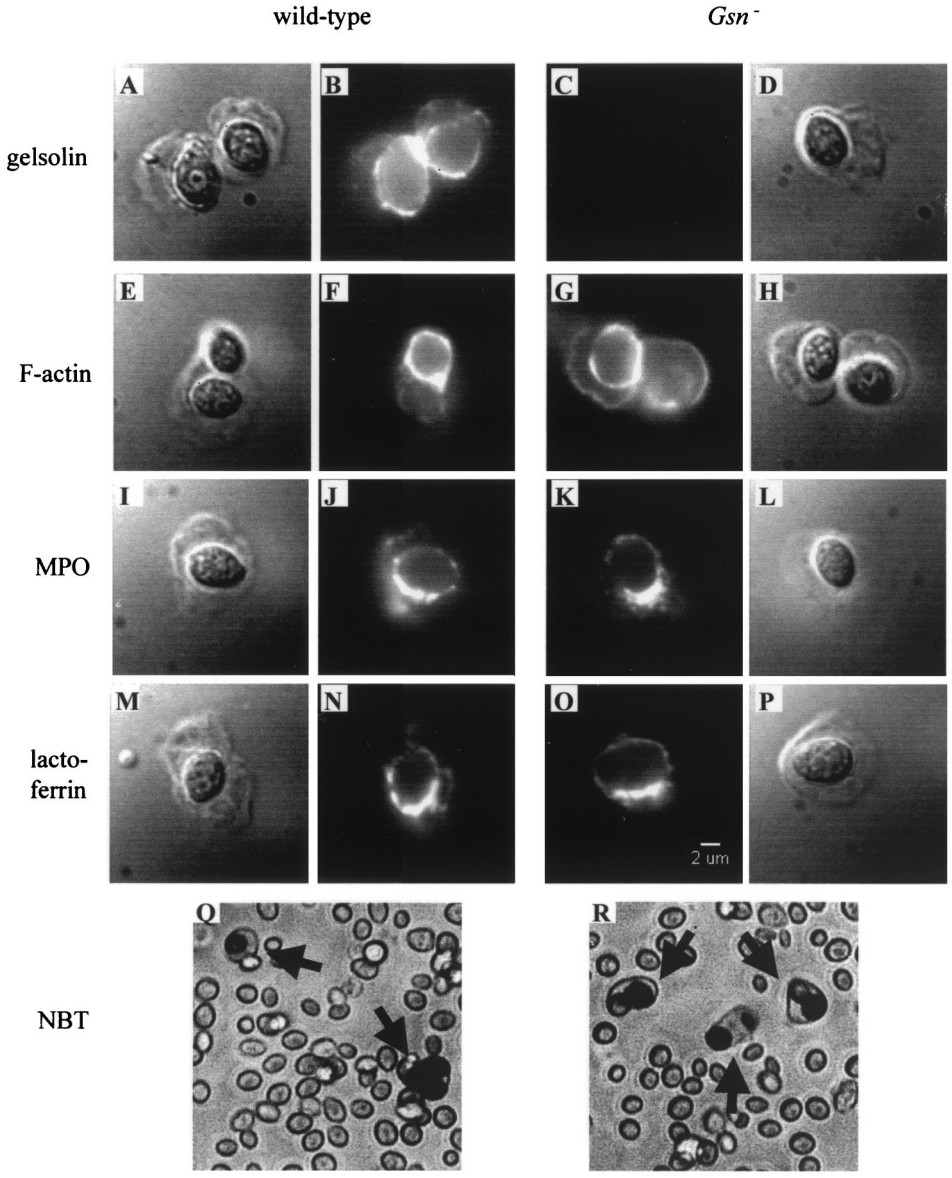

FIGURE 5. Translocation of gelsolin, actin, MPO, or lactoferrin to the phagosome in wild-type (*left*) and Gsn^- (*right*) cells during complement-mediated phagocytosis. The outer images of each row show the cells and their phagosome under differential interference contrast. The inner fluorescence images show the localization of the respective marker in the same cell. The micrographs taken through a 100× objective show representative images from three experiments where at least 50 cells were analyzed per condition. Neutrophils were adhered on glass coverslips for 5 min. After washing off nonadherent cells complement opsonized yeast was added. After10 min, cells were fixed in 4% paraformaldehyde and stained with FITC-phalloidin supplemented with 100 μg/ml lyso PC to visualize actin filaments. For immunofluorescence, neutrophils were fixed and permeabilized after 15 min of phagocytosis, labeled with polyclonal primary Ab and a secondary dichlorotriazinylaminofluorescein-labeled goat-anti rabbit Ab. *A–D*, Localization of gelsolin around the phagosome. *E—H*, Actin filaments. *I–L*, Translocation of MPO (primary granules). *M—P*, Translocation of lactoferrin (secondary granules). *Q* and *R*, NBT test on phagocytosing control and Gsn^- neutrophils. Dark precipitates of reduced NBT (formazan) reveal NADPH-oxidase activity and are visible on the phagocytosed yeast. Gsn^- (*R*) and control neutrophils (*Q*) were incubated for 20 min at 37°C with complement-opsonized yeast in the presence of NBT. *Q* and *R* show a representative phase contrast microscopy image using a 40× objective ($n = 3$). The arrows point toward neutrophils.

Which step of the phagocytic process depends on gelsolin? We found that attachment of IgG-opsonized particles is the first step in phagocytosis to be reduced, but clearly not abolished in Gsn^- neutrophils. This is also the first step that depends on the formation of new actin filaments (Fig. 2 and Ref. 43). Therefore, we hypothesize that gelsolin is specifically involved in the cytoskeletal changes associated with early pseudopod formation during IgG-mediated phagocytosis in neutrophils.

Once the cell has ingested the particle, the processing of the phagosome is generally unaffected in Gsn^- cells. Gelsolin is dispensable for formation of actin filaments around the phagosome, translocation of granules, intraphagosomal superoxide production, and granule fusion with the plasma membrane excluding gelsolin as the Ca^{2+} sensor in secretion from neutrophils. If rac is important for NADPH-oxidase function (44), it does not require gelsolin downstream to activate the oxidase. Other actin severing and capping proteins may compensate for the lack of gelsolin in these processes. We have dissected phagocytosis and phagosomal processing and adjusted well-established methods to probe each function. However, it remains possible, that other assay conditions (e.g., cells in suspension as for phagocytosis) reveal a role of gelsolin in one of these functions.

The choice of the phagocytic receptor influences the fate of the phagocytosed particle. Two examples illustrate this point. The protozoan parasite *Toxoplasma gondii* actively invades mammalian cells (45). The parasitophorous vacuole does not fuse with lysosomes and the parasite avoids killing. Rerouting the parasite to IgG-mediated phagocytosis is sufficient to render the vacuole fusogenic (46). CRs mediate phagocytosis of *Mycobacterium tuberculosis*, but the pathogen effectively avoids killing by unknown mechanisms. However, coating *M. tuberculosis* with Ab redirects the bacterium to fusion-competent phagosomes (reviewed in Ref. 47). Thus IgG-mediated phagocytosis enhances killing of the phagocytosed pathogens. Understanding the molecular differences between IgG- and complement-mediated phagocytosis will identify potential targets for pharmaceutical intervention. We have identified gelsolin as part of the IgG-specific phagocytic machinery. Therefore, it will be interesting to see whether gelsolin knockout mice are susceptible to infections that involve IgG-mediated phagocytosis.

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References

1. Weeds, A., and S. Maciver. 1993. F-actin capping proteins. *Curr. Opin. Cell. Biol. 5:63.*
2. Lin, K. M., E. Wenegieme, P. J. Lu, C. S. Chen, and H. L. Yin. 1997. Gelsolin binding to phosphatidylinositol 4,5-bisphosphate is modulated by calcium and pH. *J. Biol. Chem. 272:20443.*
3. Janmey, P. A. 1994. Phosphoinositides and calcium as regulators of cellular actin assembly and disassembly. *Annu. Rev. Physiol. 56:169.*
4. Witke, W. , A. H. Sharpe, J. H. Hartwig, T. Azuma, T. P. Stossel, and D. J. Kwiatkowski. 1995. Hemostatic, inflammatory, and fibroblast responses are blunted in mice lacking gelsolin. *Cell 81:41.*
5. Azuma, T. , W. Witke, T. P. Stossel, J. H. Hartwig, and D. J. Kwiatkowski. 1998. Gelsolin is a downstream effector of rac for fibroblast motility. *EMBO J. 17:1362.*
6. Wang, J. S., J. P. Coburn, A. I. Tauber, and K. S. Zaner. 1997. Role of gelsolin in actin depolymerization of adherent human neutrophils. *Mol. Biol. Cell 8:121.*
7. Vidarsson, G., and J. G. J. Van de Winkel. 1998. Receptor and complement receptor-mediated phagocytosis in host defence. *Curr. Opin. Infect. Dis. 11:271.*
8. Allen, L. A., and A. Aderem. 1996. Molecular definition of distinct cytoskeletal structures involved in complement- and Fc receptor-mediated phagocytosis in macrophages. *J. Exp. Med. 184:627.*
9. Kaplan, G. 1977. Differences in the mode of phagocytosis with Fc and C3 receptors in macrophages. *Scand. J. Immunol. 6:797.*
10. Bengtsson, T., M. E. E. Jaconi, J. M. Theler, D. P. Lew, O. Stendahl, M. Gustafson, and K.-E. Magnusson. 1993. Actin dynamics in human neutrophils during adhesion and phagocytosis are partly controlled by changes in intracellular free calcium. *Eur. J. Cell Biol. 62:49.*
11. Stendahl, O., K. H. Krause, J. Krischer, P. Jerström, J. M. Theler, R. A. Clark, J. L. Carpentier, and D. P. Lew. 1994. Redistribution of intracellular Ca^{2+} stores during phagocytosis in human neutrophils. *Science 265:1439.*
12. Jaconi, M. E. E., D. P. Lew, J. L. Carpentier, K. E. Magnusson, M. Sjogren, and O. Stendahl. 1990. Cytosolic free calcium elevation mediates the phagosome-lysosome fusion during phagocytosis in human neutrophils. *J. Cell Biol. 110: 1555.*
13. Muallem, S., K. Kwiatkowska, X. Xu, and H. L. Yin. 1995. Actin filament disassembly is a sufficient final trigger for exocytosis in nonexcitable cells. *J. Cell Biol. 128:589.*
14. Miyamoto, S., T. Funatsu, S. Ishiwata, and S. Fujime. 1993. Changes in mobility of chromaffin granules in actin network with its assembly and Ca^{2+}-dependent disassembly by gelsolin. *Biophys. J. 64:1139.*
15. Borovikov, Y. S., J. C. Norman, L. S. Price, A. Weeds, and A. Koffer. 1995. Secretion from permeabilised mast cells is enhanced by addition of gelsolin: Contrasting effects of endogenous gelsolin. *J. Cell Sci. 108:657.*
16. Hed, J., and O. Stendahl. 1982. Differences in the ingestion mechanisms of IgG and C3b particles in phagocytosis by neutrophils. *Immunology 45:727.*
17. Hed, J. 1986. Methods for distinguishing ingested from adhering particles. *Methods Enzymol. 132:198.*
18. Serrander, L., M. Fällman, and O. Stendahl. 1996. Activation of phospholipase D is an early event in integrin-mediated signalling leading to phagocytosis in human neutrophils. *Inflammation 20:439.*
19. Johansson, B., T. Sundqvist, and K. E. Magnusson. 1987. Regulation of the lateral diffusion of WGA-labeled glycoconjugates in human leukocytes. Comparison between adult granulocytes and differentiating promyelocytic HL60 cells. *Cell Biophys. 10:233.*
20. Axelrod, D., D. E. Koppel, J. Schlessinger, E. Elson, and W. W. Webb. 1976. Mobility measurement by analysis of fluorescence photobleaching recovery kinetics. *Biophys. J. 16:1055.*
21. Jacobson, K., Z. Derzko, E. S. Wu, Y. Hou, and G. Poste. 1976. Measurement of the lateral mobility of cell surface components in single, living cells by fluorescence recovery after photobleaching. *J. Supramol. Struct. 5:565.*
22. Nüβe, O., L. Serrander, D. P. Lew, and K. H. Krause. 1998. Ca^{2+}-induced exocytosis in individual human neutrophils: high- and low-affinity granule populations and submaximal responses. *EMBO J. 17:1279.*
23. Nüβe, O., and M. Lindau. 1988. The dynamics of exocytosis in human neutrophils. *J. Cell Biol. 107:2117.*
24. Crowley, M. T., P. S. Costello, C. J. Fitzer-Attas, M. Turner, F. Meng, C. Lowell, V. L. Tybulewicz, and A. L. DeFranco. 1997. A critical role for Syk in signal transduction and phagocytosis mediated by Fcgamma receptors on macrophages. *J. Exp. Med. 186:1027.*
25. Araki, N., M. T. Johnson, and J. A. Swanson. 1996. A role for phosphoinositide 3-kinase in the completion of macropinocytosis and phagocytosis by macrophages. *J. Cell Biol. 135:1249.*
26. Lew, D. P., T. Andersson, J. Hed, F. Di Virgilio, T. Pozzan, and O. Stendahl. 1985. Ca^{2+}-dependent and Ca^{2+}-independent phagocytosis in human neutrophils. *Nature 315:509.*
27. Griffin, F. M., Jr., and P. J. Mullinax. 1981. Augmentation of macrophage complement receptor function in vitro. III. C3b receptors that promote phagocytosis migrate within the plane of the macrophage plasma membrane. *J. Exp. Med. 154:291.*
28. Mukherjee, G., B. Rasmusson, J. G. Linner, M. T. Quinn, C. A. Parkos, K. E. Magnusson, and A. J. Jesaitis. 1998. Organization and mobility of CD11b/CD18 and targeting of superoxide on the surface of degranulated human neutrophils. *Arch. Biochem. Biophys. 357:164.*
29. Paccaud, J. P., W. Reith, B. Johansson, K. E. Magnusson, B. Mach, and J. L. Carpentier. 1993. Clathrin-coated pit-mediated receptor internalization. Role of internalization signals and receptor mobility. *J. Biol. Chem. 268:23191.*
30. Jacobson, K., E. D. Sheets, and R. Simson. 1995. Revisiting the fluid mosaic model of membranes. *Science 268:1441.*
31. Stossel, T. P. 1992. The mechanical responses of white blood cells. In *Inflammation: Basic Principles and Clinical Correlates.* J. I. Gallin, I. M. Goldstein, and R. Snyderman, eds. Raven Press, New York, p. 459.
32. Zurier, R. B., S. Hoffstein, and G. Weissmann. 1973. Cytochalasin B: effect on lysosomal enzyme release from human leukocytes. *Proc. Natl. Acad. Sci. USA 70:844.*
33. Brown, A. M., A. J. O'Sullivan, and B. D. Gomperts. 1998. Induction of exocytosis from permeabilized mast cells by the guanosine triphosphatases Rac and Cdc42. *Mol. Biol. Cell 9:1053.*
34. Stossel, T. P. 1993. On the crawling of animal cells. *Science 260:1086.*
35. Greenberg, S., J. el Khoury, F. Di Virgilio, E. M. Kaplan, and S. C. Silverstein. 1991. Ca^{2+}-independent F-actin assembly and disassembly during Fc receptor-mediated phagocytosis in mouse macrophages. *J. Cell Biol. 113:757.*
36. Caron, E., and A. Hall. 1998. Identification of two distinct mechanisms of phagocytosis controlled by different Rho GTPases. *Science 282:1717.*
37. Greenberg, S. 1995. Signal transduction of phagocytosis. *Trends Cell Biol. 5:93.*
38. Hawkins, P. T., A. Eguinoa, R. G. Qiu, D. Stokoe, F. T. Cooke, R. Walters, S. Wennstrom, L. Claesson-Welsh, T. Evans, and M. Symons. 1995. PDGF stimulates an increase in GTP-Rac via activation of phosphoinositide 3-kinase. *Curr. Biol. 5:393.*
39. Cox, D., C. C. Tseng, G. Bjekic, and S. Greenberg. 1999. A requirement for phosphatidylinositol 3-kinase in pseudopod extension. *J. Biol. Chem. 274:1240.*
40. Cox, D., P. Chang, Q. Zhang, P. G. Reddy, G. M. Bokoch, and S. Greenberg. 1997. Requirements for both Rac1 and Cdc42 in membrane ruffling and phagocytosis in leukocytes. *J. Exp. Med. 186:1487.*
41. Hackam, D. J., O. D. Rotstein, A. Schreiber, W. J. Zhang, and S. Grinstein. 1997. Rho is required for the initiation of calcium signaling and phagocytosis by Fc-gamma receptors in macrophages. *J. Exp. Med. 186:955.*
42. Arcaro, A. 1998. The small GTP-binding protein Rac promotes the dissociation of gelsolin from actin filaments in neutrophils. *J. Biol. Chem. 273:805.*
43. Peterson, P. K., J. Verhoef, and P. G. Quie. 1977. Influence of temperature on opsonization and phagocytosis of staphylococci. *Infect. Immun. 15:175.*
44. Bokoch, G. M. 1994. Regulation of the human neutrophil NADPH oxidase by the Rac GTP-binding proteins. *Curr. Opin. Cell Biol. 6:212.*
45. Sibley, L. D. 1995. Invasion of vertebrate cells by *Toxoplasma gondii*. *Trends Cell Biol. 5:129.*
46. Joiner, K. A., S. A. Fuhrman, H. M. Miettinen, L. H. Kasper, and I. Mellman. 1990. *Toxoplasma gondii*: fusion competence of parasitophorous vacuoles in Fc receptor-transfected fibroblasts. *Science 249:641.*
47. Clemens, D. L. 1996. Characterization of the *Mycobacterium tuberculosis* phagosome. *Trends Microbiol. 4:113.*