The proinflammatory effects of CD95L have raised questions about its contribution to immune privilege (*3, 5, 16*), tolerance, and graft survival (*8–10*). Although it triggers apoptosis in T lymphocytes (*2*) (Fig. 2D), CD95L unexpectedly stimulated PMN activation. As described for other PMN stimulants (*17*), this activity is dependent on its ability to enhance p38 MAPK activity (Fig. 3). PMNs directly mediate cytolysis of $CD95L^+$ cells, and this effect is inhibited by TGF-β, which is present in the aqueous humor (*13*). TGF-β also plays a role in immune tolerance through this mechanism and its effect on T cell proliferation. Although it inhibits p38 MAPK activity in other cells (*18*), its effect on innate immune responses mediated by neutrophils was previously unknown. Together CD95L and TGF-β promote lymphocyte clonal deletion and suppress inflammation. Thus, providing a microenvironment that includes both of these elements may aid in amelioration of allograft rejection at nonprivileged sites. Both CD95L and TGF-β1 have also been detected in tumors, particularly in the extracellular matrix, where they may inhibit immunologic recognition of malignancies (*6, 19*). Successful immune therapies for cancer are likely to require strategies to reverse this mechanism of immune suppression in vivo.

References and Notes

1. N. Itoh *et al*., *Cell* **66**, 233 (1991); T. Suda, T. Takahashi, P. Golstein, S. Nagata, *ibid.* **75**, 1169 (1993).
2. T. Brunner *et al*., *Nature* **373**, 441 (1995); J. Dhein, H. Walczak, C. Baumler, K.-M. Debatin, P. H. Krammer, *ibid*., p. 438; S. Ju *et al*., *ibid*., p. 444, S. Nagata and P. Golstein, *Science* **267**, 1449 (1995).
3. T. S. Griffith, T. Brunner, S. M. Fletcher, D. R. Green, T. A. Ferguson, *Science* **270**, 1189 (1995).
4. H. Arai, S. Y. Chan, D. K. Bishop, G. J. Nabel, *Nature Med.* **3**, 843 (1997).
5. D. Bellgrau *et al*., *Nature* **377**, 630 (1995).
6. J. O'Connell, G. C. O'Sullivan, J. K. Collins, F. Shanahan, *J. Exp. Med.* **184**, 1075 (1996); M. Hahne *et al*., *Science* **274**, 1363 (1996); S. Strand *et al*. *Nature Med.* **2**, 1361 (1996).
7. H. T. Lau, M. Yu, A. Fontana, C. J. Stoeckert Jr., *Science* **273**, 109 (1996).
8. H. Arai, D. Gordon, E. G. Nabel, G. J. Nabel, *Proc. Natl. Acad. Sci. U.S.A.* **94**, 13862 (1997).
9. K.-I. Seino, N. Kayagaki, K. Okumura, H. Yagita, *Nature Med.* **3**, 165 (1997).
10. J. Allison, H. M. Georgiou, A. Strasser, D. L. Vaux, *Proc. Natl. Acad. Sci. U.S.A.* **94**, 3943 (1997); A. V. Chervonsky *et al*., *Cell* **89**, 17 (1997); S.-M. Kang *et al*., *Nature Med.* **3**, 738 (1997); S.-M. Kang *et al*., *Science* **278**, 1322 (1997).
11. D. R. Green and C. F. Ware, *Proc. Natl. Acad. Sci. U.S.A.* **94**, 5986 (1997).
12. Eight-week-old female BALB/c mice from Jackson Laboratory were injected with CT26-CD95L or CT26-neo cells (10^5) in the right anterior chamber of the eye or subcutaneously into the flank after cells were harvested with trypsin, incubated in growth medium at 37°C for 1 hour, washed with phosphate-buffered saline, and resuspended in saline.
13. J. W. Streilein, G. A. Wilbanks, A. Taylor, S. Cousins, *Curr. Eye Res.* **11**, 41 (1992); M. Muhaya *et al*., *Clin. Exp. Immunol.* **111**, 123 (1998).
14. J. J. Chen, Y. Sun, G. J. Nabel, unpublished observations.
15. J. Raingeaud *et al*., *J. Biol. Chem.* **270**, 7420 (1995).
16. P. M. Stuart *et al*., *J. Clin. Invest.* **99**, 396 (1997).
17. N. Nahas, T. F. Molski, G. A. Fernandez, R. I. Sha'afi, *Biochem. J.* **318**, 247 (1996); Y. L. Zu *et al*., *J. Immunol.* **160**, 1982 (1998); J. A. Nick *et al*., *J. Clin. Invest.* **99**, 975 (1997).
18. E. Berrou *et al*., *Biochem. J.* **316**, 167 (1996); D. M. Rose, B. W. Winston, E. D. Chan, D. W. Riches, P. M. Henson, *Biochem. Biophys. Res. Commun.* **238**, 256 (1997); M. Lee, C. Hwang, J. Lee, H. Slavkin, D. Warburton, *Am. J. Physiol.* **273**, L374 (1998); K. Pazdrak, L. Justement, R. Alam, *J. Immunol.* **155**, 4454 (1998).
19. W. H. Daughaday and T. F. Deuel, *Endocrinol. Metab. Clin. North Am.* **20**, 539 (1991).
20. W. C. Liles, J. A. Ledbetter, A. W. Waltersdorph, S. J. Klebanoff, *J. Immunol.* **155**, 2175 (1995).
21. J. Varani *et al*., *Am. J. Pathol.* **135**, 435 (1989); D. G. Okrent, A. K. Lichtenstein, T. Ganz, *Am. Rev. Respir. Dis.* **141**, 179 (1990).
22. K. Horgan and S. Shaw, *Curr. Protoc. Immunol.* **7**, 4 (1998).
23. Y. Luo and M. E. Dorf, *ibid.* **3**, 20 (1998).
24. Z. Yang *et al*., *Science* **279**, 1034 (1998).
25. B. A. Arrick *et al*., *J. Cell Biol.* **118**, 715 (1992).
26. T. M. Kinsella and G. P. Nolan, *Hum. Gene Ther.* **7**, 1405 (1996).

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Identification of Two Distinct Mechanisms of Phagocytosis Controlled by Different Rho GTPases

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The complement and immunoglobulin receptors are the major phagocytic receptors involved during infection. However, only immunoglobulin-dependent uptake results in a respiratory burst and an inflammatory response in macrophages. Rho guanosine triphosphatases (molecular switches that control the organization of the actin cytoskeleton) were found to be essential for both types of phagocytosis. Two distinct mechanisms of phagocytosis were identified: Type I, used by the immunoglobulin receptor, is mediated by Cdc42 and Rac, and type II, used by the complement receptor, is mediated by Rho. These results suggest a molecular basis for the different biological consequences that are associated with phagocytosis.

Phagocytosis is the process by which cells recognize and engulf large particles (>0.5 μm) and is important to host defense mechanisms as well as to tissue repair and morphogenetic remodeling. Two of the best characterized phagocytic receptors in macrophages, the complement receptor 3 (CR3) and Fc gamma receptors (FcγRs), are involved in the uptake of opsonized microorganisms during infection. CR3 binds C3bi on complement-opsonized targets, whereas FcγRs bind to immunoglobulin G (IgG)–coated targets. Phagocytosis by both types of receptors is driven by the reorganization of filamentous actin (F-actin), but the mechanisms of uptake appear to be different (*1, 2*). First, FcγR-mediated uptake is accompanied by pseudopod extension and membrane ruffling, whereas complement-opsonized targets sink into the cell, producing little protrusive activity (*3*). Second, FcγR ligation is accompanied by the activation of the respiratory burst (to produce reactive oxygen species) and by the production of arachidonic acid metabolites and cytokines, such as tumor necrosis factor–α. C3bi-dependent uptake occurs in the absence of any of these proinflammatory signals (*4–6*).

The Rho family of small guanosine triphosphatases (GTPases) is involved in the reorganization of filamentous actin structures in response to extracellular stimuli (*7*). Rho induces the assembly of contractile actomyosin filaments, whereas Rac and Cdc42 control actin polymerization into lamellipodial and filopodial membrane protrusions, respectively (*8, 9*). In addition, these GTPases can affect gene transcription [through the activation of nuclear factor kappa B, through the c-Jun NH_2-terminal kinase (JNK), and through the p38 mitogen-activated protein kinase (MAPK)], and Rac regulates the reduced form of nicotinamide adenine dinucleotide phosphate (NADPH) oxidase enzyme complex that is responsible for the respiratory burst (*10, 11*). We have, therefore, analyzed the relative roles of Rho, Rac, and Cdc42 in FcγR- and CR3-mediated phagocytosis.

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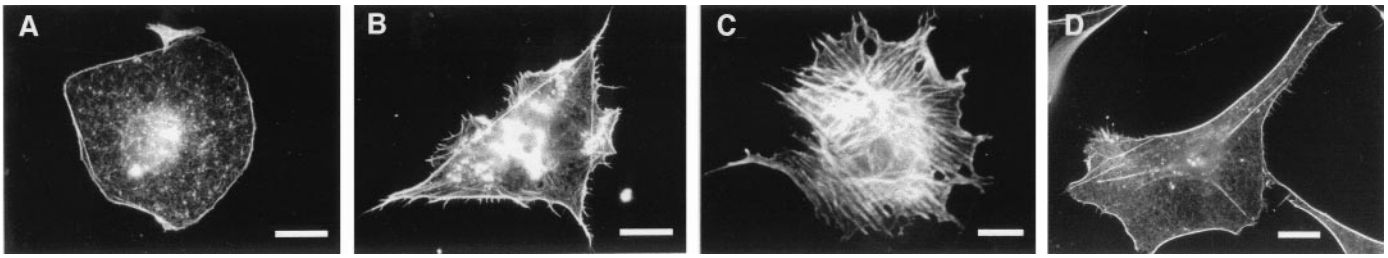
Quiescent, serum-starved Swiss 3T3 fibroblasts provide a simple model for studying the formation of polymerized actin structures that are induced by extracellular stimuli (*8, 9*). To analyze the effects of FcγR and CR3 receptor activation on actin, we microinjected quiescent cells with either a plasmid encoding the single FcγRIIA chain or with a combination of plasmids encoding the two chains of the CR3 integrin receptor (CD11b and CD18) (*12*). Surface expression of the receptors, visualized by immunofluorescence 2 hours after microinjection, did not in itself affect cell morphology or F-actin distribution (Fig. 1A). However, antibody cross-linking of FcγRII resulted in the formation (within 10 min) of filopodia, accompanied by localized ruffles (Fig. 1B). After 30 min, cells harboring cross-linked FcγR showed a contracted morphology and contained stress fibers. Antibody cross-linking of CR3 induced rapid cell contraction and F-actin reorganization into stress fibers with no evidence of filopodia or lamellipodial protrusions (Fig. 1C).

To determine whether changes in the actin cytoskeleton were mediated by the activation of Rho GTPases, we expressed the phagocytic receptors along with inhibitors of Rho, Rac, or Cdc42. Coinjection of a dominant negative Cdc42 construct blocked the formation of all filamentous actin structures upon FcγRIIA ligation (Fig. 1D), whereas dominant negative Rac blocked ruffling and stress fibers but not filopodia. With CR3, dominant negative Cdc42 or Rac constructs had no effect on the induced actin changes, whereas the Rho inhibitor (C3 transferase) blocked all actin changes. Thus, FcγRIIA cross-linking specifically results in the activation of Cdc42, which in turn activates a previously described Cdc42-Rac-Rho cascade (*8, 9*). However, cross-linking of the integrin receptor CR3 activates only Rho.

As a first step toward analyzing the role of Rho GTPases in phagocytosis, plasmids encoding FcγRII or CR3 receptors were transfected into COS cells. Cells were then presented with red blood cells (RBCs) that were opsonized with either IgG (for FcγR) or C3bi (for CR3), and the efficiency of particle internalization was determined (*13, 14*). Ninety-seven percent of FcγR-expressing COS cells were able to bind IgG-opsonized cells, and ~60% of expressing cells contained one or more internalized particles (Fig. 1E). Cotransfection of the two chains of the CR3 receptor resulted in 70% of CR3-expressing cells binding to opsonized particles, and ~50% of these showed one or more internalized particles. Both chains of the CR3 receptor were needed for the efficient binding and ingestion of complement-opsonized cells (Fig. 1E). Next, both phagocytic receptors were cotransfected with dominant negative versions of Rho, Rac, or Cdc42 or with the specific Rho inhibitor (C3 transferase). None of the inhibitors had any substantial effect on the binding of opsonized RBCs to either CR3- or FcγR-expressing COS cells (Fig. 1E). Dominant negative Cdc42 and dominant negative Rac (Fig. 1E) showed no inhibitory effect on the phagocytic behavior of CR3-transfected COS cells, whereas both dominant negative Rho and C3 transferase showed essentially complete inhibition of CR3-mediated phagocytosis (Fig. 1E). For FcγR-mediated phagocytosis, dominant negative Rho and C3 transferase had no effect, whereas dominant negative Cdc42 and dominant negative Rac abolished particle internalization (Fig. 1E). Thus, CR3-mediated phagocytosis in COS cells is mediated by Rho, and FcγR-mediated phagocytosis is mediated by a combination of Cdc42 and Rac.

To determine whether Rho GTPases control phagocytosis in professional phagocytic cells, we treated the mouse macrophage cell line J774 with toxin B from *Clostridium difficile*, an inhibitor of all members of the Rho family (*15*). A pretreatment (2 hours) with toxin B inhibited (in a dose-dependent manner) both CR3- and FcγR-mediated phagocytosis but had no effect on the initial binding of opsonized targets to J774 macrophages (*15*). To identify the specific GTPases involved, we microinjected J774 cells with plasmids that encoded inhibitors of Rho, Rac, and Cdc42 (*16*). Representative examples of the results that were obtained for FcγR-mediated phagocytosis are shown (Fig. 2, A through H). C3 transferase–expressing cells (Fig. 2, C, D, and I) or dominant negative Rho–expressing cells were as competent as control cells (Fig. 2, A, B, and I) at internalizing IgG-opsonized RBCs, which appear as swollen particles in vacuoles (*14*). In contrast, macrophages expressing dominant negative Rac (Fig. 2, E, F, and I) or dominant negative Cdc42 (Fig. 2G, H and I) were unable to carry out FcγR-mediated phagocytosis, as attested by the crenated morphology of the RBCs (*14*). As an alternative to using dominant negative proteins, the Cdc42-binding domain of a specific Cdc42 target protein, Wiscott-Aldrich syndrome protein (WASP) (*17*), was also used, which completely prevented FcγR-mediated phagocytosis (Fig. 2I). In agreement with the results presented here, an earlier report showed that both Rac



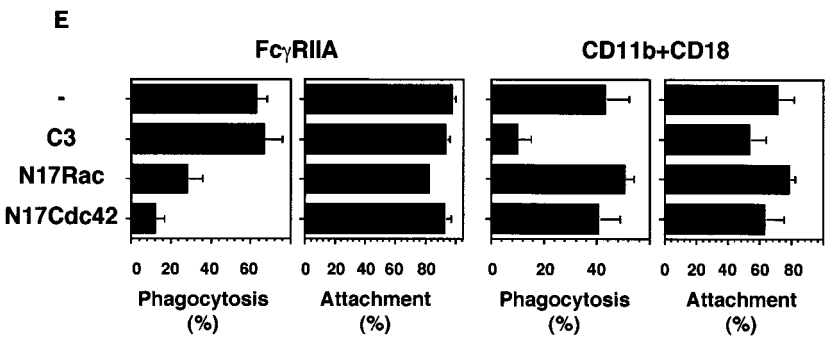


Fig. 1. Activation of Rho GTPases by FcγR and CR3 receptors in Swiss 3T3 fibroblasts and in COS cells. (**A** through **D**) Cross-linking of FcγR or CR3 receptors activates Cdc42 or Rho in Swiss 3T3 fibroblasts. F-actin distribution in microinjected (*12*) subconfluent serum-starved fibroblasts expressing FcγRII [(A), (B), and (D)] or CR3 (C), which were subjected to receptor crosslinking for 10 min [(B) and (D)] or 30 min (C), is shown. In (D), cells were coinjected with N17Cdc42. Scale bars, 10 μm. (**E**) Rho GTPases control FcγR- and CR3-mediated phagocytosis in COS cells. COS cells were cotransfected (*13*) with phagocytic receptors [that is, FcγRIIA or CR3 (CD11b+CD18)] and with either an empty vector (−) or with vectors expressing Myc-tagged versions of C3 transferase (C3), dominant negative Rac (N17Rac), or dominant negative Cdc42 (N17Cdc42). Phagocytosis assays were then performed ($n \geq 3$) with appropriately opsonized RBCs; ≥100 transfected cells were scored for their ability to bind (right) or phagocytose (left) RBCs (mean ± SEM).

and Cdc42 are required for FcγR uptake (*18*). Another report, however, suggested that Rho is also required for FcγR uptake, but in those experiments, Rho inhibition prevented even the binding of opsonized particles (*19*).

Representative examples of the results that were obtained for CR3-mediated phagocytosis are shown (Fig. 3). In this case, macrophages expressing dominant negative Rac (Fig. 3, E, F, and I) or dominant negative Cdc42 (Fig. 3, G through I) were as competent as control cells [Fig. 3, A, B, and I (left)] at internalizing complement-opsonized RBCs. C3 transferase–expressing cells (Fig. 3I) or dominant negative Rho–expressing cells (Fig. 3, C and D), however, were unable (up to 90% inhibition) to carry out CR3-mediated phagocytosis. Thus, FcγR receptors activate Cdc42 and Rac, and both these GTPases (but not Rho) are required for phagocytosis. CR3-mediated phagocytosis occurs through a distinct cellular mechanism and is dependent on Rho (but not dependent on Rac or Cdc42).

To determine whether Rho GTPases are differentially recruited to phagosomes surrounding IgG- or complement-opsonized particles, we analyzed their association with internalized particles. In the absence of antibody reagents that were sufficiently sensitive to visualize all three endogenous Rho GTPases in J774 macrophages, the behavior of wild-type, tagged GTPases was analyzed in the COS cell phagocytosis assay. Opsonized particles that were internalized through either CR3 or FcγR expressed in COS cells were both associated with F-actin. Coexpression of the FcγR receptor with Myc-tagged versions of wild-type Rho (Fig. 4B), Rac (Fig. 4D), or Cdc42 revealed that all three GTPases were recruited along with actin (Fig. 4, A and C) to the IgG-opsonized particles. However, when the experiment was repeated with CR3-expressing COS cells, only Rho (Fig. 4F), and not Rac (Fig. 4H) or Cdc42, colocalized with F-actin (Fig. 4, E and G).

Thus, Cdc42 is activated after FcγRII ligation, and this results in the activation of a Rac-Rho cascade and the concomitant association of all three GTPases with the phagosome. In this case, both Cdc42 and Rac, but not Rho, activities are required for particle internalization. By contrast, CR3-induced phagocytosis only results in the activation of Rho, and only Rho is recruited to phagosomes surrounding the complement-opsonized particles. The fact that Rho, Rac, and Cdc42 each interact with distinct effector molecules and affect the assembly and organization of filamentous actin in very differ-

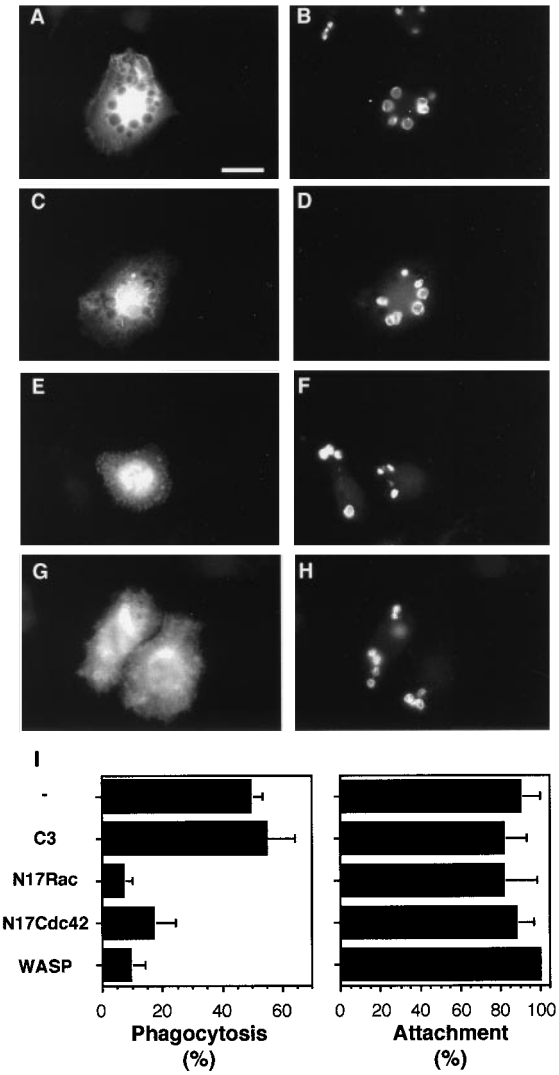


Fig. 2 (left). Cdc42 and Rac are necessary for FcγR-mediated phagocytosis in macrophages. (**A** through **H**) FcγR-mediated phagocytosis in J774.A1 cells that were microinjected with biotin dextran and control plasmid [(A) and (B)], Myc-tagged C3 transferase [(C) and (D)], N17Rac [(E) and (F)], or N17Cdc42 [(G) and (H)]. Microinjected cells were detected by costaining with cascade blue–conjugated avidin (A) or 9E10 anti-Myc [(C) (E), and (G)], and RBCs were visualized with anti-rabbit IgG [(B), (D), (F), and (H)]. Scale bar, 10 μm. (**I**) Quantitation of the J774.A1 cell ability to bind (right) and phagocytose (left) IgG-opsonized RBCs and the effect of an empty vector (−) or the effect of microinjected expression vectors encoding C3 transferase (C3), dominant negative Rac (N17Rac), dominant negative Cdc42 (N17Cdc42), or Cdc42-binding domain (amino acids 201 through 321) of WASP. Data shown are the mean ± SEM of three to five independent experiments.

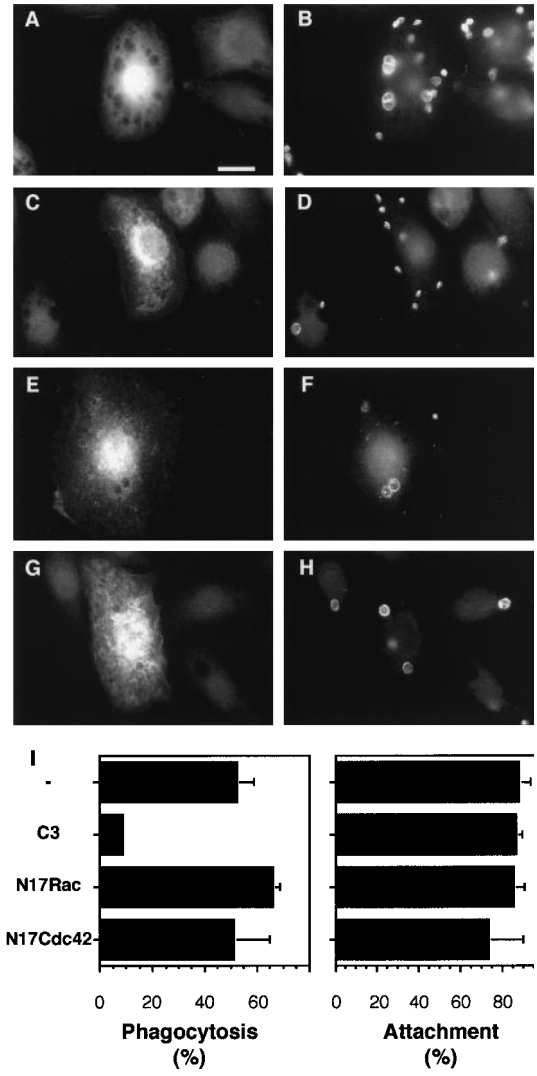


Fig. 3 (right). Rho is necessary for CR3-mediated phagocytosis in macrophages. (**A** through **H**) CR3-mediated phagocytosis in J774.A1 cells that were microinjected with biotin dextran and control plasmid [(A) and (B)], Myc-tagged N19Rho [(C) and (D)], N17Rac [(E) and (F)], or N17Cdc42 [(G) and (H)]. Microinjected cells were detected by costaining with cascade blue–conjugated avidin (A) or 9E10 [(C), (E), and (G)], and RBCs were visualized with anti-rabbit IgG [(B), (D), (F), and (H)]. Scale bar, 10 μm. (**I**) Quantitation of the microinjected J774.A1 cells ability to bind (right) and phagocytose (left) C3bi-opsonized RBCs and the effect of an empty vector (−) or the effect of microinjected constructs encoding C3 transferase (C3), dominant negative Rac (N17Rac), or dominant negative Cdc42 (N17Cdc42). Data shown are the mean ± SEM of three to five independent experiments.

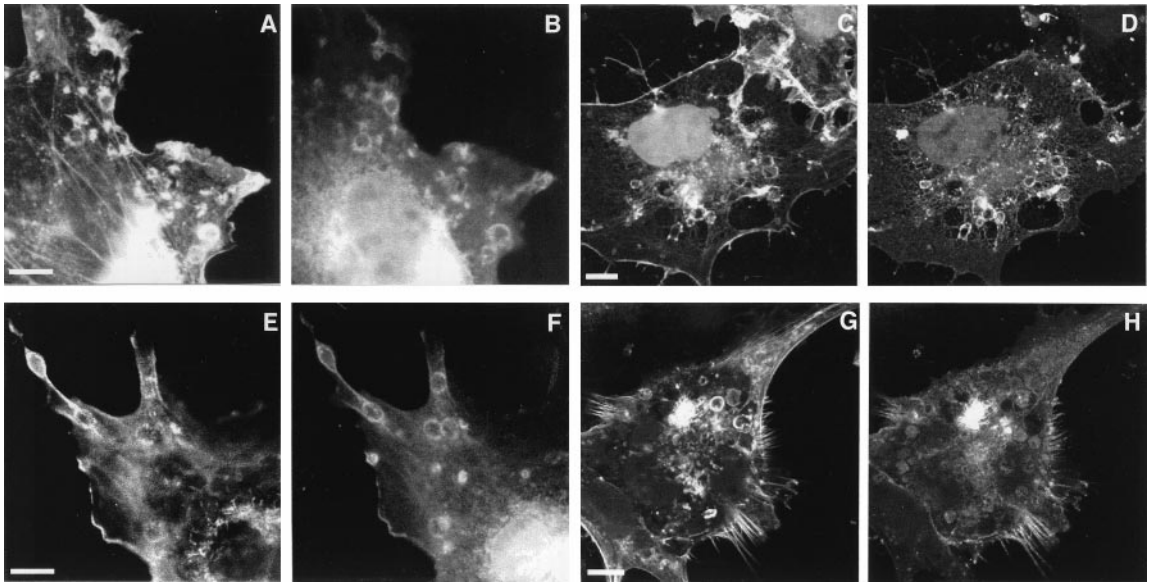


Fig. 4. Differential recruitment of Rho and Rac to nascent phagosomes during complement- and IgG-dependent phagocytosis. COS cells were cotransfected with (**A** through **D**) FcγRIIA or (**E** through **H**) CR3 and wild-type Myc-tagged versions of Rho [(A), (B), (E), and (F)] and Rac [(C), (D), (G), and (H)]. Cells were challenged with the appropriate opsonized targets, and immunofluorescence was performed to detect Myc, that is, Rho GTPases [(B), (D), (F), and (H)] and F-actin [(A), (C), (E), and (G)]. Cells that were cotransfected with wild-type Rac [(C), (D), (G), and (H)] were examined with confocal microscopy. Scale bar, 10 μm.

ent ways suggests that the biochemical mechanisms of particle uptake are quite different (*20*). We propose that the Cdc42/Rac-dependent uptake (typified by FcγR) be termed type I phagocytosis and the Rho-dependent uptake (typified by CR3) be termed type II. The recruitment of Rho, Rac, and Cdc42 in type I (but only Rho in type II) phagocytosis suggests a molecular explanation not only for the well-known morphological differences observed between FcγR- and CR3-mediated phagocytosis but, more important, for the different associated biological responses (*2*–*6*). Because Rac is an essential regulatory component of the NADPH oxidase enzyme complex and because both Rac and Cdc42 activate the JNK and p38 MAPK pathways, the reason why type I, but not type II, mediated phagocytosis is accompanied by an inflammatory response could be explained (*11, 21*). Finally, type II phagocytosis provides a possible explanation for the lack of an inflammatory response associated with the uptake of apoptotic cells and with the invasion of macrophages by pathogenic microorganisms such as *Leishmania major* and *Mycobacterium leprae* (*22*).

References and Notes

1. L.-A. H. Allen and A. Aderem, *Curr. Opin. Immunol.* **8**, 36 (1996).
2. S. L. Newman, L. K. Mikus, M. A. Tucci, *J. Immunol.* **146**, 967 (1991); L.-A. H. Allen and A. Aderem, *J. Exp. Med.* **184**, 627 (1996).
3. G. Kaplan, *Scand. J. Immunol.* **6**, 797 (1977).
4. A. A. Aderem, S. D. Wright, S. C. Silverstein, Z. A. Cohn, *J. Exp. Med.* **161**, 617 (1985).
5. S. D. Wright and S. C. Silverstein, *ibid.* **158**, 2016 (1983); K. Yamamoto and R. B. Johnston, *ibid.* **159**, 405 (1984).
6. P. R. Scholl, D. Ahern, R. S. Geha, *J. Immunol.* **149**, 1751 (1992); M. Stein and S. Gordon, *Eur. J. Immunol.* **21**, 431 (1991).
7. A. Hall, *Science* **279**, 509 (1998).
8. A. J. Ridley and A. Hall, *Cell* **70**, 389 (1992); A. J. Ridley, H. F. Paterson, C. L. Johnston, D. Diekmann, A. Hall, *ibid.*, p. 401; R. Kozma, S. Ahmed, A. Best, L. Lim, *Mol. Cell. Biol.* **15**, 1942 (1995).
9. C. D. Nobes and A. Hall, *Cell* **81**, 53 (1995).
10. R. Perona *et al.*, *Genes Dev.* **11**, 463 (1997).
11. O. A. Coso *et al.*, *Cell* **81**, 1137 (1995); A. Minden, A. Lin, F.-X. Claret, A. Abo, M. Karin, *ibid.*, p. 1147; H. Teramoto *et al.*, *J. Biol. Chem.* **271**, 25731 (1996); A. Abo *et al.*, *Nature* **353**, 668 (1991); T. G. Gabig, C. D. Crean, P. L. Mantel, R. Rosli, *Blood* **85**, 804 (1995).
12. cDNAs encoding human FcγRIIA, CD11b, and CD18 were subcloned into pRK5. Subconfluent, serum-starved Swiss 3T3 cells were prepared as described [N. Lamarche *et al.*, *Cell* **87**, 519 (1996)]. Eukaryotic expression vectors (0.1 mg/ml) encoding phagocytic receptors and Myc-tagged GTPase constructs were microinjected into the nucleus of at least 50 cells over a period of 10 min, in a temperature- and CO_2-controlled chamber. Cells were returned to the incubator for 3 hours for optimal expression, incubated for 20 min at 4°C with monoclonal antibodies (mAbs) (10 μg/ml) to human CD11b (clone 44, Pharmingen, San Diego, CA) or to human FcγRIIA (clone IV.3, Medarex, Annandale, NJ), washed in ice-cold serum-free medium, and further incubated at 37°C with donkey anti-mouse IgG (15 μg/ml). Cells were rinsed in serum-free medium, fixed for 10 min in freshly prepared cold 4% (w/v) paraformaldehyde, and processed for immunofluorescence as described (*9*). Biotin-conjugated mAb 9E.10, followed by cascade blue–conjugated avidin, was used to detect Myc-tagged constructs. Anti-CD11b or anti-FcγRIIA, followed by fluorescein isothiocyanate (FITC)–conjugated $F(ab')_2$ antimouse IgG, was used to detect receptor expression. Rhodamine-labeled phalloidin (Sigma) was used to visualize F-actin. To block the nonspecific binding of antibodies to the Fc receptor, we performed incubations with antibodies when appropriate in the presence of excess human IgGs.
13. COS-1 cells were transfected by the DEAE-dextran method as described [M. F. Olson, A. Ashworth, A. Hall, *Science* **269**, 1270 (1995)]. Plasmid amounts in each 10-cm petri dish were as follows: 4 μg of pRK5-FcγRIIA, or 2 μg of pRK5-CD11b and 2 μg of pRK5-CD18, with either 1 μg of pRK5myc, 1 μg of pEFmyc-C3 transferase, or one of the Rho, Rac, and Cdc42 constructs (1 μg). Twenty-four hours later, cells were serum-starved for 16 hours before phagocytic challenge and immunofluorescence. Sheep RBCs (Cappel, Thame, UK) were coated with IgG (IgG-RBCs) or with C3bi (C3bi-RBCs) as follows. RBCs in gelatin veronal buffer were incubated in the presence of subagglutining concentrations of IgG or IgM antibodies to rabbit RBCs (Cappel). RBCs were then washed, and IgM-coated RBCs were further opsonized by incubation with 10% (v/v) C5-deficient human serum for 20 min at 37°C. Under these conditions, C3b is rapidly fixed to IgM-coated RBCs and is completely converted into C3bi [S. L. Newman and L. K. Mikus, *J. Exp. Med.* **161**, 1414 (1985)]. Opsonization was checked by fluorescence after incubation with FITC-conjugated anti-rabbit IgG or with goat anti-C3 (Sigma) followed by FITC-conjugated anti-goat IgG. All IgG-RBCs and C3bi-RBCs appeared to be uniformly labeled. After a final wash, the RBCs were suspended in Hepes-buffered Dulbecco's modified Eagle's medium (DMEM) (Gibco-BRL) and added to cells at a ratio of ~10 RBCs per cell. For phagocytosis assays, complement-opsonized RBCs were allowed to interact for 20 min at 37°C with transfected COS cells. IgG-opsonized RBCs were allowed to adhere to cells for 15 min at 4°C, then unbound RBCs were washed off and phagocytes were further incubated for 15 min at 37°C in Hepes-buffered DMEM. After fixation and immunofluorescence, transfected COS cells were scored for their ability to bind or phagocytose RBCs (*14*). COS cells were examined for phagocytic receptor (control) or Myc expression (cotransfection with Myc-tagged constructs), and only the positive cells were analyzed for RBC binding or phagocytosis. Positive cells represented ~20% of the total number of COS cells per cover slip, that is, ≥100 cells per cover slip. Rhodamine-conjugated donkey anti-rabbit IgGs were used to detect RBCs. An attachment was defined by the ability of injected or transfected cells to bind one or more RBCs. Phagocytosis was defined by the ability of transfected cells to internalize one or more RBCs. No difference was observed in the total number of particles binding to the different transfectant populations.
14. S. Greenberg, J. el Khoury, E. Kaplan, S. C. Silverstein, *J. Immunol. Methods* **139**, 115 (1991). Surface-bound particles appear to be small, have a crenated appearance, are readily distinguished from internalized particles that are enclosed in a vacuole, and have a swollen circular appearance. In our hands, this method and the counting of RBC stained pre- and post-permeabilization with different conjugates resulted in similar quantitative results in COS-1 and J774 cells.
15. I. Just *et al.*, *Nature* **375**, 500 (1995). Pretreatment (2 hours) of J774.A1 cells with toxin B inhibited (in a dose-dependent manner) both CR3- and FcγR-mediated phagocytosis [maximal reductions of 92 and 88%, respectively, with toxin B (10 ng/ml)] but had no effect on the initial binding of opsonized targets to J774 macrophages.
16. J774.A1 cells, grown in DMEM and supplemented

with 10% heat-inactivated fetal calf serum (Sigma) and 5% penicillin/streptomycin (Gibco-BRL), were plated overnight on acid-washed glass cover slips (13 mm in diameter) in four-well plates at a density of 10^5 cells per milliliter in each well. Cells were serum-starved immediately before microinjection. Biotin dextran (2.5 mg/ml) (Molecular Probes, Eugene, OR) was injected alone or with eukaryotic expression vectors (0.1 mg/ml) encoding Myc-tagged GTPase constructs into the nucleus of at least 50 cells over a period of 10 min. Cells were returned to the incubator for 3 hours for optimal expression. RBCs were opsonized, and phagocytic assay and immunofluorescence were performed as described (*13*). The two modifications that we introduced were (i) the preactivation of J774 cells for 15 min at 37°C with phorbol 12-myristate 13-acetate (150 ng/ml) in serum-free medium before the phagocytic challenge with CR3 targets and (ii) the visualization of microinjected cells after staining with cascade blue–conjugated avidin (Molecular Probes). To block nonspecific binding of antibodies to the Fc receptor, we performed incubations with antibodies in the presence of excess human or murine IgGs. All injected (cascade blue positive) J774.A1 control cells or all Myc-expressing macrophages were assessed (that is, ≥50 cells per condition). Microinjection did not affect viability or morphology nor did it interfere with the cell's ability to bind targets. The percentage of phagocytosis-competent cells was similar in uninjected cells and cells that were injected with biotin dextran.

17. K. Nagata, M. Driessens, N. Lamarche, J. Gorski, A. Hall, *J. Biol. Chem.* **273**, 15453 (1998).
18. D. Cox *et al*., *J. Exp. Med.* **186**, 1487 (1997).
19. D. J. Hackam, O. D. Rotstein, A. Schreiber, W.-J. Zhang, S. Grinstein, *ibid.*, p. 955.
20. L. Van Aelst and C. D'Souza-Schorey, *Genes Dev.* **11**, 2295 (1997).
21. K. L. Rhoades, S. H. Golub, J. S. Economou, *J. Biol. Chem.* **267**, 22102 (1992); J. C. Lee *et al*., *Nature* **372**, 739 (1994).
22. V. A. Fadok *et al*., *J. Clin. Invest.* **101**, 890 (1998); D. M. Mosser and P. J. Edelson, *J. Immunol.* **135**, 2785 (1985); *Nature* **327**, 329 (1987); T. J. Holzer, K. E. Nelson, R. G. Crispen, B. R. Andersen. *Infect. Immun.* **51**, 514 (1986); L. S. Schlesinger and M. A. Horwitz, *J. Clin. Invest.* **85**, 1304 (1990).
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Control of Cyclin Ubiquitination by CDK-Regulated Binding of Hct1 to the Anaphase Promoting Complex

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Proteolysis of mitotic cyclins depends on a multisubunit ubiquitin–protein ligase, the anaphase promoting complex (APC). Proteolysis commences during anaphase, persisting throughout G_1 until it is terminated by cyclin-dependent kinases (CDKs) as cells enter S phase. Proteolysis of mitotic cyclins in yeast was shown to require association of the APC with the substrate-specific activator Hct1 (also called Cdh1). Phosphorylation of Hct1 by CDKs blocked the Hct1-APC interaction. The mutual inhibition between APC and CDKs explains how cells suppress mitotic CDK activity during G_1 and then establish a period with elevated kinase activity from S phase until anaphase.

Entry into anaphase and exit from mitosis are promoted by APC-dependent proteolysis of regulatory proteins (*1*). Sister chromatid separation requires Pds1 degradation shortly before anaphase onset, whereas Cdk1 inactivation during late anaphase involves proteolysis of mitotic cyclins such as Clb2. How activity of the APC toward different substrates is regulated during the cell cycle is unclear. The APC itself might be regulated, because the cyclin ubiquitination activity associated with purified APC fluctuates during the cell cycle (*2, 3*). APC-dependent proteolysis requires two related proteins containing Trp-Asp repeats which function as substrate-specific activators. Cdc20 promotes degradation of "early" substrates such as Pds1 and Hct1 promotes degradation of "late" substrates such as Clb2 (*4–6*). In yeast, there is an inverse correlation between Cdk1 activity and degradation of mitotic cyclins (*7*). Ectopic inhibition of Cdk1 induces precocious cyclin degradation, suggesting a role for Cdk1 in the inhibition of cyclin proteolysis from S phase until anaphase (*8*). However, the relevant Cdk1 substrate has not been identified.

To test whether Hct1 is needed for cyclin ubiquitination, we incubated extracts from G_1-arrested wild-type and *hct1* mutant cells with Clb2 and Clb3 (*9*). Wild-type extracts supported destruction box–dependent cyclin ubiquitination, whereas *hct1* mutant extracts were as defective in this reaction as extracts from a *cdc16-123* mutant that contains a defective APC subunit (Fig. 1). Thus, Hct1 was required for APC-mediated cyclin ubiquitination.

To test whether Hct1 associated with the APC, we constructed *CDC16-HA3* strains containing Hct1 variants with Myc epitopes at the NH_2-terminus (Myc9-Hct1) or the COOH-terminus (Hct1-Myc9) (*10*). *HCT1-myc9* strains were defective in the degradation of Clb2 and Clb3, whereas Myc9-Hct1 was fully functional. Cdc16-HA3 coprecipitated with Myc9-Hct1 but not with Hct1-Myc9 in extracts prepared from cycling or G_1-arrested cells (Fig. 2A) (*11*). Another APC subunit, Cdc23-HA3, also coprecipitated with Myc9-Hct1 but not with Hct1-Myc9 (*12*). The correlation between Hct1 function and coprecipitation with APC subunits suggests that cyclin ubiquitination depends on an Hct1-APC interaction.

Myc9-Hct1 was not associated with Cdc16-HA3 in extracts from cells arrested in S phase by hydroxyurea or in M phase by nocodazole (Fig. 2A). Cdc20, in contrast, was associated with APC subunits in both extracts (Fig. 2B). To test whether the Hct1-APC interaction was regulated during an unperturbed cell cycle, we measured the association between Myc18-Hct1 and Cdc16-HA3 in cells synchronized by centrifugal elutriation (Fig. 2C) (*13*). Hct1 was associated with Cdc16 during G_1 but not during the S, G_2, and M phases (*14*). Dissociation of Hct1 from the APC correlated with appearance of the S phase promoting Clb5-Cdk1 activity. Thus, the Hct1-APC interaction was cell cycle–regulated.

The Hct1-APC interaction occurred only

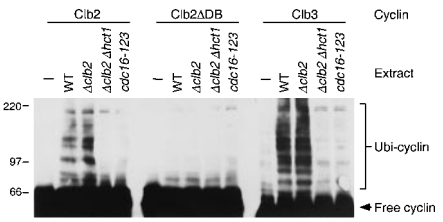

Fig. 1. Requirement of Hct1 for ubiquitination of mitotic cyclins. Strains (*MATa Δpep4 Δbar1*) were arrested in G_1 with α factor at 25°C and shifted to 37°C for 30 min. Protein extracts were incubated with adenosine 5′-triphosphate (ATP) and HA3-tagged cyclins (*9*). Clb2ΔDB lacks the destruction box. Cyclin-ubiquitin conjugates were detected by immunoblotting with an antibody to the HA epitope. Molecular sizes in kilodaltons are indicated on the left. Δ*hct1* mutants are partially resistant to α factor. To allow complete arrest in G_1, *CLB2* was deleted (*4*).

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