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Chimeric Receptors Composed of Phosphoinositide 3-Kinase Domains and Fcγ Receptor Ligand-binding Domains Mediate Phagocytosis in COS Fibroblasts*

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Receptors for the Fc portion of IgG (FcγR) initiate phagocytosis of IgG-opsonized particles by a process involving the assembly of a multi-molecular signaling complex. Several members of this complex have been identified, including Src family kinases, Syk/ZAP 70 family kinases, and phosphoinositide 3-kinase (PI3-K). To test directly the role of PI3-K in mediating phagocytosis, we assessed the phagocytic ability of chimeric receptors composed of FcγR extracellular and transmembrane domains fused to regions of the p85 subunit of PI3-K. We found that chimeric receptors with cytoplasmic tails composed of the entire p85 subunit of PI3-K or the inter-Src homology 2 portion of p85 triggered phagocytosis in transfected COS fibroblasts. These two chimeras also showed phosphoinositide kinase activity *in vitro* when immunoadsorbed. In contrast, a chimera containing only the carboxyl-terminal Src homology 2 domain of p85 that does not interact with the catalytic p110 subunit of PI3-K did not trigger phagocytosis, nor did it show kinase activity *in vitro*. These data suggest that localization and direct activation of PI3-K at the site of particle attachment is sufficient to trigger the process of phagocytosis.

Fc receptors for IgG (FcγR)[1] trigger cellular processes that mediate the crucial protective functions of antibodies. Our understanding of one such cellular process, namely phagocytosis, by which IgG-coated pathogens are engulfed and degraded, is beginning to yield to intense molecular dissection. This process is initiated by ligation of FcγR with IgG-coated particles and proceeds by the spreading of pseudopods around the particle, with receptors binding ligands in a zipper-like fashion (1). A multi-molecular signaling complex assembles beneath the membrane at the site of particle attachment that induces remodeling of the actin cytoskeleton required to complete the internalization process (1, 2).

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[1] The abbreviations used are: FcγR, Fc receptor for IgG; ITAM, immunoreceptor tyrosine activation motif; PI3-K, phosphoinositide 3-kinase; PI, phagocytic index; PtdIns, phosphatidylinositol; RBC, red blood cell; HA, hemagglutinin; SH2, Src homology 2; iSH2, inter-SH2; PKC, protein kinase C; SH2-C, carboxyl-terminal SH2; DMEM, Dulbecco's modified Eagle's medium; FITC, fluorescein isothiocyanate; PBS, phosphate-buffered saline.

The earliest identifiable signal associated with receptor clustering is tyrosine kinase activity, which is necessary for phagocytosis to proceed (3, 4). The cytoplasmic portions of FcγR do not contain intrinsic tyrosine kinase activity. Instead these receptors either contain (FcγRIIa) or associate with an auxiliary molecule that contains (FcR γ-chain) a conserved amino acid motif that upon phosphorylation of critical tyrosine residues serves as a docking site for Src homology 2 (SH2) domain-containing proteins (5). This docking site is designated immunoreceptor tyrosine activation motif (ITAM).

Our major working hypothesis explaining signal transduction leading to phagocytosis states that, immediately after receptor clustering, the ITAM is phosphorylated by one or another member of the Src family of tyrosine kinases. Src kinases have been co-isolated with FcγR in the resting state (6), and their activities appear to increase following receptor clustering (7–11). The phosphorylated ITAM then recruits other SH2 domain-containing signaling molecules including the tyrosine kinase Syk (9–11), a member of the Syk/Zap70 family of non-receptor tyrosine kinases central to the signal generation of several other immunoreceptor molecules (12, 13).

A variety of observations confirm the requirement of Syk kinase in the signal pathway leading to phagocytosis. First, Syk-deficient lymphocytes failed to trigger significant F-actin assembly after clustering of ITAM-containing receptors (14). Second, chimeric receptors composed of FcγR extracellular domains fused to Syk kinase cytoplasmic tails triggered phagocytosis quite successfully in COS fibroblasts (15). Recently, macrophages derived from Syk-deficient mice were found to be incapable of completing phagocytosis, manifesting only partial pseudopods that contained F-actin beneath the attached particles but failed to mature into enveloping pseudopods and internalized phagosomes (16). Of special interest to our study, these Syk-deficient cells showed no association of the p85 subunit of phosphoinositide 3-kinase (PI3-K) with tyrosine-phosphorylated proteins, indicating that recruitment of PI3-K to receptor complexes did not occur. Furthermore, the block in phagocytosis exhibited by these Syk-deficient macrophages morphologically resembled the effect of the PI3-K enzyme inhibitor wortmannin on normal macrophages (16). These data place Syk kinase upstream of PI3-K in the signaling pathway and would suggest that the role of Syk kinase in phagocytosis is to allow the recruitment and activation of PI3-K.

FcγR clustering leads to the association of PI3-K with receptor complexes and to an increase in PI3-K activity in various cell types (11, 17). PI3-K has been implicated in the process of FcγR-mediated phagocytosis in neutrophils (17) and macrophages (18) through the use of inhibitors of PI3-K catalysis such as wortmannin. Wortmannin not only blocks phagocytosis in these cells, it also blocks granule exocytosis and cell killing

directed through FcγR in NK cells (19), indicating a conserved role for PI3-K in FcγR signaling. The enzyme phosphorylates phosphoinositides at the D-3 position of the inositol ring, producing phosphatidylinositol (3,4)-bisphosphate and phosphatidylinositol (3,4,5)-trisphosphate that act as second messengers (20, 21). The particular isoform of PI3-K associated with FcγR is a heterodimer composed of a regulatory subunit designated p85 and a catalytic subunit designated p110. The several functional domains of the p85 subunit include a Src homology 3 domain, a breakpoint cluster region, two SH2 domains, and an inter-SH2 (iSH2) domain (Fig. 1). The two SH2 domains direct the interaction of PI3-K with activated receptors (22, 23), whereas the breakpoint cluster region domain may bind small G-proteins such as Rac (24). The iSH2 domain, required for the enzymatic activity of PI3-K, binds to the p110 subunit to form a constitutively active enzyme (25).

To test directly the role of PI3-K in mediating phagocytosis, we assessed the phagocytic capacity of three chimeric receptors composed of the extracellular and transmembrane domains of FcγRIa fused to regions of the p85 subunit of PI3-K. The chimeras were designed to mimic the recruitment of PI3-K to immune complex-induced FcγR clusters without the need for additional proteins of the proximal signal cascade, specifically, FcR γ-chain and its ITAM, members of the Src family that phosphorylate FcR γ-chain, and Syk. The three chimeras are depicted in Fig. 1. The cytoplasmic portion of the first chimera is composed of the wild type p85, which contains several known functional domains of PI3-K. The tail of the second contains only the iSH2 region, which is responsible for binding the catalytic p110 subunit of PI3-K causing constitutive kinase activity (26). The third chimera includes only the carboxyl-terminal SH2 region (SH2-C) of p85, which is not capable of binding the p110 subunit. These chimeras allow us to separate the contributions of p85 adaptor functions from enzymatic induced functions of PI3-K. Using a model system that we have characterized extensively (27), we transiently transfected these chimeric receptors into COS cells to examine their capacity to mediate phagocytosis. We herein show that chimeric receptors that localize PI3-K enzymatic activity to the site of particle attachment mediate phagocytosis in COS fibroblasts and circumvent the need for ITAMs and associated Src and Syk kinases acting upstream.

EXPERIMENTAL PROCEDURES

Cells—COS-7 cells (ATCC, Rockville, MD) were maintained in Dulbecco's modified Eagle's medium (DMEM, Life Technologies, Inc.) supplemented with 10% fetal calf serum (HyClone Laboratories, Logan, UT), 2 mM L-glutamine, 100 IU/ml penicillin, and 100 μg/ml streptomycin.

Antibodies—The anti-FcγRI monoclonal antibody 32 was kindly supplied by Medarex (Annandale, NJ). The anti-HA epitope monoclonal antibody 12CA5 was obtained from Boehringer Mannheim. $F(ab)'_2$ fragments of goat anti-mouse IgG and FITC-conjugated forms were obtained from Pierce.

cDNAs and Transfection of COS-7 Cells—The cDNA for FcγRIA (28) was cloned into the pCDM expression vector for expression in COS-7 cells. The FcγRIIA cDNA cloned in the pCEXV3 expression vector was kindly provided by Dr. J. Ravetch (Rockefeller University, New York, NY). The three chimeric receptors composed of the FcγRIA extracellular and transmembrane regions fused to constructs of the p85 subunit of PI3-K were constructed by joining the constructs at a *Xba*I site introduced into FcγRIA by PCR. Two different primers were used to introduce the *Xba*I site into human FcγRIA to allow the reading frame to be compatible with the different p85 constructs, which all contain a *Xba*I site near the start of the protein sequence. The first primer, 5′ CGT *C*TA GAC ACC CAG AGA AC 3′ on the antisense strand, was used to construct the FcγRIA *Xba*I segment joined to create the Ia-p85 and Ia-SH2-C chimeras. The second primer, 5′ GTT CTA *G*AC GTA TTG TCA CCC 3′ on the antisense strand, was used to construct the FcγRIa segment joined to create the Ia-iSH2 chimera. Nucleotides changed with respect to the wild type FcγRIA sequence are italicized. The PCR-modified FcγRIA was first cloned into T-vector (Invitrogen, San Diego, CA), and then shuttled into pBluescriptSK (Stratagene, La Jolla, CA). The wild type murine p85, iSH2–2 construct, and SH2-C construct (29) used to create the chimeras were kindly provided by Dr. A. Klippel and Dr. L. Williams (University of California, San Francisco, CA). The chimeras were shuttled into the pCDNA-1 vector (Invitrogen) for expression in COS-7 cells. The reading frame of the chimeras was confirmed by DNA sequencing. COS-7 cells were transfected with 3 μg of plasmid DNA/10-cm dish of cells by the diethylaminoethyldextran method as described previously (30) and were used for functional assays 2 days after transfection.

Preparation of Phagocytic Targets—Sheep RBC (Colorado Serum, Denver, CO) were washed three times in PBS (145 mM NaCl, 20 mM phosphate buffer, pH 7.4) to remove serum, and labeled by incubation with 0.1 mg/ml FITC in PBS overnight at 4 °C. After four washes with PBS, FITC-labeled RBC were incubated with a sub-agglutinating dose of rabbit anti-sheep RBC IgG (Diamedix, Miami, FL) at 37 °C for 1 h. The RBC were washed four times to remove excess IgG and were then used in phagocytosis assays. Non-fluorescent beads, 2 μm in diameter (Polysciences, Warrington, PA), with a carboxylate-coupled surface were coated with human IgG or bovine serum albumin using a carbodiimide procedure as specified by the manufacturer.

PI3-K Assay—Transfected COS cells were removed from culture plates by trypsinization, and were resuspended in DMEM. The cells were incubated with anti-FcγRI mAb32 for 20 min on ice, and were then warmed to 37 °C for 10 min. A secondary $F(ab)'_2$ goat anti-mouse IgG (Pierce) was added to cross-link the receptors for 3 min at 37 °C, followed by addition of 1% Triton lysis buffer containing PBS (145 mM NaCl, 20 mM phosphate buffer, pH 7.4), 10 mM EDTA, 1 mM sodium orthovanadate, 2 mM phenylmethylsulfonyl fluoride (Sigma) to lyse the cells. The lysates were clarified by centrifugation at 13,000 × *g* for 10 min, and the resulting supernatants incubated with anti-HA antibody (Boehringer Mannheim) and protein G-Sepharose to immunadsorb the chimeric receptors. As a control, COS cell supernatants were also incubated with anti-p85 serum to adsorb native PI3-K. The adsorbates were washed four times in lysis buffer, followed by three washes in 10 mM Hepes-KOH, pH 7.4. Substrate phosphatidylinositol liposomes were prepared by resuspending vacuum-dried phosphatidylinositol in assay buffer (30 mM Hepes-KOH, pH 7.4, 30 mM $MgCl_2$, 1 mM EDTA, 50 μM ATP) and sonicating on ice for 5 min at 50 MHz. The washed adsorbates were resuspended in 40 μl of assay buffer to which 10 μCi of [γ-^{32}P]ATP in 20 μl of phosphatidylinositol liposomes was added. The reactions were allowed to proceed at RT for 30 min and were terminated by the addition of 100 μl of 1 N HCl. Lipids were extracted with 300 μl of $CHCl_3$:CH_3OH (1:1), removing the organic phase, which was then dried under vacuum. The lipids were resuspended in 50 μl of $CHCl_3$:CH_3OH (2:1) and were spotted on Silica Gel-60 HP-TLC plates (Merck, Darmstadt, Germany). The lipid products were separated by thin layer chromatography in a chloroform:pyridine:boric acid:formic acid:water solvent system as described previously (31) and were visualized by autoradiography. PI3-K products were identified by their sensitivity to wortmannin and by their migration R_F values in this solvent system. The solvent system allows separation of the singly phosphorylated forms PtdIns3P and PtdIns4P, based on the ability of borate to retard the migration of the 4P form.

Phagocytosis Assay—Transfected COS cells were removed from culture plates by trypsinization and were resuspended in DMEM. FITC-labeled sheep RBC opsonized with IgG were added, and the cells were gently pelleted by low speed centrifugation for 3 min. To study binding of the RBC targets to COS cells, the cells were incubated at 4 °C for 1 h and were then washed in PBS and fixed in 1% paraformaldehyde in PBS. Three hundred cells were counted per condition by phase microscopy, and the percentage of cells binding 4 or more RBC was defined as rosetting activity. To study phagocytosis cells were incubated at 37 °C for 1 h. The cells were then washed in PBS and subjected to a 30-s hypotonic shock to lyse externally bound RBC; this treatment does not lyse COS cells or internalized RBC. The cells were then fixed in 1% paraformaldehyde and were analyzed by phase and fluorescence microscopy. One hundred COS cells per condition that had bound RBC targets identified by fluorescence microscopy were scored for the number of internalized RBC. The data were expressed as a phagocytic index defined as the number of RBC internalized by 100 FcγR-expressing cells. To determine statistical significance of the results, the data were analyzed using a Student-Newman-Keuls multiple comparisons test. In experiments using cytochalasin D (Sigma), the inhibitor was added at 1 μg/ml just prior to the addition of the RBC targets. In experiments using wortmannin (Sigma), the inhibitor was added at a final concentration of 100 nM 15 min prior to addition of the RBC targets.

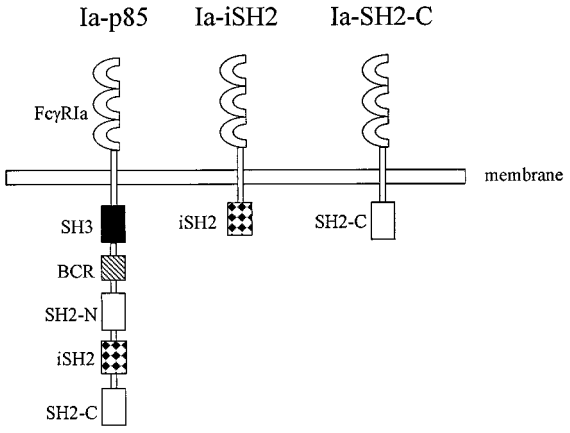

FIG. 1. **Schematic diagram of the three receptor chimeras.** The diagram depicts the chimeras formed by fusing the extracellular and transmembrane domains of FcγRIa with various regions of the p85 subunit of PI3-K. Domains depicted: *FcγRIa*, extracellular and transmembrane regions of FcγRIa; *SH3*, Src homology 3; *BCR*, breakpoint cluster region; *SH2-N*, amino-terminal SH2 domain; *iSH2*, inter-SH2 domain; *SH2-C*, carboxyl-terminal SH2 domain.

Measurement of Receptor Expression—Transfected COS cells were removed from culture plates and incubated with anti-FcγRI mAb32 at 5 μg/ml for 1 h on ice. The cells were washed three times in PBS supplemented with 0.1% bovine serum albumin, and were then incubated with a FITC-labeled $F(ab)'_2$ goat anti-mouse IgG for 1 h on ice. Following three washes, the cells were fixed in 1% paraformaldehyde in PBS and were analyzed for receptor expression on an Elite EPICS fluorescence-activated cell sorter (Coulter, Hialeah, FL). Data from 10,000 cells per condition were recorded yielding the percentage of cells expressing receptor as compared with IgG isotype controls, as well as the mean fluorescence intensity of the expressing cells. As the same primary antibody was used to detect the chimeric receptors, the mean fluorescence intensities indicate relative receptor densities of the various constructs as shown in Table II.

F-actin Staining—Transfected COS cells were grown overnight on glass coverslips in 24-well plates prior to addition of nonfluorescent IgG-coated beads. Cells were incubated at 37 °C with the bead targets for 15 min and were then washed twice in DMEM and fixed in 3% paraformaldehyde in PBS for 15 min at 4 °C. The cells were then permeabilized with 0.001% Triton X-100 in PBS for 7 min at room temperature, washed three times in PBS, and stained with FITC-phalloidin (Molecular Probes) diluted 1:20 in PBS for 45 min at room temperature. The cells were washed three times in PBS and were mounted in Mowiol medium (Polysciences) and viewed by confocal microscopy.

RESULTS

The three chimeras depicted in Fig. 1 were transiently transfected into COS cells to examine phagocytosis in this model system. First, we tested the functional ability of the expressed chimeras to bind IgG-coated particles in a rosette assay. Cells expressing all three types of chimeras were capable of binding FITC-labeled IgG-coated RBC to form robust and stable rosettes that were morphologically indistinguishable from rosettes seen with COS cells expressing wild type FcγRIa. Thus, the chimeras were functional by the criterion of ligand binding.

Because linking the p85 subunit to a receptor at the membrane introduces a new physical constraint on the p85 subunit that could affect the assembly with the catalytic p110 subunit of PI3-K, we assayed whether the chimeric receptors were still capable of forming a functionally active inositol kinase. COS cells cotransfected with the chimeras and the p110 subunit were incubated with anti-FcγRI antibody, the receptors were clustered with a secondary antibody, and the cells were then lysed. The chimeric receptors were immunoadsorbed with an anti-HA epitope antibody recognizing the HA tag present at the carboxyl terminus of each chimera, and the adsorbates were subjected to a PI3-K assay using phosphatidylinositol lipid micelles and ^{32}P-labeled ATP. We found that both the Ia-p85 and the Ia-iSH2 chimeras reconstituted enzymatic activity (Fig. 2), as predicted by their described ability to bind the p110 subunit (29). The Ia-SH2-C chimera, which cannot bind the p110 subunit (29), did not show PI3-K activity. Wild type FcγRIa also did not show PI3-K activity (data not shown), as expected because FcγRIa in the absence of γ-chain does not mediate phagocytosis.

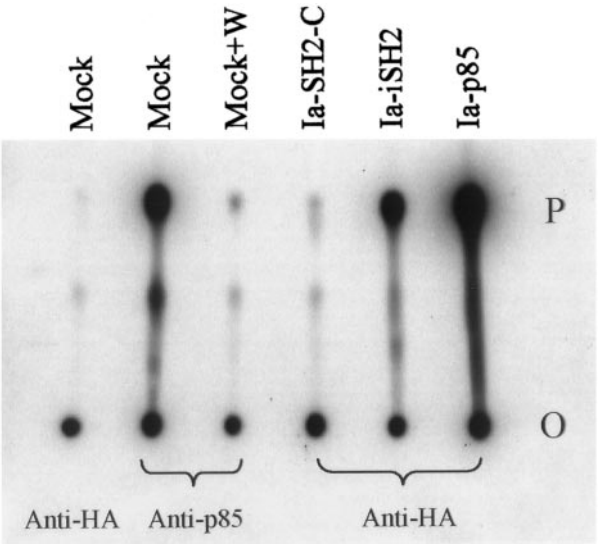

FIG. 2. **PI3-K assay of chimeric receptors expressed in COS cells.** Transfected COS cells were activated by clustering the chimeric receptors with an anti-FcγRI antibody and a cross-linking secondary antibody and were then lysed. The chimeras were immunoadsorbed with the antibody indicated beneath the lanes and the adsorbates were then subjected to a PI3-K assay. The adsorbates were incubated with phosphatidylinositol micelles in the presence of [γ-^{32}P]ATP, and the lipid products were extracted and separated by thin layer chromatography. The resulting autoradiograph is shown, with *O* indicating the origin and *P* indicating the PI3P product. In *lane 1*, mock-transfected COS cells were adsorbed as a control for specificity. In *lane 2*, wild type p85 was immunoadsorbed from mock-transfected COS as a positive control for PI3-K activity. In *lane 3*, 100 nM wortmannin (*W*) was added to the adsorbed wild type p85 *in vitro* to inhibit PI3-K activity and its product (*P*) as a standard. In *lanes 4–6*, the chimeras were co-transfected with the p110 subunit and the indicated chimeric receptors were adsorbed with the anti-HA antibody. Only the Ia-iSH2 and the Ia-p85 chimeras show PI3-K activity (*lanes 5* and *6*). We have not identified the intermediate spots.

To examine the phagocytic ability of the chimeras, we quantified by microscopy the capacity of transfected COS cells to phagocytose RBC opsonized with IgG. Cells were incubated at 37 °C with FITC-labeled RBC opsonized with IgG and were then subjected to a brief hypotonic shock to lyse externally bound RBC. The samples were fixed and examined by phase and fluorescence microscopy for internalized RBC. Phagocytosed RBC were readily apparent in cells transfected with the Ia-p85 and Ia-iSH2 chimeras but not in cells expressing the Ia-SH2-C chimera (Fig. 3). Internalized RBC were counted for each transfectant and expressed as a phagocytic index (PI). The data are tabulated in Table I. The chimeras were compared in each experiment to wild type FcγRIa, which shows little or no phagocytosis, and to FcγRIIa, which shows efficient phagocytosis. FcγRIa expressed alone does not contain an ITAM required for phagocytosis and thus serves as a base line, while FcγRIIa contains an ITAM and can recruit Syk kinase family members as well as PI3-K (11). Comparing the Ia-p85 chimera to FcγRIIa showed that the chimera was capable of mediating phagocytosis at levels similar to FcγRIIa (PI 170 *versus* 153). Cotransfection of the p110 subunit with the Ia-p85 chimera raised the phagocytic index to levels significantly above that of

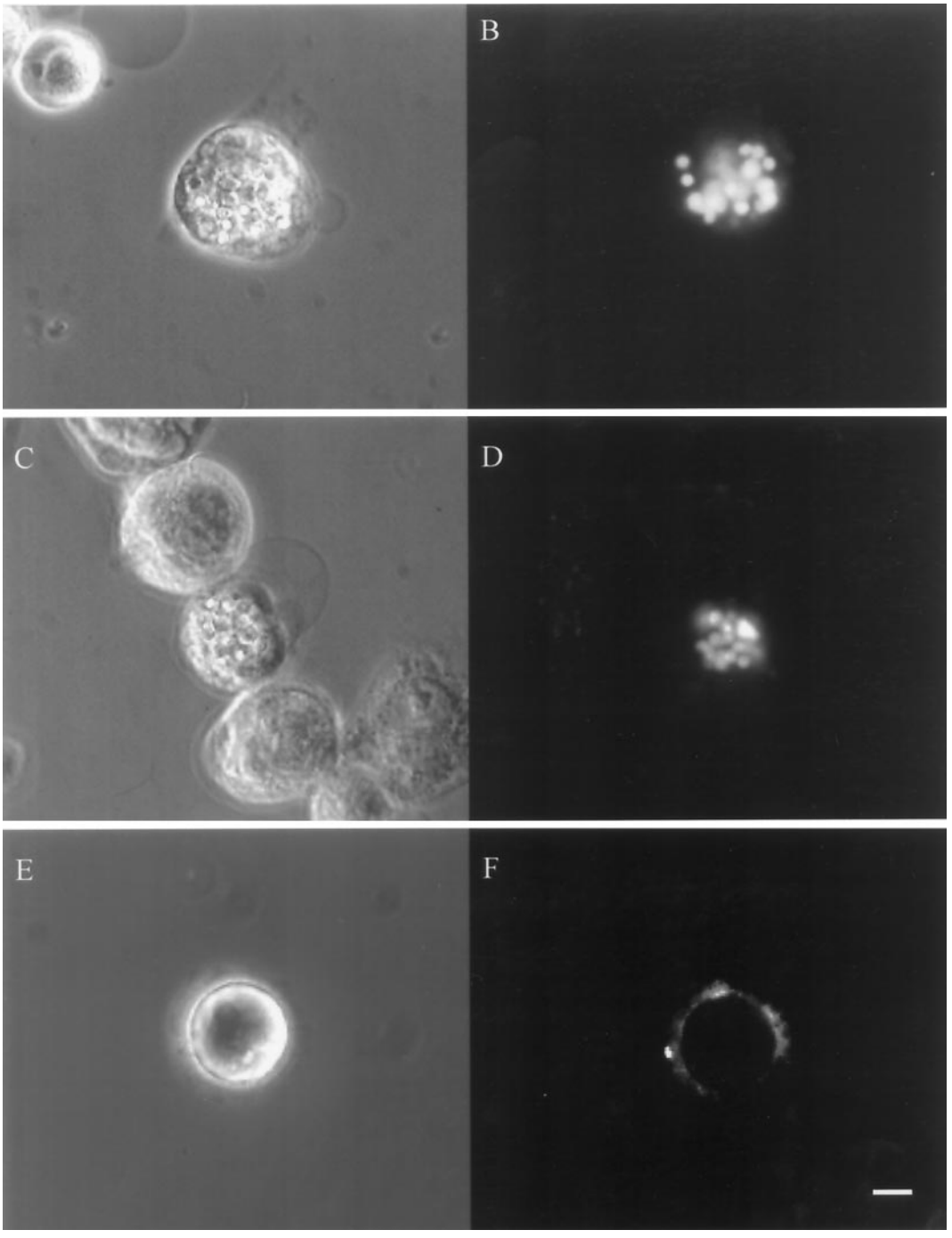

FIG. 3. **Phagocytosis of the receptor chimeras.** COS cells transfected with the three receptor chimeras were incubated with FITC-labeled RBC opsonized with IgG at 37 °C for 1 h to allow phagocytosis to occur. The cells were then subjected to a brief hypotonic shock to lyse externally bound RBC and were fixed and examined by phase and fluorescence microscopy. *Panels A*, *C*, and *E* are phase contrast micrographs; *panels B*, *D*, and *F* are fluorescence micrographs of the same cells detecting the FITC- labeled RBC targets. In *panels A* and *B*, internalized RBC targets can be seen clearly in a COS cell cotransfected with the Ia-p85 chimera and p110 subunit. In *panels C* and *D*, internalized RBC are also readily apparent in a cell cotransfected with the Ia-iSH2 chimera and p110 subunit. In contrast, *panels E* and *F* show a COS cell transfected with the Ia-SH2-C chimera and p110 subunit that does not show any internalized RBC but shows lysed RBC ghosts bound to the surface visible only by fluorescence microscopy. *Bar* = 10 μm.

FcγRIIa (PI 268 *versus* 153), indicating very efficient phagocytosis. The Ia-iSH2 chimera also triggered phagocytosis, although at levels slightly lower than that of FcγRIIa (PI 108 *versus* 153) or the Ia-p85 chimera. Cotransfection of the p110 subunit with the Ia-iSH2 chimera increased the phagocytic index to levels similar to FcγRIIA (PI 153 *versus* 153). The Ia-SH2-C chimera did not support phagocytosis except at baseline levels, much like FcγRIa alone (PI 16 and 26). Co-transfection of the Ia-SH2-C chimera with p110 only slightly increased the mean phagocytic index (PI 36).

To consider whether receptor densities of the expressed chimeras accounted for their phagocytic capacities, we measured in parallel both receptor expression by flow cytometry and phagocytosis. Surface expression of the chimeras was analyzed using an antibody that recognizes the extracellular region of FcγRIa. The data from two experiments are shown in Table II. We found that wild type FcγRIa, which mediates minimal phagocytosis, is expressed in a higher percentage of cells and at a greater relative density than any of the phagocytic chimeras. The expression of the p110 subunit with the Ia-p85 chimera

TABLE I

Phagocytic capacity of the receptor chimeras expressed in COS cells

COS cells transfected with the above noted constructs were incubated with IgG-opsonized RBC targets for 1 h at 37 °C to allow phagocytosis to occur. The cells were subjected to a brief hypotonic shock to lyse externally bound RBC and were then fixed and viewed by phase and fluorxescence microscopy. The number of RBC internalized by 100 receptor-expressing cells (mean ± S.D.) was expressed as the phagocytic index. *, the phagocytic indices of these receptors are significantly greater than that of FcγRIa ($p < 0.01$ for all indicated receptors). ‡, the increase in phagocytic index upon co-transfection of the p110 subunit with Ia-p85 is significant ($p < 0.001$).

COS transfectant	Phagocytic index	No. of experiments
FcγRIa	26 ± 17	8
FcγRIIa	153 ± 55*	8
Ia-p85	170 ± 47*‡	6
Ia-p85 + p110	268 ± 67*‡	6
Ia-iSH2	108 ± 19*	5
Ia-iSH2 + p110	153 ± 34*	5
Ia-SH2-C	16 ± 5	5
Ia-SH2-C + p110	36 ± 19	4

TABLE II

Parallel analysis of phagocytosis and receptor expression

COS cells transfected with receptor chimeras were analyzed in parallel for phagocytic capacity and receptor expression. Receptor expression was measured by flow cytometry using an anti-FcγRIa antibody. The mean fluorescence intensities of the expressing cells of each chimera were compared to estimate relative receptor densities. The same cells were analyzed for phagocytic capacity expressed as a phagocytic index as described in Table I. The data from two experiments are shown.

COS transfectant	Phagocytic index	Percentage of cells stained positive	Mean fluorescence Intensity
Experiment 1			
FcγRIa	20	41	59
Ia-iSH2	112	10	4
Ia-iSH2 + p110	184	10	4
Ia-p85 + p110	127	20	22
Experiment 2			
FcγRIa	10	30	34
Ia-p85	83	14	4
Ia-p85 + p110	119	11	12
Ia-SH2-C	19	18	6
Ia-SH2-C + p110	59	18	8

appears to increase the density of the Ia-p85 chimera, but has no apparent effect on the expression of either the Ia-iSH2 or the Ia-SH2-C chimeras. Parallel phagocytosis experiments show that a high density of receptor does not correlate with phagocytic ability. Specifically, FcγRIa-transfected cells show the lowest phagocytic index despite having the highest relative density of receptor. In contrast, the Ia-iSH2 chimera coexpressed with p110 confers a high phagocytic index while expressing only a very low receptor density. The Ia-SH2-C chimera transfected with or without p110 confers a low phagocytic index similar to FcγRIa while expression is commensurate with the other chimeras. The phagocytic index of 59 for the Ia-SH2-C chimera transfected with p110 noted in experiment 2 was the highest observed value of four experiments, which averaged 36, not significantly different from FcγRIa (Table I). Thus, the differences found in the phagocytic capacity are attributable to the signaling capacity of the receptors and not simply to receptor density. We suggest that receptor density plays a role in influencing the ability of the transfected cells to bind IgG-coated targets. Cells with a density of receptor too low to permit RBC binding are not considered functional in this assay as only cells capable of binding the IgG-coated RBC are examined.

TABLE III

Cytochalasin D and wortmannin block chimera-mediated phagocytosis

Cells were incubated with IgG-opsonized RBC at 37 °C with or without cytochalasin D in A or wortmannin in B for 1 h. The cells were then subjected to a brief hypotonic shock to lyse externally bound RBC and were analyzed by phase and fluorescence microscopy to measure phagocytosis. Binding of the RBC to the cells was measured by rosetting of the RBC in parallel samples incubated at 4 °C.

A. COS transfectant	Percentage of cells forming rosettes with cytochalasin D		Phagocytic index for cytochalasin D	
	−	+	−	+
FcγRIa	37	34	27	0
FcγRIIa	32	29	174	0
Ia-p85	25	24	164	0
Ia-p85 + p110	26	27	284	0

B. COS transfectant	Percentage of cells forming rosettes with wortmannin		Phagocytic index for wortmannin	
	−	+	−	+
FcγRIa	38	40	38	18
FcγRIIa	27	26	107	32
Ia-iSH2 + p110	12	10	122	24
Ia-p85 + p110	21	20	116	26

To determine whether actin was polymerized during chimera-mediated phagocytosis, we evaluated the effect of cytochalasin D, an inhibitor of F-actin polymerization that blocks phagocytosis in professional phagocytes and in COS cells expressing functional FcγR (32). As seen in Table III, part A, cytochalasin D blocked internalization of IgG-coated RBC while leaving unaffected the binding of IgG-coated RBC to the chimera-expressing cells. We also analyzed directly whether actin was polymerized in response to particle binding by staining actin filaments with FITC-phalloidin early in the course of phagocytosis and examining cells by confocal microscopy. Transfected COS cells were incubated with IgG-coated beads for 15 min at 37 °C and were then fixed and stained to detect actin filaments. As illustrated in Fig. 4, COS cells cotransfected with the Ia-p85 chimera and p110 subunit show accumulations of F-actin in cuplike structures beneath bound beads, while wild type FcγRIa alone does not show any F-actin accumulations due to bead binding. Thus, the chimeric receptors, like competent FcγR, activate the actin cytoskeleton during the course of phagocytosis.

Inhibitors of the enzymatic activity of PI3-K have been shown to block FcγR-mediated phagocytosis in professional phagocytes such as neutrophils (17) and macrophages (18). To examine whether phagocytosis mediated by the chimeric receptors was also sensitive to PI3-K inhibitors, we evaluated the effect of wortmannin. Wortmannin blocked the phagocytic capacity of the chimeric receptors and of FcγRIIa, which has been shown previously to be wortmannin-sensitive (17). The degree of inhibition varied between a 70% to 85% decrease in the phagocytic index as compared with untreated cells (Table III, part B). Binding of the IgG-opsonized RBC as measured by rosetting was unaffected by wortmannin treatment (0–2% change).

DISCUSSION

We have demonstrated that chimeric Fcγ receptors with cytoplasmic tails composed of domains of the p85 subunit of PI3-K trigger phagocytosis in COS fibroblasts. Specifically, chimeric FcγR containing either the entire p85 subunit of PI3-K or the inter-SH2 portion that conveys constitutive enzymatic activity to the p110 subunit of PI3-K (26) were phagocytically active when expressed in COS cells and showed phosphoinositide kinase activity *in vitro* when immunoadsorbed. In

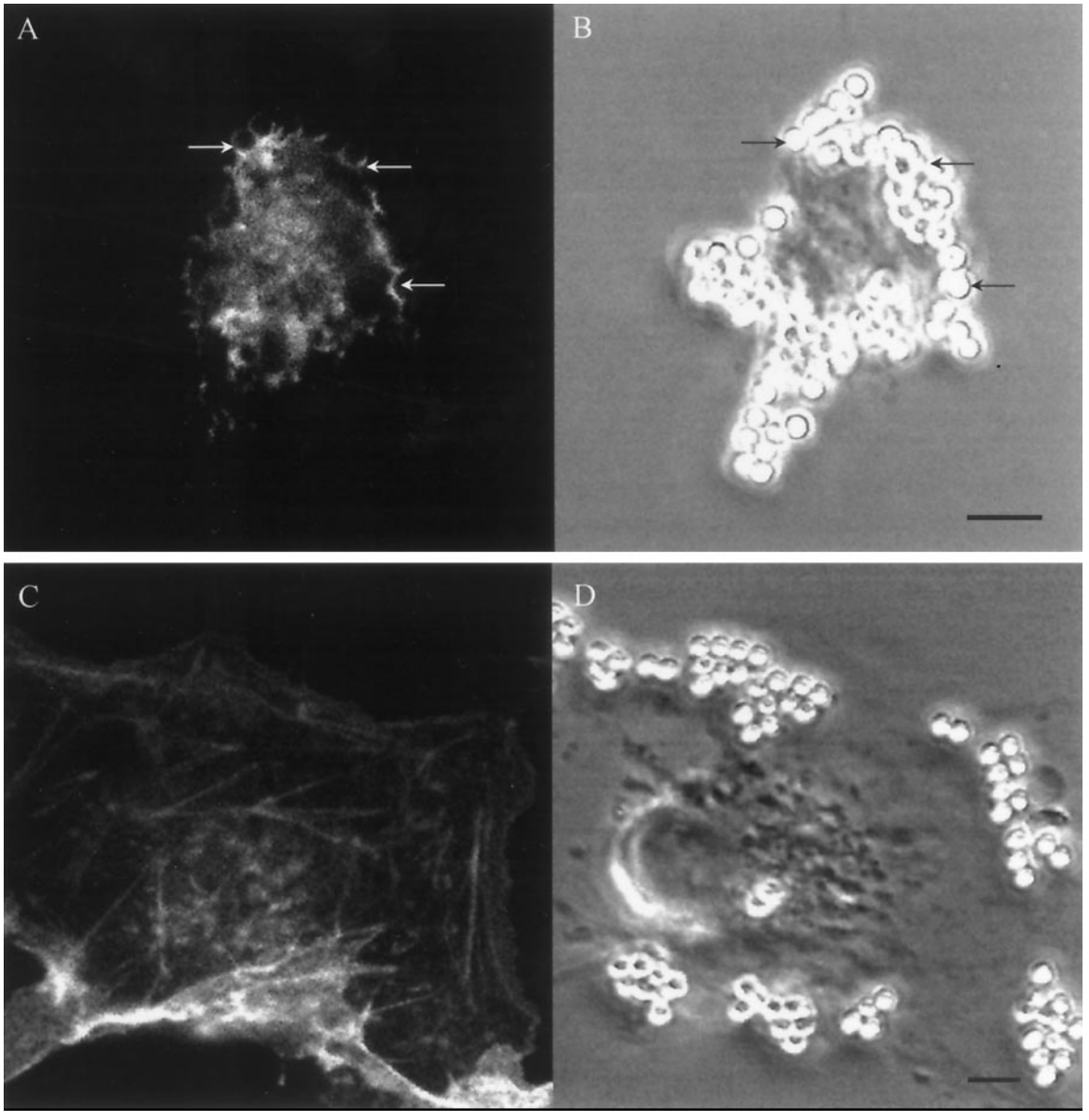

FIG. 4. **Confocal microscopy of F-actin triggering following particle binding.** Transfected COS cells were incubated with IgG-coated beads for 15 min at 37 °C to allow the initial steps of phagocytosis to proceed. The cells were then fixed and stained with FITC-phalloidin to detect actin filaments and were examined by confocal microscopy. *Panels A* and *C* show the fluorescent actin filament staining of the cells, while *panels B* and *D* show companion non-confocal phase contrast images to locate the beads. In *panels A* and *B*, a COS cell cotransfected with the Ia-p85 chimera and p110 subunit displays actin filaments accumulating beneath bound beads in cuplike structures (*arrows* in *A*, corresponding *beads* in *B*), indicating triggered actin polymerization. In *panels C* and *D*, a COS cell transfected with FcγRIa alone does not show any triggered actin polymerization beneath bound beads, reflecting the inability of the receptor to mediate internalization. *Bar* = 5 μm.

contrast, a chimera composed of the SH2-C region of p85 that does not interact with the p110 subunit did not trigger significant phagocytosis nor did it show PI3-K activity *in vitro*. These data, combined with our observation that the mediation of phagocytosis by both of these chimeras was inhibited by wortmannin, would indicate that the enzymatic activity of PI3-K is a critical mediator of phagocytosis and that the other adaptor domains of p85 are not essential for this function. Furthermore, our results would indicate that specific upstream signaling molecules essential for PI3-K recruitment to the active receptor complex can be effectively bypassed. Specifically, neither FcR γ-chain with its ITAM, nor the Src kinase responsible for ITAM phosphorylation, nor the non-receptor tyrosine kinase Syk would appear to be required for the phagocytic responses that we have measured. Simple localization and direct activation of PI3-K at the site of particle attachment appears to be sufficient to trigger the process of phagocytosis in COS cells. Our findings thus implicate the products of PI3-K enzymatic activity as central messengers in activating the process of phagocytosis.

For the purpose of this study, we have defined the term phagocytosis to be particle internalization inhibited by cytochalasin D as judged by phase and fluorescence microscopy. We did not examine further subtleties of the phagocytic process such as the ultrastructural details of phagosome formation, the movement of targets to lysosomes, and the possible modulation of phosphoinositide kinase activity. Our data, therefore, do not rule out the contributions of other signaling molecules of the FcγR complex in mediating these more subtle details of phagocytosis.

How the enzymatic products of PI3-K might mediate phagocytosis is largely unknown. Studies using macrophages treated with wortmannin suggest that PI3-K acts to promote closure of apposing pseudopods during phagocytosis (18). This step may involve actin polymerization leading to remodeling of

the cytoskeleton, and it may require active contraction of the cytoskeleton. Additionally, the membrane lipid fusion step required for pseudopod closure may require PI3-K, as suggested by the implication of PI3-K in endosome fusion events (33).

Other biological systems suggest how PI3-K might initiate FcR-mediated phagocytosis. For example, transient expression of a constitutively activated form of the p110 subunit of PI3-K in fibroblasts leads to F-actin membrane ruffling and stress fiber breakdown that mimics the effects of insulin treatment (34). Similarly, in adipocytes, an activated p110 is capable of inducing membrane ruffles, while a kinase-deficient form of p110 cannot (35). Treatment of 3T3 fibroblasts with the lipid product of PI3-K, PtdIns(3,4,5)P_3, increases cell motility in a chemotaxis assay, implicating directly the lipid products as mediators of cytoskeletal changes (36). The lipid products of PI3-K may regulate several molecules that modify the F-actin cytoskeleton, such as gelsolin and profilin. These two proteins regulate F-actin filament growth and stability. In studies measuring F-actin elongation rates, PtdIns(3,4)P_2 shows a high affinity for profilin that results in removal of profilin from F-actin filaments and a net increase in filament growth (37). Similarly, PtdIns(3,4)P_2 is an effective inhibitor of the actin severing activity of gelsolin (38). The lipid products of PI3-K have different affinities for profilin, with PtdIns(3,4)P_2 showing the highest affinity, followed by PtdIns(3,4,5)P_3 and PtdIns(4,5)P_2 (37) found in resting cells, implying that the levels of the various lipids may modulate the elongation and capping of F-actin filaments. Thus, several effects of PI3-K on the cytoskeleton can be correlated with our observations showing mediation of phagocytosis.

There are several effectors downstream of PI3-K that may transduce the signals needed to remodel the actin cytoskeleton. Two proteins identified as essential for phagocytosis in leukocytes are the small GTPases Rac1 and Cdc42. Dominant negative mutants of both these molecules expressed in a mouse macrophage line block FcγR-mediated phagocytosis (39). These members of the Rho family have been shown previously to have distinct properties in regulating the actin cytoskeleton in fibroblasts, with Cdc42 promoting filopodia and Rac1 promoting lamellipodia (40). The dominant negative mutants of Rac1 and Cdc42 display different abilities to inhibit the formation of F-actin rich pseudopods, implying that they have non-overlapping functions during phagocytosis. As the inhibition of F-actin polymerization by Rac1 and Cdc42 is incomplete, likely other molecules are involved in transducing the signals to remodel the cytoskeleton.

Members of the PKC family have also been implicated in the process of phagocytosis. Studies in monocytes have shown that PKC is enriched in phagosomes during FcγR-mediated phagocytosis, and that PKC inhibitors block phagocytosis in these cells (41), as well as in macrophages (42). A related observation in fibroblasts implicates PKC family members in mediating chemotaxis induced by treatment with the lipid products of PI3-K (36). This and other observations in cells stimulated through the platelet-derived growth factor receptor indicate that some PKC family members are activated downstream of PI3-K (43–45).

The pathway from PI3-K enzymatic activity to activation of these potential downstream effectors is not clear. However, the activation of Rac1 through the exchange of GDP for GTP has been shown to be dependent on PI3-K function (46). One possible model that may link PI3-K to the Rho family GTPases involves recruitment of guanine nucleotide exchange factors containing pleckstrin homology domains. In this model, production of Ptdlns(3, 4, 5)-P3 at the membrane leads to recruitment of an exchange factor through the binding of its pleckstrin homology domain to the lipid in a specific manner. The exchange factor in turn activates the Rho family member which then leads to effector function. One such family of proteins has been identified that includes the proteins Grp-1, cytohesin-1, ARNO, and Cts18 (47). These proteins contain not only pleckstrin homology domains but also Sec7 domains that stimulate GDP exchange by small GTPases. It is possible that related proteins may provide a similar link between PI3-K and downstream mediators of phagocytosis such as Rac1 and Cdc42. Localization of effector proteins by binding to the lipid products of PI3-K at the membrane may be a general strategy used to recruit the machinery needed to complete the process of phagocytosis.

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