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Integrin Clustering Drives Phagocytosis Coupled to Collagenase 1 Induction Through RhoA GTPase and Superoxide Production

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ABSTRACT

Integrin-mediated phagocytosis in fibroblasts is associated to collagenase 1 induction when the particles are coated with high-affinity binding ligands. This study shows that the high density of ligand coating on the particle elicits RhoA-dependent particle uptake coupled to signal transduction. Integrin clustering induced by anti-integrin antibodies or cell surface-binding lectins is sufficient to trigger the pathway. The GTPase RhoA is recruited in response to integrin aggregation at the plasma membrane when uptake is inhibited at 4ºC and is necessary for particle engulfment, as function interference with the dominant negative mutant RhoAN19, but not with RacN17, abrogates particle ingestion. Phagocytosis driven by clustering is associated with signal transduction through a transient rise in cellular hydrogen peroxide production to induce a proinflammatory cascade leading to collagenase 1 induction. *Antioxid. Redox Signal.* 7, 318–326.

INTRODUCTION

INVADING PATHOGEN AND DEAD CELL PHAGOCYTOSIS is part of the repertoire of defense mechanisms used by the host. Particle uptake by professional phagocytes is a multistep process initiated by receptor-dependent binding of the particle (for reviews, see 7, 21). In phagocytes, Fc receptors mediate attachment of immunoglobulin-coated particles, whereas members of the integrin family, the C3 complement receptors, mediate attachment of complement opsonized particles. The receptor mediating the internalization determines the engulfment mechanism, which can be morphologically and biochemically distinguished by new membrane addition and cortical actin cytoskeleton reorganization via the small GTPases of the RhoA family (2, 17). All three GTPases, Rac1, Cdc42, and RhoA, are recruited to Fc receptor-generated phagosomes (6). Fc-mediated phagocytosis proceeds through the extension of ruffles and pseudopodia-like protrusions to engulf the particle and then fuse to generate the phagosome. Functional interference experiments suggest that whereas membrane extension to surround the particle is Cdc42-dependent, protrusion fusion to seal the phagocytic cup into a phagocytic vesicle is Rac-dependent (20). The role for RhoA remains controversial in Fc receptor-mediated phagocytosis (6, 15). C3-dependent phagocytosis proceeds through particle sinking into the cell with translocation and an essential role for RhoA (6, 17). In both cases, particle engulfment is coupled to proinflammatory and proapoptotic signaling; however, the role of the GTPases has not been evaluated in this phase of phagocytosis.

In addition to polymorphonuclear cells, several other cell types actively ingest particles, although at a lower efficiency. These cells include fibroblasts, which phagocytize collagen fibers during tissue remodeling (32), tumor cells (19), retinal pigment epithelial cells, which phagocytize spent photoreceptor outer segment fragments (12), and endothelial cells, which phagocytize apoptotic cells (24).

The mechanisms involved in particle ingestion by non-professional phagocytes remain largely unexplored. Even though the basic events of particle binding, membrane protrusion, and engulfment are expected to be conserved, the molecules recruited to mediate particle uptake could be different, and

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variations of the steps of binding, ingestion, and actin reorganization could exist. In retinal epithelial cells, for example, different molecules fulfill the steps of binding and internalization of outer segments of spent rods and cones (12). Thus, when photoreceptors bind to αvβ5 integrins, focal adhesion kinase recruitment to the focal adhesion complex signals for CD36- and MerTK-mediated internalization (11).

We have previously shown that integrin occupancy by high-affinity ligands drives collagenase expression and that these events are coupled to efficient bead uptake (36). Thus, whereas the high-affinity ligand for integrins, *Yersinia pseudotuberculosis* surface protein invasin, mediates integrin-dependent particle uptake and gene expression, low-affinity binding ligands, such as fibronectin or a mutant low-affinity binding invasin, mediate less efficient phagocytosis without associated collagenase induction. Invasin competes with the endogenous ligands of several β1 integrins at a 100-fold higher affinity than RGD peptides and mediates bacterial internalization determined by ligand affinity and receptor density (33). We have previously observed that anti-integrin antibodies, which in contrast to invasin induce integrin cross-linking without occupation, mediate bead phagocytosis with collagenase gene expression. This suggests that integrin clustering is sufficient to trigger phagocytosis with collagenase expression and gives clues to the involved mechanism because it is known that integrin clustering and occupation recruit different molecules to the adhesion complex, affecting signal transduction (22, 38). The current study examines whether integrin aggregation is sufficient to drive phagocytosis with signal transduction and its effects on the underlying phagocytosis mechanism.

MATERIALS AND METHODS

Reagents

The mutant GTPases RhoAN19 and RacN17 were kindly provided by Dr. Marc Symons (North Shore–LIJ Research Institute, Manhasset, NY, U.S.A.); the histone2B–green fluorescent protein (GFP) construct is from Dr. Jennifer Lippincott-Schwartz (National Institutes of Health, Bethesda, MD, U.S.A.). The anti-α5β1 antibody BIIG2 was kindly provided by Dr. Carolyn Damsky (University of California, San Francisco, CA, U.S.A.). Anti-vinculin and anti-activator protein-2 (AP-2) antibody were obtained from Sigma–Aldrich (St. Louis, MO, U.S.A.), anti-human transferrin receptor from Zymed (South San Francisco, CA, U.S.A.), anti-RhoA from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.), and Rac1 from Upstate (Waltham, MA, U.S.A.). The Alexa-conjugated phalloidin and secondary antibodies anti-rat and anti-mouse immunoglobulins were obtained from Molecular Probes (Eugene, OR, U.S.A.). Interleukin-1 (IL-1) receptor antagonist is from R&D Systems (Minneapolis, MN, U.S.A.). All nonspecified chemicals were from Sigma–Aldrich.

Cells

Rabbit synovial fibroblasts (RSFs) were isolated from 2-month-old New Zealand White female rabbits, as described previously (1), cultured in Dulbecco's modified Eagle medium H-21 (DME) (Cellgro, Hendon,VA, U.S.A.) supplemented with 10% fetal bovine serum (FBS; Hyclone, Logan, UT, U.S.A.), penicillin, streptomycin, and 2 m*M* glutamine (Sigma–Aldrich) in 5% CO_2 at 37ºC, and used between passages 3 and 10. For the experiments, RSFs were plated in serum-free media, with DME 0.2% lactoalbumin hydrolysate (LH) (Fisher, Pittsburgh, PA, U.S.A.) on dishes previously coated for 2 h at room temperature with 20 ug/ml human fibronectin (Molecular Biochemicals, Indianapolis, IN, U.S.A.) in phosphate-buffered saline (PBS), washed twice with PBS, and blocked with DME 0.2% LH. Cells were transfected with Effectene (Qiagen, Valencia, CA, U.S.A.) following the recommendations of the manufacturer.

Bead coating

Magnetic beads (Dynal, Oslo, Norway) of 4.5 μm in diameter were coated following the manufacturer's instructions by adsorption of the antibody in 0.1 *M* borate buffer, pH 8, at 4ºC overnight. For the coating density curve, 10 μg/ml bovine serum albumin (BSA) was added to decreasing amounts of antibody. For low-density anti-α5β1-coated bead immunofluorescence, instead of BSA, 10 μg of mouse IgG was added. Lectin-coated beads were prepared by incubating overnight with 2 μg/ml streptavidin (Calbiochem, Darmstadt, Germany). After three washes with PBS, the beads were incubated for 2 h at room temperature with 1 μg/ml biotinylated concanavalin A (ConA) or wheat germ agglutinin (WGA) (Vector Laboratories, Burlingame, CA, U.S.A.) in PBS. After coating, the beads were washed 3× in PBS and stored for no more than 2 weeks in PBS with 1 μg/ml LH. They were added to the cells at a 4:1 ratio after 1 h of plating.

Collagenase 1 (CL-1) expression analysis

CL-1 expression was measured as described previously (36). In brief, serial dilutions of the cell culture supernatants were analyzed by slot blot or western blot using a mixture of mouse anti-rabbit collagenase monoclonal antibodies, which recognize a single band by western blot, and horseradish peroxidase-conjugated anti-mouse secondary antibody (Zymed). Horseradish peroxidase activity was detected by chemiluminescence.

Bead-associated membrane isolation and western blot

Membrane fractions associated with the beads were isolated as previously described (36). In brief, after incubation with magnetic beads, cells were disrupted in hyposmotic buffer (100 m*M* MES, pH 6.8, 1 m*M* EDTA, 10 m*M* $MgCl_2$, antiproteases) by three sequential freeze-thaw cycles using ethanol/dry ice and 37ºC incubations. The beads were recovered with a magnet and washed three times in hyposmotic buffer. Equal amounts of protein were separated by sodium dodecyl sulfate electrophoresis and analyzed by western blot.

Immunofluorescence

RSFs were plated on fibronectin-coated glass coverslips. After treatment, the cell monolayer was washed with PBS and fixed for 20 min in 4% paraformaldehyde in PBS, permeabi-

lized with PBS 0.02% saponin for 5 min, and blocked for 1 h in PBS 15% FBS. Primary and secondary antibodies were diluted in 15% FBS and incubated for 1 h. The coverslips were embedded in gelvatol, analyzed, and photographed using a fluorescence microscope (Leica model DMR-E).

Phagocytosis assay

Coated beads were added at a ratio of four per cell on well spread cell monolayers cultured on fibronectin-coated glass coverslips. The cells were incubated for 2 h, washed with 1 ml/well PBS, and fixed for 20 min in PBS 4% paraformaldehyde at room temperature. After a PBS wash, they were incubated with 15% FBS in PBS. The cells were incubated for 1 h with Alexa 468 nm-conjugated anti-rat (for high-density coated beads) or anti-mouse (for anti-transferrin receptor or low-density coated beads) immunoglobulin. The beads remaining at the cell surface were visualized as green, whereas all the beads were visualized as red in a fluorescence microscope. Both frames were superimposed using Adobe Photoshop software.

Hydrogen peroxide (H_2O_2) measurement

Extracellular H_2O_2 was determined using the peroxidase-dependent oxidation of homovanillic acid, described previously (34, 35).

Sample handling for scanning electron microscopy

Fibroblasts cultured on Si-wafer supports were fixed with 2.5% glutaraldehyde in 0.1 *M* sodium cacodylate buffer, pH 7.4, for 4 h and then washed 3× in ddH_2O. Samples were postfixed with 1% OsO_4 in ddH_2O for 30 min, followed by 3× washes in ddH_2O and dehydration through a graded series in ethanol to 100%. Cell wafers were critical point dried from CO_2, mounted onto Al stubs, and sputter-coated with 3-nm Au/Pd (60:40). Specimens were staged in-lens of a DS-130 FESEM operated at 5 kV. Images were digitally recorded (5 Mbytes) in 16 s.

RESULTS

The dependence between integrin clustering and signal transduction was examined using magnetic beads of 4.5 μm diameter coated with decreasing amounts of anti-α5β1 antibody in the presence of a constant amount of BSA, as detailed in Materials and Methods. Western blot analysis of the protein adsorbed onto the beads shows that this coating procedure yields beads with decreasing amounts of surface-bound anti-α5β1 antibody (Fig. 1A). Equal amounts of beads with different coating densities were used to challenge RSFs. The number of beads associated with the monolayer was determined after 2 h, and collagenase production was measured in the supernatant after 24 h. Figure 1B shows that bead association, which includes bound and ingested beads, and collagenase induction are a direct function of anti-α5β1 bead coating density. This dependency was observed also with invasin-coated beads (data not shown).

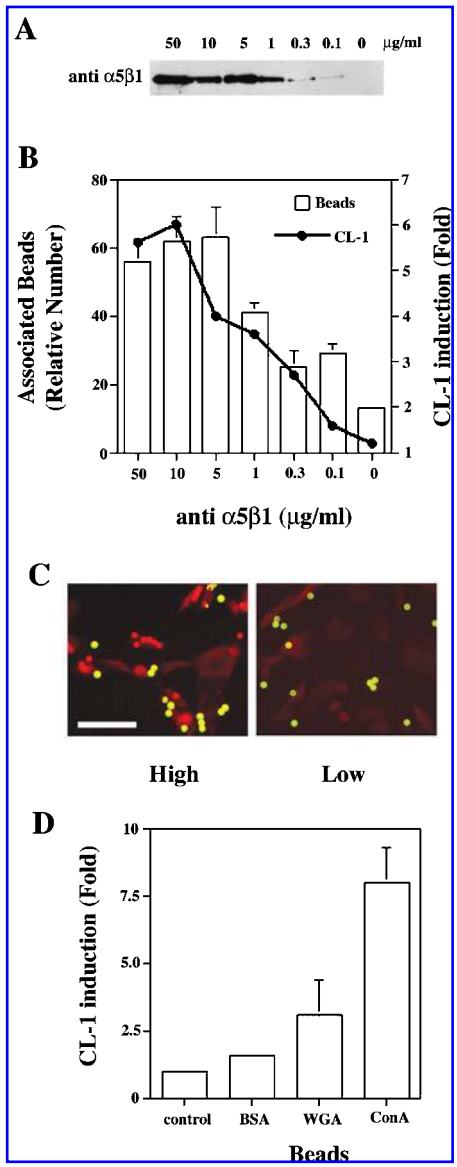

FIG. 1. Integrin clustering drives phagocytosis coupled to collagenase induction. (**A**) Amount of anti-α5β1 adsorbed onto beads after coating with decreasing antibody concentration was determined by western blot analysis of 12 × 10^6 beads. (**B**) Anti-α5β1 coating concentration-dependent bead association to cell monolayer and collagenase induction. Four hundred thousand beads coated at the indicated antibody concentrations were added to cell monolayer (4:1 ratio). The relative number of associated beads was determined after 2 h, and collagenase was measured in the culture supernatant by slot blot analysis after 24 h. This is one representative experiment of three. (**C**) Phagocytosis driven by high-concentration anti-α5β1-coated beads. After 2 h of high (10 μg/ml) or low (0.1 μg/ml) concentration coated beads challenge, the extracellular (green) vs. internalized (red) beads were determined in a phagocytosis assay (see Materials and Methods). This is one representative experiment of two. Scale bar = 50 μm. (**D**) Other surface protein clustering agents induce collagenase. Fibroblasts were challenged with beads coated with 10 μg/ml BSA, WGA, or ConA, and collagenase production was detected in the culture supernatants by slot blot analysis after 24 h. This is one representative experiment of two.

At low anti-α5β1 density (0.1 μg/ml), association to the monolayer is observed without collagenase induction, suggesting that, at low ligand density, bead binding occurs without phagocytosis and/or coupled signal transduction to induce collagenase. To test whether particle uptake was impaired, particle ingestion was determined by a phagocytosis assay, which involves comparing the particles remaining on the surface to the total number of beads by means of immunofluorescence directed to the bead coat in nonpermeabilized cells. Noninternalized beads appear green-yellow due to the immunodetection, and the internalized beads appear red after image overlay. As shown in Fig. 1C, whereas low-density (0.1 μg/ml) anti-α5β1-coated beads bind to RSFs, they remain at the cell surface (green), contrasting with high-density (10 μg/ml) coated beads, which are ingested (red) within 2 h. This result suggests that binding is not sufficient to drive collagenase induction and phagocytosis, but that efficient integrin clustering is necessary. It also indicates that clustering without occupation is sufficient; thus, lectins were used to coat beads and tested as an alternative mechanism to induce cell-surface molecule clustering. ConA and WGA are lectins known to bind cell-surface proteins and induce their clustering and capping. RSF monolayers were challenged with ConA- and WGA-coated beads, and collagenase production was measured 24 h later. Figure 1D shows that ConA- and WGA-coated beads are potent collagenase inducers. Soybean and peanut agglutinin did not bind or mediate phagocytosis and collagenase production (data not shown).

These results suggest that close contact with the membrane and efficient receptor clustering drive phagocytosis. Because Fc- and C3-dependent phagocytosis display distinctive morphological and biochemical features in neutrophils (17), morphological cues about the anti-α5β1 bead phagocytic mechanism were sought using scanning electron microscopy. RSFs were challenged with high-density anti-α5β1-coated beads for 30 min, fixed in 4% glutaraldehyde in cacodylate buffer, and processed for high-resolution scanning electron microscopy (see Materials and Methods). At this time, all the stages of the phagocytosis were observed, indicating that complete particle engulfment takes <30 min. The beads bound mostly, although not exclusively, at the cell periphery (Fig. 2D), establishing an initially small area of contact (Fig. 2A), which

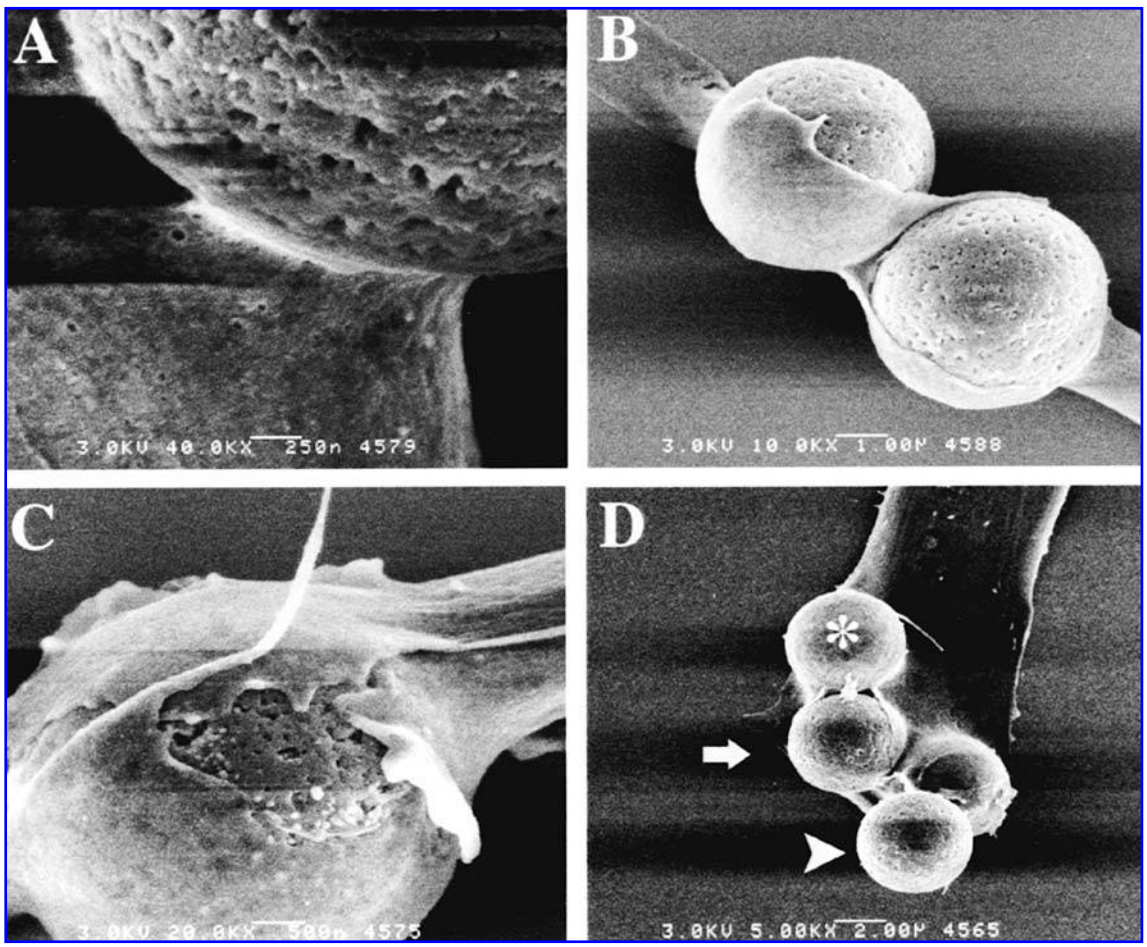

FIG. 2. Scanning electron microscopy of anti-α5β1-coated bead phagocytosis. Fibroblasts were challenged for 30 min with high-concentration anti-α5β1-coated beads (4.5 μm diameter). (**A**) Area of bead contact with the membrane at an early step of ingestion. (**B**) Phagocytosis proceeds through the advance of the plasma membrane in close apposition to the bead. (**C**) Engulfment is completed by a zipper-like mechanism. (**D**) Image including several stages of the process at the edge of a cell: binding (arrowhead), ingestion (arrow), and complete engulfment (*).

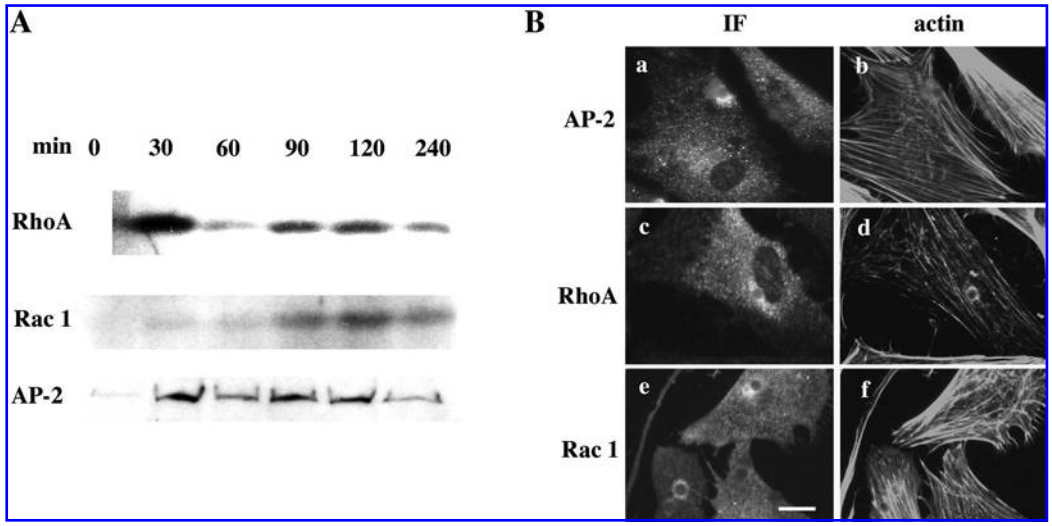

FIG. 3. Rho GTPase recruitment to the plasma membrane upon bead phagocytosis. (**A**) Fibroblasts were incubated with high-concentration anti-α5β1-coated beads for different periods. Recruitment of RhoA, Rac1, and AP-2 was assessed by western blot analysis of the bead-associated membranes. This is one representative experiment of two. (**B**) After 90 min of bead addition, AP-2 (**a**), RhoA(**c**), and Rac1 (**e**) recruitment was visualized by immunofluorescence (IF) and actin filaments (**b**, **d**, **f**) by rhodamine-labeled phalloidin staining. Scale bar = 25 μm.

increases upon membrane extensions. These scarcely elaborated membrane protrusions advance in close apposition to the bead surface (Fig. 2B) to surround and enclose the particle by a zipper-like mechanism (Fig. 2C), reminiscent of Fc-mediated phagocytosis. Figure 2D shows all stages of the phagocytic process.

In neutrophils and macrophages, the type of GTPase required for phagocytosis defines the mechanism of internalization. Thus, recruitment of small GTPases to the plasma membrane was analyzed by western blot of bead-associated membranes. After bead challenge for different time periods, western blot analysis demonstrates that RhoA translocates to the membrane as early as 30 min and remains associated with membrane fractions for up to 4 h (Fig. 3A), following a pattern similar to the adaptor AP-2. This pattern contrasts with Rac1, which translocates at low levels at early time points and reaches a transient maximum at 2 h. These results suggest that these two GTPases are recruited at different steps of phagocytosis. The western blot results were corroborated by immunofluorescence analysis of GTPase localization during phagocytosis. Immunofluorescence analysis shows that RhoA localizes diffusely to the plasma membrane in the vicinity of the beads (Fig. 3B). This staining pattern, which is similar to that of AP-2, suggests recruitment to the plasma membrane before it is completely surrounding the bead. At this step, fibers staining for actin radiate from the bead, indicating phagocytic cup formation. Rac staining was less diffuse, frequently surrounding the beads and colocalizing with actin.

Because cell spreading on high-density coated substrates activates RhoA (9), it was tested whether integrin clustering is sufficient to recruit RhoA to the plasma membrane. High-density coated beads were added for 1 h to cells incubated at 37ºC and 4ºC (which arrests particle ingestion, but not binding), and the bead-associated membrane fraction was separated as described in Materials and Methods. Western blot analysis of the isolated proteins (Fig. 4A) shows that when phagocytosis is halted at 4ºC, RhoA translocates selectively to membranes associated with high-density and ConA-coated beads, but not to beads coated at low density with anti-α5β1 or antibodies directed to an unrelated cell-surface protein, the transferrin receptor. Vinculin associated to membranes was used as a loading control, because it is known that fibronectin bead phagocytosis in fibroblasts recruits selectively α-actinin, but not vinculin (14). These results suggest that integrin clustering at the plasma membrane, before internalization, is sufficient to induce RhoA translocation to the membrane and thus places this GTPase at an early step of particle internalization.

The role of the GTPases during phagocytosis was examined by expressing dominant negative mutants of RhoA (N19) or Rac1 (N17) together with histone 2B–green fluorescent protein (GFP) to mark the transfected cells. The phagocytosis assay shows that RhoA is required for bead internalization because RhoAN19-expressing cells, although able to bind beads, showed a reduced number of phagocytosed beads (Fig. 4Ba) in contrast to RacN17-expressing cells (Fig. 4Bb). Anti-transferrin receptor-coated beads bound to the cells, but were not internalized, corroborating that bead binding is not sufficient to promote phagocytosis. Particle internalization is dependent on temperature as 4ºC incubation abrogates particle ingestion (Fig. 4Bc) and is dependent on an intact actin cytoskeleton, because cytochalasin D treatment inhibits phagocytosis (Fig. 4Bd). These experiments demonstrate that integrin clustering is sufficient to induce RhoA translocation to the membrane, where it mediates particle internalization by an actin polymerization-dependent and a temperature-sensitive mechanism, both typical hallmarks in phagocytosis.

We have previously shown that phagocytosis induces collagenase expression through IL-1 production (36). Because nuclear factor-κB is a redox-sensitive transcription factor, we examined whether bead ingestion induces an oxidative burst.

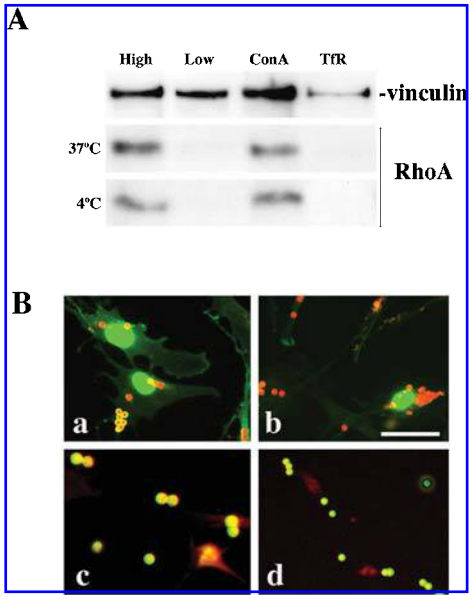

FIG. 4. RhoA-dependent ingestion upon integrin clustering. (**A**) RhoA, but not vinculin, is selectively recruited to the membrane upon integrin clustering. Fibroblasts were challenged with high- and low-concentration anti-α5β1-, ConA-, and anti-transferrin receptor (TfR)-coated beads at 37ºC or at 4ºC for 30 min. Bead-associated membranes were isolated, and an equal amount of protein was analyzed by western blot for the presence of RhoA and vinculin. This is one representative experiment of two. (**B**) RhoA-dependent bead ingestion. Phagocytosis assays of high-concentration anti-α5β1-coated beads added to cells, transiently expressing histone 2B-GFP and RhoAN19 (**a**) or RacN17 (**b**), cooled to 4ºC (**c**), or treated for 30 min with 0.5 ug/ml cytochalasin D (**d**) are presented. Scale bar = 50 μm.

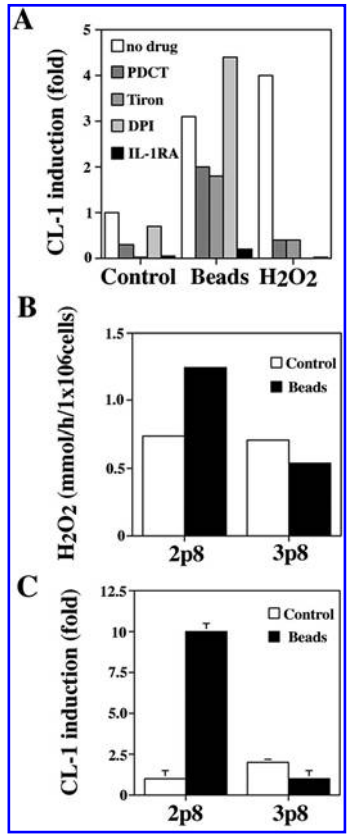

FIG. 6. Superoxide-dependent collagenase induction. (**A**) Antioxidant-sensitive collagenase induction. RSFs were incubated for 30 min with 100 μ*M* PDCT, 1 m*M* Tiron, or 10 μ*M* DPI and then challenged without or with beads or 50 μ*M* H_2O_2 in the presence or absence of 100 ng/ml IL-1 receptor antagonist (IL-1RA). After 24 h, collagenase was measured in the cell culture supernatant by slot blot analysis. This is one representative experiment of two. (**B**) H_2O_2 produced by two different cell strains upon challenge with high-density coated beads. (**C**) Collagenase production detected by slot blot analysis of cell culture supernatant after 24 h of bead addition to the cell strains used in B.

As intracellular superoxide ($O_2^{\bullet -}$) rapidly dismutates to the readily diffusible H_2O_2, extracellular H_2O_2 was measured after bead addition at various intervals (34). A transient rise in H_2O_2 production was detected between 60 and 120 min after high-density anti-α5β1-bead challenge (Fig. 5A). Extracellular addition of catalase abrogated this H_2O_2 peak (Fig. 5B), and Tiron (4,5-dihydroxy-1,3-benzenedisulfonic acid), a

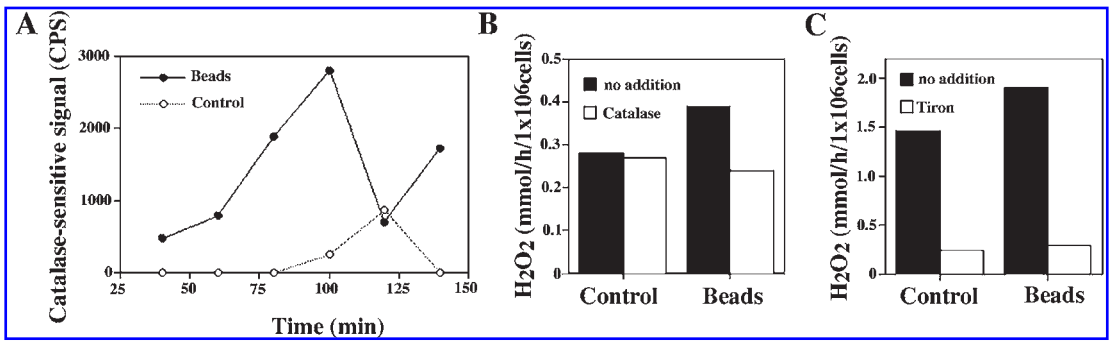

FIG. 5. Phagocytosis-induced superoxide production. (A) Time course of phagocytosis-induced H_2O_2 production. After various intervals of bead addition, H_2O_2 was measured in the extracellular beads in the presence and absence of 200 U/ml catalase. The graph displays the observed catalase-sensitive signal. **(B)** The H_2O_2 rise is abrogated by 200 U/ml catalase addition or 1 m*M* Tiron **(C)** 30 min prior to bead addition.

superoxide scavenger, reduced H_2O_2 production (Fig. 5C). These findings implicate a superoxide-generating enzyme as a source for the H_2O_2 rise induced by phagocytosis.

The role of oxidant production in bead-triggered signal transduction was evaluated with several antioxidants used to treat the cells before bead challenge. RSFs treated with 1-pyrrolidinecarbodithioic acid (PDCT) (30), Tiron, a superoxide scavenger (18), or diphenyleneiodonium (DPI), a NADPH membrane oxidase inhibitor (23) were challenged with high-density anti-α5β1-coated beads for 24 h. CL-1 determination (Fig. 6A) shows that bead-induced CL-1 expression was reduced in cells treated with the antioxidants PDCT and Tiron, but not with DPI, suggesting that although superoxide is required for high levels of collagenase induction, the source is not the NADPH membrane oxidase. Further proof of the importance of H_2O_2 production in signal transduction emerges from the finding that only cell strains able to produce ROS in response to bead phagocytosis (Fig. 6B) induce CL-1 expression (Fig. 6C). Thus, these results implicate superoxide production in response to phagocytosis as intermediaries in signal transduction.

DISCUSSION

We have previously shown that high-affinity integrin-binding ligands drive efficient phagocytosis associated with a cellular proinflammatory response (36). The current study demonstrates that integrin clustering is sufficient to drive phagocytosis coupled to signal transduction through transient superoxide production in fibroblasts. Integrin occupation is not necessary, as integrin-binding antibodies (this work and 36), as well as lectins, promote signaling coupled to phagocytosis. However, the mechanism requires the selective engagement of a restricted pool of surface proteins, because beads coated with anti-transferrin receptor antibody or polylysine (data not shown) did not induce either phagocytosis or signaling. Cell-surface protein clustering with lectins was sufficient to mediate phagocytosis and CL-1 induction. However, although WGA has been used to purify the fibronectin receptor (27), and thus binds integrins, these results do not exclude phagocytosis mediated by other cell-surface proteins as well. Nevertheless, like high-density anti-α5β1, ConA-coated beads induced RhoA recruitment, phagocytosis, and collagenase production, indicating convergence to a common pathway after protein clustering at the cell surface.

This study shows that high ligand density on the particle's surface actively drives ingestion with associated signal transduction. A clustering threshold is necessary because bivalent cross-linking by the antibody adsorbed at low concentration on the bead mediated bead binding and uptake at a slow rate (similar to fibronectin-coated beads; data not shown) without signal transduction. Integrin clustering facilitates ligand binding and outside-in signaling (16) and triggers a subset of cellular responses (22). Thus, the results of this work suggest that integrin clustering specifically recruits RhoA to mediate signal transduction and phagocytosis.

The small GTPases of the Rho family have been involved in different phagocytosis mechanisms in several cell types of myeloid origin using overexpressed constructs of the GTPases (5, 20, 22). However, few studies have examined the direct activation and recruitment of the endogenous GTPases during phagocytosis. The study of endogenous GTPases would contribute to understanding the role of RhoA in phagocytosis, which has been difficult to prove, because function interference with C3 toxin leads to overall reduced ligand binding, adhesion, and spreading (25). The time-course analysis for endogenous GTPase recruitment to the plasma membrane showed predominant RhoA recruitment at an early step during integrin-mediated phagocytosis, whereas Rac recruitment increased later, possibly on engulfment progression. Addition of phagocytosis competent beads at 4ºC, to allow membrane protein aggregation but prevent membrane fusion events, induced RhoA membrane translocation, indicating that particle ingestion is by itself not necessary to recruit this GTPase, but forming a signaling and phagocytosis competent complex. Dominant negative RhoA inhibited phagocytosis likely because of interference with the function of the endogenous GTPase in assembly and maintenance of adhesion complexes required for phagocytosis and signaling. Similarly, RhoA is involved in focal adhesion complex maturation and tension development during cell spreading (8, 29).

Cell spreading, which has been compared to phagocytosis of a particle too big to be ingested (13), induces initially Cdc42 and Rac activation with an accompanying inhibition in RhoA activity during ruffle formation and spreading (26, 28). However, the results shown in this article propose that RhoA is recruited and activated by the high density of the ligand presented to the integrins. RhoA activation in response to high-density coated beads is consistent with studies showing that cells plated on a high-density coated substratum activate RhoA along with changes in cell behavior, including cell protrusion inhibition with a halt in cell migration (9). An additional variable that could determine selective RhoA recruitment and activation is the stiffness of the bead. Interestingly, the rigidity of the particle changes the phagocytic mechanism as Rac regulates Fc-dependent phagocytosis of soft beads, but not of rigid beads (4).

Rac immunolocalized surrounding the beads and was translocated to the membrane at low levels at all times during phagocytosis, but reached a peak at a later time point than RhoA, when the beads are still surrounded with actin. Function interference with Rac by expression of RacN17 mutant did not impair complete bead ingestion, further pointing to a role for this GTPase at a later step in the process and excluding a significant role of this GTPase before 30 min, which was the earliest time point analyzed. This mechanism contrasts with the reported Rac involvement in collagen phagocytosis (3) and in invasin-dependent bacterial uptake (37). Given the findings presented here, Rac could be involved in a mechanism to ingest low-affinity ligands such as fibronectin or low-density antibody-coated beads. These beads are ingested over time, but uptake could proceed through an alternate pathway without RhoA recruitment and CL-1 gene expression induction.

The morphological analysis showed that, upon bead binding, the membrane crawls over the bead engulfing the particle by a zipper-like phagocytic mechanism. Discrete protrusions different from ruffles and similar to microvilli lead the advancing membrane. Although Cdc42 could mediate protrusion extension (further studies are needed to evaluate the par-

ticipation of this GTPase), RhoA can mediate apical protrusion formation via ERM proteins (ezrin, radixin, and moesin) in response to lysophosphatidic acid (31). Ezrin associates with phagosomes during latex bead ingestion and can promote *de novo* actin assembly (10); however, the role of ERM proteins during phagocytosis remains unknown. Further studies are needed to test whether RhoA is involved in the morphological phenotype of phagocytosis.

In addition to orchestrating the actin cytoskeleton changes implicated in phagocytosis, RhoA could mediate signal transduction to induce the superoxide burst independently of Rac activity. This role for RhoA is supported by the results showing that RacN17 does not interfere with phagocytosis (this work) or with phagocytosis-coupled signaling (36). We have previously shown that expression of constitutively activated Rho is sufficient to induce CL-1 and H_2O_2 production in this cell type (35); thus, this GTPase is an attractive intermediary to couple phagocytosis to signal transduction.

Phagocytosis driven by integrin clustering triggered a superoxide burst. This transient change in the cellular redox state was necessary for collagenase induction through IL-1 production, as antioxidants reduced protease induction without affecting bead uptake (data not shown). The superoxide production significance is underscored by the absence of respiratory burst in cells unable to induce collagenase in response to bead ingestion and by the observation that exogenous H_2O_2 is sufficient to induce CL-1. The observations that phagocytosis and signaling occur independently of Rac activity and the insensitivity to DPI suggest an alternative source for ROS production to a membrane-associated NADPH oxidase. Further experiments are required to demonstrate this novel mechanism.

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ABBREVIATIONS

AP-2, activator protein-2; BSA, bovine serum albumin; CL-1, collagenase 1; ConA, concanavalin A; DME, Dulbecco's modified Eagle medium H-21; DPI, diphenyleneiodonium; FBS, fetal bovine serum; GFP, green fluorescent protein; H_2O_2, hydrogen peroxide; IL-1, interleukin-1; LH, lactoalbumin hydrolysate; PBS, phosphate-buffered saline; PDCT, 1-pyrrolidinecarbodithioic acid; RSF, rabbit synovial fibroblast; WGA, wheat germ agglutinin.

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