Brief Definitive Report

Tyrosine Phosphorylation Is Required for Fc Receptor–mediated Phagocytosis in Mouse Macrophages

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Summary

Although Fc receptor–mediated phagocytosis is accompanied by a variety of transmembrane signaling events, not all signaling events are required for particle ingestion. For example, Fc receptor–mediated phagocytosis in mouse inflammatory macrophages (Di Virgilio, F., B. C. Meyer, S. Greenberg, and S. C. Silverstein. 1988. *J. Cell Biol.* 106:657; Greenberg, S., J. El Khoury, F. Di Virgilio, and S. C. Silverstein. 1991. *J. Cell Biol.* 113:757) and neutrophils (Della Bianca, V., M. Grzeskowiak, and F. Rossi. 1990. *J. Immunol.* 144:1411) occurs in the absence of cytosolic calcium transients. We sought to identify transmembrane signaling events that are essential for phagocytosis. Here we show that tyrosine phosphorylation is an early event after Fc receptor ligation in mouse inflammatory macrophages, and that the formation of tyrosine phosphoproteins coincides temporally with the appearance of F-actin beneath phagocytic cups. The distribution of tyrosine phosphoproteins that accumulated beneath phagocytic cups was punctate and corresponded to areas of high ligand density on the surface of the antibody-coated red blood cells, which provided the phagocytic stimulus. A tyrosine kinase inhibitor, genistein, but not several inhibitors of protein kinase C, blocked the appearance of tyrosine phosphoproteins as assessed by immunofluorescence, the focal accumulation of F-actin beneath immunoglobulin G-opsonized particles, and the ingestion of these particles as well. We suggest that tyrosine phosphorylation is a critical signaling event that underlies Fc receptor–mediated phagocytosis in mouse macrophages, and is necessary for the engulfment per se.

Phosphorylation of tyrosine residues in proteins is a well-characterized event after ligation of growth factor receptors containing intrinsic tyrosine kinase domains, and after activation of other receptors that lack such domains, such as the TCR (reviewed in reference 1). Signal transduction mediated by Fc receptors is less well characterized. Recent studies suggest that increased tyrosine phosphorylation accompanies activation of Fc receptors in neutrophils (2) and platelets (3), and is required for Fc receptor–mediated cytotoxicity in NK cells (4). It is not known, however, whether tyrosine phosphorylation is necessary for any of the physiological functions mediated by Fc receptors in phagocytic leukocytes. Of the various other kinase activities that are associated with Fc receptor activation, including protein kinase C (5), casein kinase II (6), and cAMP-dependent protein kinase (7), only protein kinase C has been directly implicated in Fc receptor–mediated phagocytosis (8). To examine the roles of tyrosine kinases and protein kinase C in the ingestion of IgG-coated particles by mouse macrophages, we used genistein, a specific tyrosine kinase inhibitor, and various inhibitors of protein kinase C. We show that genestein, but not inhibitors of protein

tein kinase C, inhibits both the focal accumulation of tyrosine phosphoproteins and F-actin beneath sites of attachment of IgG-coated SRBC to mouse macrophages, and reversibly blocks the ingestion process.

Materials and Methods

Cells and Reagents. Mouse macrophages (thio-macrophages) were harvested from the peritoneal cavities of female C57BL6/J mice and maintained as adherent cultures as described (9). mAb PY-20 was obtained from ICN Biomedicals (Costa Mesa, CA). Genestein and herbimycin A were from Gibco Laboratories (Grand Island, NY). Fluorescein-phalloidin and Slow-fade were from Molecular Probes (Eugene, OR). Rhodamine-conjugated F(ab')₂ fragments of goat anti–mouse IgG were from Boehringer Mannheim Biochemicals (Indianapolis, IN). Fluorescein-conjugated F(ab')₂ fragments of donkey anti–rabbit IgG were from Jackson Immunoresearch (West Grove, PA). Rabbit anti–sheep erythrocyte IgG was from Diamedix (Miami, FL). Calphostin C, staurosporine, and H-7 were from Calbiochem-Behring Corp. (La Jolla, CA).

Immunofluorescence. Immunofluorescence staining of macrophages was performed as described (9), except that the cells were

fixed and permeabilized with acetone at room temperature. The cells were incubated with fluorescein-phalloidin, followed by PY-20 and rhodamine anti–mouse IgG. The specificity of fluorescence staining was verified for fluorescein-phalloidin by inhibition of staining with unlabeled phalloidin, and for PY-20 by both absence of staining with PY-20 in the presence of o-phosphotyrosine, and absence of staining with MOPC-141, an isotype-matched control. Slides were examined using conventional fluorescence microscopy using a photomicroscope (III; Carl Zeiss, Inc., Thornwood, NY) equipped with epifluorescence, or confocal fluorescence microscopy using a confocal laser scanning system (MRC 600; Bio-Rad Laboratories, Richmond, CA). There was no significant fluorescence of either fluorescein when viewed under rhodamine optics or rhodamine when viewed under fluorescein optics.

Phagocytosis Assays. Adherent thio-macrophages were preincubated in the presence of either genistein or vehicle (DMSO) at the indicated concentrations in a Hepes-buffered saline (HBS; reference 10) for 10 min at 37°C. 10^7 IgG-RBC were added to the genistein-containing medium and the cells were incubated for a further 15 min. For experiments using protein kinase C inhibitors, the adherent cells were preincubated with either 100 μM HA1004, 100 nM staurosporine, 100 μM H7, or 5 μM calphostin C for 10 min at 37°C, or with 100 nM PMA for 16 h at 37°C, and further incubated with IgG-RBC as described above. Quantitation of engulfment was performed using a fluorescence assay as described (11), except the concentration of acridine orange used was 2.5 μg/ml, and the coverslips were mounted using Slow-fade to minimize photobleaching.

Results and Discussion

To determine whether there is a redistribution or focal accumulation of tyrosine phosphoproteins during Fc receptor–mediated phagocytosis, we performed indirect immunofluorescence using mAb PY-20 (12) on cells undergoing phagocytosis. Thio-macrophages were incubated with IgG-RBC at 4°C, a temperature that allows binding but not ingestion of these red cells, and then were fixed and stained for F-actin and for tyrosine phosphoproteins. There were no focal accumulations of F-actin or tyrosine phosphoproteins in the submembranous region beneath the particles (not shown). Parallel cultures were shifted to 37°C for varying times. After 30 s at 37°C there were focal increases in the appearance of F-actin and PY-20 staining beneath some adherent IgG-RBC, although at this early time point most of the attached erythrocytes were not associated with detectable increases in F-actin or PY-20 staining in the macrophages (not shown). By 1 min at 37°C, there was a dramatic increase in the appearance of both F-actin and PY-20 staining in the cortical areas subjacent to the majority of attached IgG-RBC (Fig. 1, A and B). The appearance of PY-20 staining was focal, in contrast to the relatively diffuse pattern of staining with fluorescein-phalloidin. At this early (1 min) time point, all phagocytic cups showed enhanced staining for F-actin and tyrosine phosphoproteins. At later time points during phagocytosis, some particles that were completely engulfed no longer displayed the enhanced staining for F-actin (Fig. 1 C, *arrowhead*). However, the enhanced staining for tyrosine phosphoproteins persisted and retained the punctate pattern observed at the base of phagocytic cups at 1 min (Fig. 1 D, *arrowhead*). Later time
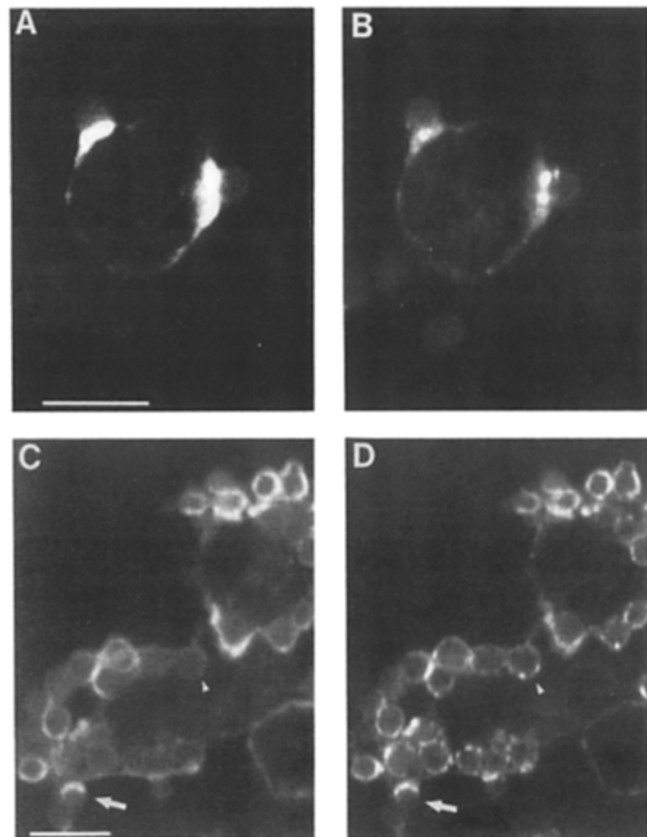

Figure 1. Tyrosine phosphoproteins and F-actin accumulate beneath phagocytic cups during phagocytosis of IgG-RBC by thio-macrophages. Confocal laser micrographs of fluorescein-phalloidin– and PY-20-stained cells after 1 min (*A* and *B*) and 3 min (*C* and *D*) of phagocytosis. (*A* and *C*) Fluorescein-phalloidin; (*B* and *D*) PY-20. Arrows show peripherally based incompletely engulfed particle surrounded by staining for both F-actin (*C*) and tyrosine phosphoproteins (*D*). Arrowheads show completely engulfed particle, which is no longer associated with enhanced staining for F-actin (*C*) but is still associated with staining for tyrosine phosphoproteins (*D*). Optical sections were 4 μm thick; bar = 10 μm.

points (e.g., 4–6 min) showed persistence of this pattern, whereas even later time points (>7 min) showed disappearance of staining for both phosphotyrosine and F-actin surrounding completely ingested particles. Thus, the appearance of polymerized actin beneath phagocytic cups coincided with the appearance of tyrosine phosphoproteins during the early stages of phagocytosis, but the disappearance of F-actin from the cytoplasm surrounding fully engulfed particles preceded
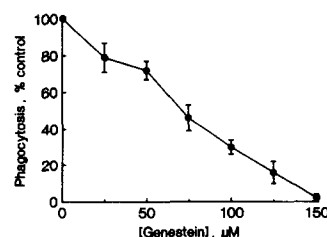

Figure 2. Genistein inhibits Fc receptor–mediated phagocytosis in thio-macrophages. Experiments were performed as described in Materials and Methods. Mean phagocytic index of controls was 857 ± 154 particles ingested per 100 macrophages. Each point represents the mean ± SEM of six experiments.
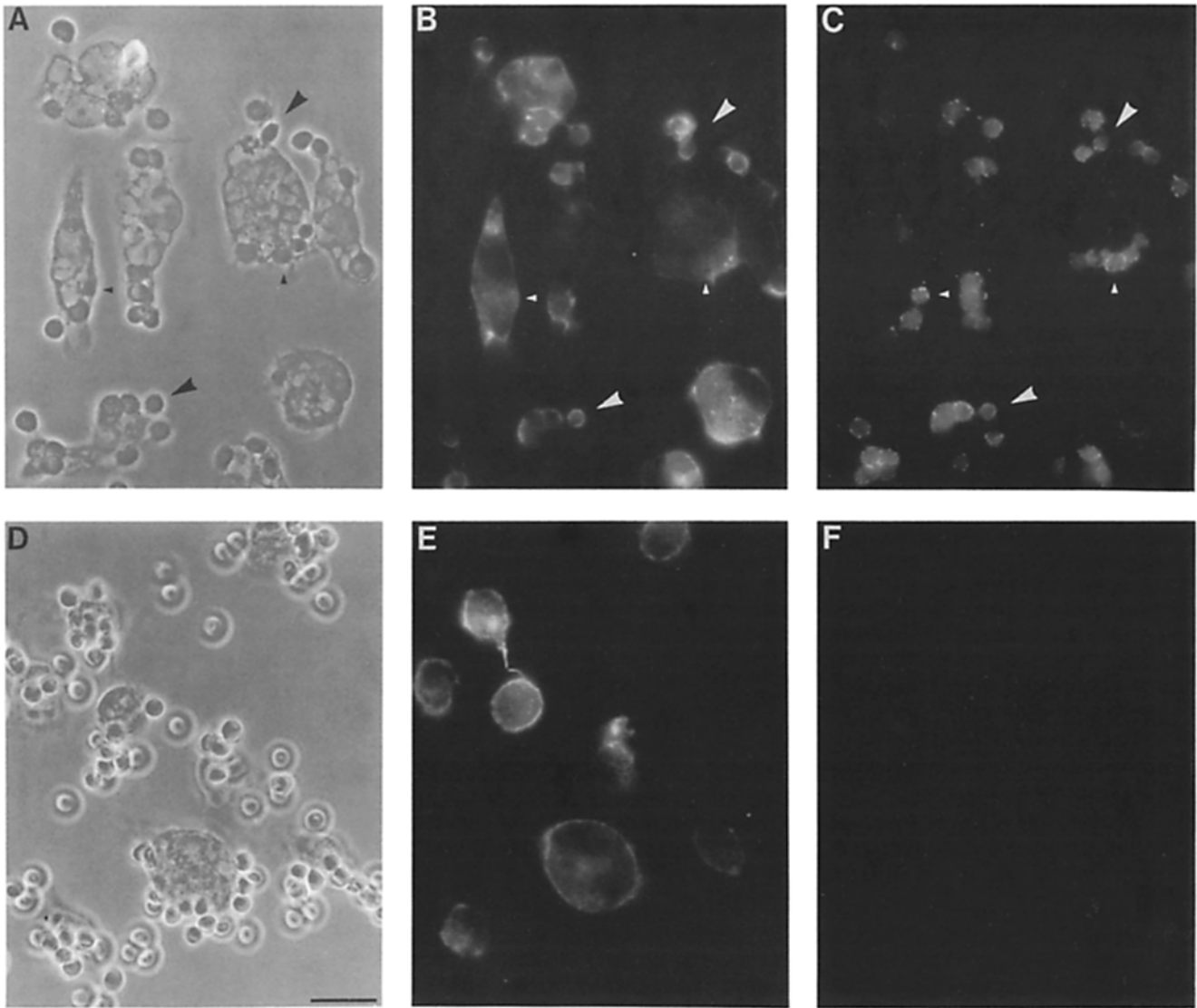
Figure 3. Genistein inhibits the focal accumulation of tyrosine phosphoproteins and F-actin beneath phagocytic particles. Phagocytosis was performed for 4 min. (*A* and *D*) Phase contrast micrographs; (*B* and *E*) fluorescein-phalloidin staining; (*C* and *F*) PY-20 staining. (*A–C*) Incubation in the presence of DMSO (vehicle); (*D–F*) incubation in the presence of 150 μM genistein. Note incompletely engulfed particles (*large arrowheads* in *A–C*) associated with both enhanced staining for F-actin and tyrosine phosphoproteins, and completely engulfed particles (*small arrowheads* in *A–C*) associated with tyrosine phosphoproteins, only. Note absence of staining for F-actin (*E*) and tyrosine phosphoproteins (*F*) beneath test particles in cells incubated in the presence of genistein. Similar results were observed in cells undergoing phagocytosis for 1 and 2 min.

the disappearance of enhanced staining for tyrosine phosphoproteins.

To ascertain whether tyrosine phosphorylation was necessary for particle engulfment, we used the tyrosine phosphorylation inhibitor genistein (13). Genistein caused a dose-dependent reversible decrease in the phagocytic index of thio-macrophages. The concentration causing 50% inhibition (IC_{50}) was 70 μM (Fig. 2), which is comparable to values reported in other systems ($\sim$111 μM for both tyrosine kinase activity in T cells [14], and EGF receptor autophosphorylation in A431 cells [13]). Genistein reduced both the number of macrophages ingesting particles as well as the number of particles ingested by those macrophages that were

capable of ingestion. Genistein did not affect the ability of the macrophages to bind IgG-RBC (Fig. 3 *D*). To confirm that genistein was inhibiting tyrosine phosphorylation, we performed immunofluorescence experiments using macrophages incubated with IgG-RBC in the presence or absence of genistein. Genistein inhibited the focal accumulation of tyrosine phosphoproteins beneath phagocytic cups (Fig. 3); in addition, genistein prevented the accumulation of F-actin beneath the test particles (compare Fig. 3, *E* with *B*). Although it was not possible to assess the absolute specificity of genistein's inhibitory effects, we tested the ability of an inhibitor of cAMP-dependent protein kinase, HA1004, and the ability of a variety of inhibitors of protein kinase C to

block phagocytosis. Neither HA1004, staurosporine, cal-phostin C, H7, nor prolonged incubation in PMA to down-regulate protein kinase C activity inhibited phagocytosis of IgG-RBC by thio-macrophages (Fig. 4). However, a struc-turally unrelated tyrosine kinase inhibitor, herbimycin A (15, 16), also inhibited phagocytosis without affecting the attach-ment of opsonized particles to the macrophages (Fig. 4). The lack of efficacy of protein kinase C inhibitors is at variance with the studies of Zheleznyak and Brown (8), who used similar concentrations of protein kinase C inhibitors in their studies of Fc receptor-mediated phagocytosis in human mono-cytes; however, this cell type has a much lower phagocytic capacity than mouse inflammatory macrophages, in part due to a lower surface expression of Fc receptors (17). In addi-tion, since these cells do not express appreciable quantities of FcRIII, whereas thio-macrophages do (18), it is difficult to compare directly these two cell types. Although staurospo-rine is a relatively nonspecific protein kinase inhibitor and, in fact, has been used as an inhibitor of tyrosine kinases (19, 20), at the concentration used (100 nM), it did not inhibit the appearance of tyrosine phosphoproteins during phagocy-tosis as assessed by immunofluorescence (data not shown).

The finding that tyrosine phosphorylation is an early signal sent by ligated Fc receptors confirms recent work by several independent investigators (2, 3, 4, 21). The results of the current work suggest that enhanced tyrosine phosphoryla-tion occurs in a highly localized area of the cell, i.e., in the submembranous region beneath phagocytic cups. This spa-tial localization coincides with the distribution of F-actin as-sembly (Figs. 1 and 3) (10), and presumably the enhanced actin nucleation sites that underlie this assembly. It is in-teresting that the appearance of staining for tyrosine phos-phoproteins is punctate and not diffuse as is staining for F-actin, which suggests that the signal for tyrosine phosphorylation

is more spatially restricted, perhaps constrained by the loca-tion of the ligated Fc receptors. Indeed, double immunofluores-cence staining of phagocytosing macrophages using PY-20 and anti-rabbit IgG to localize the distribution of the Fc receptor ligand on the surface of the sheep erythrocyte confirmed that the punctate staining of PY-20 coincided with focal areas of high ligand density (Fig. 5). This is reminiscent of the findings of Hermanowski-Vosatka et al. (22), who found that erythro-cytes whose C3 opsonin was clustered were more efficiently ingested than erythrocytes opsonized diffusely with C3, and suggests that clustered ligand serves as a potent stimulus for signal transduction. In addition, oligomerized mAb 2.4G2 was delivered efficiently to lysosomes, whereas monovalent 2.4G2 was not a potent stimulus for this intracellular pathway (23). Since genistein inhibited both tyrosine phosphoryla-tion and the focal accumulation of F-actin beneath phago-cytic cups, it is likely that tyrosine phosphorylation is neces-sary for F-actin assembly, and may serve as a proximate "signal" for actin polymerization and pseudopod extension. Although it is not clear which Fc receptor(s) is (are) responsible for phagocytosis in thio-macrophages, it is likely that FcRα, the mouse homologue of human FcRIII (24, 25), is at least partly responsible. First, treatment of the macrophage monolayers with trypsin, which eliminates the surface expression of FcRI (26), did not inhibit ingestion of IgG-RBC, or the focal ap-pearance of F-actin or tyrosine phosphoproteins beneath phago-cytic cups (data not shown). In addition, thio-macrophages express mRNA for both FcRα and its associated γ subunit, and have little if any mRNA for FcRII (18). Given the high degree of sequence homology between the γ subunit of FcRα and the ζ subunit of CD3, and the fact they both participate in tyrosine phosphorylation events during receptor activa-tion (the high affinity IgE receptor [27-29] and the TCR [1], respectively), it is possible that the γ subunit is crucial in mediating signaling in thio-macrophages during Fc receptor-mediated phagocytosis, analogous to recent findings in T and B cell lines transfected with the γ subunit (30, 31).
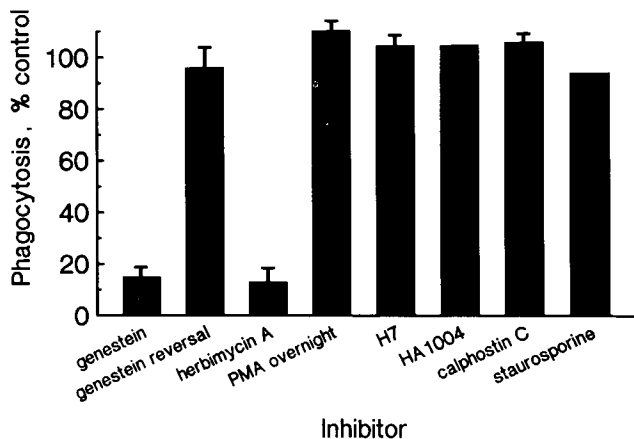

Figure 4. Inhibitors of tyrosine kinases, but not protein kinase C, in-hibit phagocytosis of IgG-RBC by thio-macrophages. Cells were prein-cubated with the indicated inhibitor for 10 min before onset of phagocy-tosis, except for PMA and herbimycin, which were added for 16 h before the onset of the experiment. For genistein reversal, the cells were washed three times after a 10-min incubation in genestein and allowed to recover for 15 min before the onset of phagocytosis. Each bar represents mean ± SEM, n = 3.
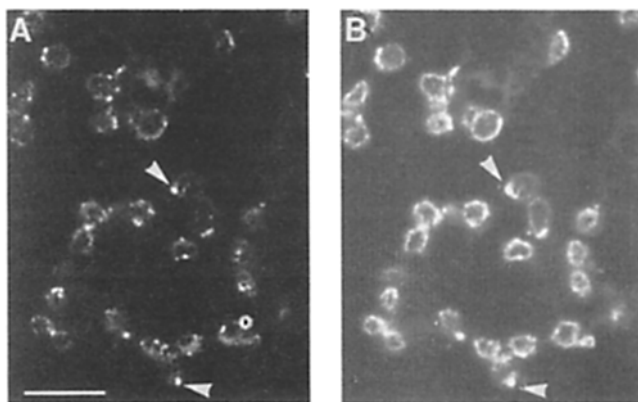

Figure 5. Focal accumulation of tyrosine phosphoproteins coincides with regions of high ligand density on the surface of the phagocytic particles. Confocal micrographs of PY-20- (A) and antiopsonin- (B) stained cells fixed and stained after 3 min of phagocytosis. Arrowheads point to colocali-zation of staining. Bar = 10 μm.

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