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FUNCTIONAL ANALYSIS OF Fcγ RECEPTOR-MEDIATED PHAGOCYTOSIS

DISSERTATION

Presented in partial fulfillment of the requirements for the degree
Doctor of Philosophy in the Graduate School of
The Ohio State University

By

Malcolm B. Lowry, B.S.

*****

The Ohio State University

1998

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ABSTRACT

The immune system protects the body from foreign pathogens through the combined action of antibodies and their respective receptors. FcR. FcR are integral membrane receptors that bind the constant Fc portion of Ig and link the identification of antigens by antibodies to protective immune responses. One of the primary mechanisms of host defense triggered by FcR is the process of phagocytosis, in which IgG-coated particles are engulfed and degraded by immune system cells. There are three classes of Fc receptors for IgG (FcγR) that differ in structure and cellular distribution. The way in which FcγR mediate the phagocytic process is the focus of this dissertation.

The process of phagocytosis can be divided into four major steps: receptor-ligand binding, pseudopod extension, internalization, and sorting and fusion of the particle with lysosomes. The first three steps of the process were previously thought to depend on a single mechanism triggered by FcγR. Through the use of a COS fibroblast model in which receptors can be manipulated genetically, I found that the steps of pseudopod extension and internalization depended on different mechanisms and signals. Pseudopod extension was mediated by mutant FcγR that lacked intracellular domains in the absence of accessory subunits required for known signaling functions. In contrast, internalization required recruitment of tyrosine kinase activity through a conserved amino acid motif.
found in the γ-chain subunit, or in the cytoplasmic domain of FcγRII. Furthermore, although inhibitors of actin polymerization were capable of blocking internalization mediated by FcγR, pseudopod extension still persisted. The functional differences found between these two steps prompts a revision in the model of how phagocytosis proceeds.

Next, I focused on the signals involved during internalization of IgG coated particles. FcγR clustering leads to the recruitment of a multi-molecular signaling complex. One member of the complex, phosphoinositide 3-kinase (PI3-K), has been implicated in phagocytosis. To test directly the role of PI3-K, I examined the phagocytic capacity of chimeric receptors composed of FcγR extracellular domains fused to intracellular regions of the p85 subunit of PI3-K. Chimeric receptors that localized PI3-K enzymatic activity to the site of particle binding were sufficient to trigger internalization.
Dedication

This dissertation is dedicated with love to my entire family for their support through the years.
ACKNOWLEDGMENTS

I would like to express my gratitude and thanks to my adviser, Dr. Clark Anderson, for the training and support I have received during my development as a scientist in his laboratory. Many members of the Anderson laboratory have been instrumental in my training as a scientist. I would especially like to thank Dr. Anne-Marie Duchemin for her patience and enthusiasm in training my skills when I first joined the lab. Dr. Duchemin provided a wonderful role model, applying infectious enthusiasm, artistic skills, and intellect to tackle scientific challenges. I would also like to thank Jeanne Osborne for her help and also her keen ability to keep order in the often chaotic world of scientific study. For their assistance and comradery through the years, I would like to thank George Chacko, Diane Maresco, Kathy Miller, Stacey Garber, Jim Leach, and Linda Ernst.

Many members of the faculty have provided ideas, critiques, and suggestions over the years that have been very helpful in my career here. I would like to thank Dr. Mark Coggeshall for his advice and ideas through the years, and to Dr. John Robinson for his training in the ways of cell biology. I would also like to thank the members of my committee, Dr. Amanda Simcox, and Dr. Thomas Byers for their helpful suggestions.
On a personal note, I would like to thank Emily Ho for her patience and caring which allowed me to remain sane during the preparation of this dissertation.
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<td>cDNA</td>
<td>complementary DNA</td>
</tr>
<tr>
<td>Fab</td>
<td>fragment antigen binding</td>
</tr>
<tr>
<td>Fc</td>
<td>fragment crystallizable, constant domain of Ig</td>
</tr>
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<td>FcR</td>
<td>Fc receptor</td>
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<tr>
<td>FcγR</td>
<td>Fc receptor for IgG</td>
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<tr>
<td>ITAM</td>
<td>immunoreceptor tyrosine activation motif</td>
</tr>
<tr>
<td>MFI</td>
<td>mean fluorescence intensity</td>
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<tr>
<td>PI</td>
<td>phagocytic index</td>
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<td>PI3-K</td>
<td>phosphoinositide 3-kinase</td>
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<tr>
<td>PKC</td>
<td>protein kinase C</td>
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<tr>
<td>PLC</td>
<td>phospholipase C</td>
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CHAPTER 1

INTRODUCTION

The human immune system serves to protect the body from foreign substances called antigens by identifying and eliminating them. The immune system identifies antigens through the action of specific antibodies or immunoglobulins (Ig) that bind antigens. Immunoglobulins are composed of a tetrameric complex of two identical peptide "heavy chains" and two "light chains" (1). The amino terminals of the peptide chains vary extensively, composing the variable region that binds antigen. The carboxy terminal regions of the heavy chains are constant and compose the Fc portion of Ig (Figure 1). Receptors for the Fc region of Ig (FcR) expressed on the membrane of immune system cells bind Ig coated antigens and activate cellular processes to eliminate antigens. In this way, FcR link the identification of antigens by Ig to the activation of protective cellular responses by the immune system.

There are FcR specific for each Ig type, such as IgA, IgE, IgG, and IgM (2). These receptors belong to the immunoglobulin superfamily that includes the Ig family, the T-cell receptor, B-cell antigen receptor, and several interleukin receptors. Members of this superfamily have a common genetic organization, sequence homology, and a common structural motif (3). The motif is based on the Ig domain that folds into a structure of two
antiparallel β-sheets linked by a disulfide bond. All receptors of this family contain copies of the Ig domain in the extracellular region which are believed to participate in ligand binding.

**FcγR**

FcR specific for IgG (FcγR) are important mediators of immune system functions. These receptors trigger a spectrum of responses including phagocytosis, antibody-dependent cytotoxicity, release of inflammatory mediators, and superoxide production (4-6). FcγR have also been demonstrated to participate in the regulation of B-cell activation and T-cell development (7:8). An unexpected role for FcγR in initiating immune complex mediated inflammation has recently been found in transgenic mice targeted to knock out FcγR expression (9:10). Type III inflammation described as the “arthus reaction” (11) was believed to be mediated by a complement system cascade (12), but with data from transgenic mice it appears FcγR are the initiating factor (13). Thus new roles for FcγR are being defined in regulating a variety of essential functions of the immune system.

FcγR comprise a family of integral membrane glycoproteins that is divided into three classes (FcγRI, II, III) which differ in structure and affinity for IgG. These receptors share a similar structural organization with a signal sequence directing membrane insertion, two or three Ig-like extracellular domains, generally a single hydrophobic transmembrane region, and a cytoplasmic tail. There are several genes of each class of
receptor that have been identified in humans along with multiple transcripts generated by alternative splicing.

FcγRI is a 70 kD integral membrane receptor constitutively expressed on monocytes and macrophages and inducibly expressed on neutrophils. FcγRI is unique among the FcγR in that it binds monomeric IgG with high affinity (2). The extracellular region is composed of three Ig-like domains, unlike FcγRII and FcγRIII, which have only two Ig-like domains and bind monomeric IgG with low affinity. FcγRI is encoded by three genes (FcγRIA, B, C) that differ by several nucleotide substitutions in the coding regions. Due to changes in the B and C genes that result in premature stop codons, only the FcγRIA gene encodes a three extracellular domain integral membrane receptor that has been characterized as the bona fide FcγRI (14).

The receptor ligand binding unit of FcγRIIa does not contain any known signaling motifs, and thus associates with a subunit to provide this function. FcγRI non-covalently associates with the γ-chain subunit, a 10 kD protein that forms disulfide linked dimers (15). The γ-chain associates with FcγRI through interactions between the transmembrane regions of the proteins (16). This subunit also associates with the FcγRIIIa form (17), the high affinity FcR for IgE (FceRI), and the FcR for IgA (FcaR). While the γ-chain is required for the signaling function of all these receptors, it is not required by FcγRIIa and FcaR for surface expression in transfected COS cells. The in vivo requirement for expression in humans is not clear, but in transgenic mice deficient in the γ-chain, FcγRI
expression is not detectable (9). The γ-chain acts as a signal transducing subunit through a conserved amino acid sequence known as an immunoreceptor tyrosine activation motif (ITAM) (18). This motif is found in other members of the Ig family of receptors including the T-cell receptor, B-cell antigen receptor, FceR, and the other FcγR.

Following receptor clustering upon ligand binding, the ITAM is phosphorylated on two conserved tyrosines. Once phosphorylated, the ITAM serves as a docking site for src homology 2 (SH2) domain bearing proteins that form a signaling complex (19).

FcγRII is a 40 kD integral membrane receptor expressed on a wide variety of immune cells including monocytes, macrophages, neutrophils, B-cells, platelets, and a subpopulation of T-cells (20). This receptor binds monomeric IgG with low affinity, but binds immune complexes readily due to multiple receptor-ligand interactions. This class of receptor is also encoded by three genes (FcγRIIA, B, C) that are highly homologous in their extracellular domains, but diverge considerably in their cytoplasmic tails (21). The FcγRIIA and C genes contain ITAM activation motifs in their cytoplasmic tails, while FcγRIIB contains an inhibitory motif designated an ITIM in its tail. FcγRIIA is unique in that it contains the ITAM signaling motif in the same molecule that binds ligand, unlike other FcγR that associate with a signaling subunit.

FcγRIII is a 50-70 kD receptor expressed in macrophages, natural killer cells, mast cells, and in a fraction of monocytes and T-cells (22). Differences in the molecular weight have been attributed to varying glycosylation of the receptor. FcγRIII is encoded by two
genes (FcγRIIIA and B) that differ in structure. FcγRIIIA encodes an integral membrane receptor that associates with the γ-chain subunit required for expression and signaling functions of the receptor complex (23). This receptor, like FcγRII, binds monomeric IgG with low affinity, but binds immune complexes of IgG readily. FcγRIIB encodes a receptor attached to the outer leaflet of the membrane by a glycosyl-phosphatidylinositol (GPI) linkage that does not associate with γ-chain (24). This form of FcγRIII is only expressed on neutrophils where it may cooperate with other transmembrane receptors to trigger functional responses.

**Phagocytosis**

The process of phagocytosis is one of the primary mechanisms of host defense triggered by FcγR to eliminate pathogens. FcγR-mediated phagocytosis results in the engulfment and degradation of IgG-coated particles by immune cells. This process is differentiated from simple endocytosis by the participation of the actin cytoskeleton, and by the size of the particles internalized which are generally larger than 1 μm. Furthermore, phagocytosis is blocked at temperatures lower than 18°C while endocytosis can proceed at temperatures above 4°C (25). Thus the basic mechanisms underlying the two processes appear to be fundamentally different.

The process of phagocytosis can be divided into four major steps: 1. receptor-ligand binding, 2. pseudopod extension, 3. internalization, and 4. fusion of the target with lysosomes for digestion (Figure 2). Several elements in this pathway have been
identified. In the first step, FcγR bind ligands on an IgG-coated particle. Next, additional FcγR are recruited to bind the IgG ligands in a sequential manner around the particle. This process termed "zippering" leads to pseudopod extension around the particle. Internalization of the particle depends on a complete circumferential coating of IgG, and on the participation of the actin cytoskeleton that remodels during the process (26). The final step, sorting of the particle to lysosomes, is speculated to involve Rab family members that control intracellular vesicle trafficking.

One of the unique aspects of phagocytosis is that it occurs as a spatially confined event at the site of particle binding to FcγR at the membrane. In classical experiments performed almost 25 years ago, macrophages were shown to internalize IgG-coated particles but not particles adjacent to them attached to the membrane by anti-macrophage F(ab')2 fragments (26). This demonstrated the segmental response of the membrane during phagocytosis in which adjacent particles attached to the surface by other means were excluded from internalization stimulated by FcγR. This initial observation of the spatially restricted nature of phagocytosis has led to many insights into how this process is regulated. With knowledge gained from the past 25 years of research, we can sketch out a model of how phagocytosis proceeds.

The engagement of an IgG-coated particle by FcγR essentially clusters the receptors which leads to signal transduction. The initial formation of pseudopods around the particle is guided by sequential receptor-ligand interactions that may or may not require signaling events to proceed. Actin polymerization occurs beneath bound particles and is
proposed to contribute to the extension of pseudopods. The role of signaling events and actin polymerization during pseudopod extension will be examined in detail in Chapter 2. One of the earliest identifiable signals following receptor clustering is tyrosine kinase activity. Inhibitors of tyrosine kinase activity block actin polymerization and subsequent internalization (27). FcγR do not possess intrinsic tyrosine kinase activity. Instead these receptors contain motifs (ITAMs) that serve as docking sites for SH2 bearing proteins including protein tyrosine kinases. Members of the src family of tyrosine kinases are found associated with FcγR in the resting state independently of the ITAM (28). Upon clustering, the activity of associated src kinases increases leading to phosphorylation of the ITAM on two conserved tyrosine residues. The phosphorylated ITAM then recruits other SH2 bearing signaling molecules including the Syk/ZAP 70 family of non-receptor tyrosine kinases. Syk kinase itself becomes tyrosine phosphorylated possibly through interactions with src kinases already present in the complex (28), allowing recruitment of phosphoinositol 3-kinase (PI3-K) (29). PI3-K is primarily a lipid kinase that acts on phosphoinositides to produce putative second messenger molecules, although a serine/threonine kinase activity has been reported in vitro. The importance of PI3-K to the process of phagocytosis will be examined in Chapter 3.

The major theme of FcγR signaling during phagocytosis is the assembly of a multimolecular signaling complex at the site of receptor clustering. This localized recruitment reflects the functional segmental response of the membrane observed during phagocytosis almost 25 years ago. The key event in transducing the signal is the clustering of receptors that leads to an increase in the local concentration of substrates for associated
kinases. One mechanism likely to be at work is trans-phosphorylation in which src kinases phosphorlyate tyrosines of nearby ITAMs. As other molecules are recruited to the complex, they too may be acted upon by a similar mechanism, thus amplifying the signal locally. The nature of associations between these recruited signaling molecules is transitory, lasting usually for only a few minutes before the signal is turned off. This model assumes there are elements that return the complex to an inactive state after the signal has been transduced. Indeed, tyrosine phosphatase activity has been shown to be involved through inhibitors that lead to signaling events in the absence of receptor-ligand binding (30). Other regulatory kinases, such as c terminal-src kinase which inactivates src activity, may also be involved in returning the complex to an inactive state.

FcγR clustering leads to the activation of numerous signaling molecules, such as src family kinases, syk, PI3-kinase, Protein kinase C forms, phospholipase Cγ1, MAP kinases, and others (31-36). A major challenge in understanding signaling is identifying specific functional responses triggered by activation of a particular pathway. Although all the molecules activated by FcγR may be important for cellular functions, I have limited my study to those involved in phagocytosis. Specifically I have focused on the ability of receptors to trigger pseudopod extension and internalization of IgG coated particles. In Chapter 2, I develop and confirm the validity of a model system using COS cell fibroblasts to study phagocytosis. This system allows the use a genetic approach to manipulate phagocytosis triggered by FcγR. Using this approach, I examine the contributions of the ligand binding and signaling functions of FcγRI to the process of pseudopod extension and internalization. In Chapter 3, analysis of the signals involved in
internalization is extended to examine the role of PI3-kinase directly. Through the study of phagocytosis in a model system, I hope to clarify the elements involved in linking receptor clustering to activation of a distinct cellular response.
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Figure 1.2. The four steps of phagocytosis. Schematic drawing showing a cell expressing FcγR on the plasma membrane engaging an IgG-coated particle. Receptor-ligand binding stimulates pseudopod extension leading to internalization of the particle within the cell. The internalized particle is then sorted to the lysosome for digestion and degradation.
1.2 LIST OF REFERENCES


CHAPTER 2

FUNCTIONAL SEPARATION OF PSEUDOPOD EXTENSION AND PARTICLE INTERNALIZATION DURING Fcγ RECEPTOR-MEDIATED PHAGOCYTOSIS

2.1 SUMMARY

Receptors for the Fc portion of IgG (Fc\γR) mediate phagocytosis of IgG opsonized particles by a process which can be divided into four major steps: receptor-ligand binding, pseudopod extension, internalization, and lysosomal fusion. We have expressed single classes of Fc\γR in COS fibroblasts to examine the structural determinants necessary to complete the four steps of phagocytosis. Using phase contrast, fluorescence, confocal, and electron microscopy we have demonstrated that Fc\γR expressing COS cells can phagocytose in a manner similar to that of professional phagocytes. We have further analyzed the capacity of the three classes of Fc\γR to phagocytose, placing special emphasis on the Fc\γRIA-\γ-chain complex which allowed us to examine independently the roles of the ligand binding unit (Fc\γRIA) and the signaling unit (\γ-chain). We found that receptor complexes containing a conserved tyrosine activation motif (ITAM), as found in the cytoplasmic domain of Fc\γRIIA and in the \γ-chain associated with Fc\γRIA and Fc\γRIIIA, readily internalized target particles. In contrast, Fc\γRIA alone, having no ITAM, was unable to internalize target particles efficiently, but did mediate pseudopod extension. Co-transfection of \γ-chain with Fc\γRIA restored the ability of the receptor to internalize target particles.
mutant FcγRIA in which the cytoplasmic domain had been deleted was also capable of mediating pseudopod extension, showing that neither the γ-chain nor the cytoplasmic domain of FcγRIA were required for this step. Cytochalasin D, an inhibitor of actin polymerization, blocked particle internalization by all FcγR but did not block pseudopod extension. Staining the FcγRIA COS cells for F-actin and for tyrosine phosphoproteins, we found that actin did not polymerize during FcγRIA mediated pseudopod extension, nor were tyrosine kinases activated. Our data suggest that pseudopod extension and internalization are functionally distinct steps mediated through different pathways.
2.2 INTRODUCTION

Fcγ receptor-mediated phagocytosis is the process whereby IgG-coated particulate pathogens are internalized and moved to lysosomes for degradation. The mechanism requires fine coordination of the action of a variety of molecules including cell membrane receptors, signaling molecules, and cytoskeleton components. The receptors initiate the process by a zipper mechanism whereby sequential interaction of particle-bound IgG with phagocyte FcγR stimulates the spreading of pseudopods around the particle resulting in its eventual engulfment (1). Complete circumferential coating of the target particle by ligand is required, and FcγR additional to those involved in the initial binding of the particle are recruited during the process (2).

Several characteristics of the intracellular mechanism of FcγR-mediated phagocytosis have been described. Engulfment of attached particles requires actin polymerization as demonstrated by the blockade of phagocytosis by inhibitors of actin polymerization such as cytochalasin D. Along with F-actin, tyrosine phosphoproteins accumulate beneath attached IgG-coated particles prior to FcγR-mediated internalization. Both F-actin accumulation and particle internalization can be blocked by tyrosine kinase
inhibitors, indicating that the signal pathway requires tyrosine kinases (3). Indeed, several non-receptor tyrosine kinases of the src and syk family have been co-isolated with FcγR, and their activities have been shown to increase after FcγR clustering (4-6). Other signaling molecules such as protein kinase C and phosphoinositide-3 kinase have also been implicated in FcγR-mediated phagocytosis (7.8).

FcγR comprise a family of integral membrane glycoproteins with three standard components: i.e., an extracellular ligand-binding portion consisting of two or three Ig-like domains, a short hydrophobic transmembrane region, and a cytoplasmic tail. In humans, three classes (I, II, III) of these receptors have been characterized, differing in fine structure and affinity for IgG, and within these classes a total of eight subclasses (A, B, C) exist, each encoded by a separate gene (9). Of the eight FcγR in humans, three have convincingly been shown to mediate phagocytosis (FcγRIA, FcγRIIA, and FcγRIIIA) (10). Two of these three associate noncovalently with a common subunit, γ-chain. A common amino acid sequence is present in the γ-chain associating with FcγRIA and FcγRIIIA and in the cytoplasmic tail of FcγRIIA. This conserved motif, designated immunoreceptor tyrosine activation motif (ITAM), links receptor clustering to the activation of tyrosine kinases (11-13). Upon FcγR clustering, the tyrosines of the ITAM are phosphorylated (6,14,15) and in turn serve as docking sites for signaling proteins containing src homology 2 domains (16,17).
Analyzing the phagocytic mechanism of a single FcγR class has been difficult because monocytes and macrophages generally express multiple classes of FcγR. Therefore, structure-function studies of individual receptor classes have required expression of single FcγR classes into FcγR negative cells such as COS cell fibroblasts. This approach has allowed analysis of the phagocytic capacities of each receptor, the identification of receptor associated molecules required for function (12,18,19,20), and of receptor structural sequences necessary for phagocytosis (21-24).

However, we have questioned two aspects of this model: first, whether phagocytosis by COS cells mimics the process as it occurs in a professional phagocyte, and second whether phase contrast and fluorescence microscopy alone are adequate indicators of bona fide phagocytosis. Therefore, before embarking intently upon the study of the COS cell model of phagocytosis, we critically scrutinized in these cells the four major steps of FcγR-mediated phagocytosis - receptor-ligand binding, pseudopod extension, internalization, and fusion of the phagosome with lysosomes - with four microscopic methods - phase contrast, fluorescence, confocal and electron microscopy. Here we show that COS cells indeed complete these four steps in a manner morphologically similar to professional phagocytes, and we validate that phase contrast and fluorescence microscopy are reasonably accurate methods for estimating phagocytosis.
We then focused on the signaling complex of FcγRIA with γ-chain to examine independently the effects of the ligand binding unit, FcγRIA, and the signaling unit, γ-chain, in driving pseudopod extension and internalization. We found that although efficient particle internalization by the receptor complex requires γ-chain, pseudopod extension is mediated in the absence of γ-chain and by a tailless FcγRIA. Moreover, pseudopod extension mediated by FcγRIA is not blocked by cytochalasin D: and F-actin does not polymerize nor do phosphotyrosine residues accumulate adjacent to pseudopod extensions. These data suggest that these two phases of phagocytosis - pseudopod extension and internalization - are functionally distinct steps mediated by different mechanisms.
2.3 MATERIALS AND METHODS

Cells and cultures: COS-7 cells (ATCC, Rockville, MD) were maintained in Dulbecco's Modified Eagle's medium (DMEM, Life Technologies, Gaithersburg, MD) supplemented with 10% fetal calf serum (Hyclone, Logan, UT), 2mM L-glutamine, 100 IU/ml penicillin, and 100µg/ml streptomycin. The THP-1 monocytic cell line (a gift from Dr. Paul Guyre, Dartmouth Univ., Lebanon, NH) was grown in RPMI 1640 supplemented as above with the addition of 2-mercaptoethanol at 5.5 x 10^-5 M. Forty eight hours prior to experiments with THP-1 cells, 100 µ/ml recombinant human interferon γ (Genentech, S. San Francisco, CA) was added to the culture medium to enhance FcγRI expression.

cDNAs and transfection of COS-7 cells: The FcγRIA cDNA (25) was cloned into the pCDM expression vector for transfection into COS-7 cells. The human FcεRI γ-chain cDNA cloned into the expression vector pSVL was a kind gift from Dr. J.-P. Kinet (Harvard Univ., Cambridge, MA). The FcγRIIA cDNA cloned in the expression vector pCEXV3 and the FcγRIIIA cDNA were kindly provided by Dr. J. Ravetch (Rockefeller Univ., NYC, NY). FcγRIIIA cDNA was further cloned into the
expression vector pCDNA-1 (Invitrogen. San Diego. CA). The FcγRIA tailless mutant was constructed by deletion of the 3' end of the cDNA corresponding to the 61 amino acids from the c-terminus of the protein (26). COS-7 cells were transfected with 3μg of plasmid DNA per 10 cm dish by the diethyaminoethyl-dextran method as described previously (12) and analyzed for receptor expression and phagocytosis after 2 days.

**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 labelled by incubation with 0.1 mg/ml FITC in PBS overnight at 4°C. After 4 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 4 times to remove excess IgG and were then used in phagocytosis assays.

Fluorescent latex beads. 2 μm in diameter. with a carboxylate coupled surface (Molecular Probes. Eugene. OR) were covalently coated with streptavidin (Pierce. Rockford. IL) using a carbodiimide procedure as specified by Molecular Probes. Purified human IgG (Cappel. Cochranville. PA) and BSA (Life Technologies) were biotinylated using NHS-LC-biotin (Pierce. Rockford. Il) as specified by the manufacturer. The streptavidin-coated latex beads were mixed with either biotinylated
IgG or BSA in PBS and incubated for 4 h. The beads were then washed 4 times in PBS to remove unbound protein, resuspended at 1% solids in PBS with 1 mg/ml BSA, and stored at 4°C until use. Non-fluorescent beads, 2μm in diameter. (Polysciences, Warrington, PA) with a carboxylate coupled surface were coated with human IgG or BSA using a carbodiimide procedure as specified by the manufacturer.

**Phagocytosis assay - Phase contrast and fluorescence microscopy:** One day post transfection COS cells were seeded and grown overnight on glass coverslips in 24 well plates for analysis of phagocytosis. In other experiments, cells were grown on tissue culture plates for 2 days and then removed by trypsinization for analysis in suspension. FITC-labeled RBC opsonized with IgG were gently pelleted onto the COS cells in culture medium by low speed centrifugation for 3 min. To study binding of RBC to COS transfectants, cells were incubated together at 4°C for 1 h and were then washed in PBS and fixed with 1% paraformaldehyde in PBS. Three hundred cells were counted per condition by phase microscopy and the percentage of cells binding 3 or more RBC was defined as rosetting activity. To study phagocytosis, cells were incubated with RBC at 37°C for 1 h. The cells were then washed in PBS and subjected to a 45 s hypotonic shock to lyse externally bound RBC: this treatment does not lyse COS cells or internalized RBC. The cells were fixed in 1% paraformaldehyde and stained for fluorescence microscopy with 20 μg/ml ethidium bromide in PBS for 20 s. The cells
were then mounted in glycerin-PBS media on slides and examined by phase contrast or fluorescence microscopy with a Zeiss microscope. Three hundred cells were examined per condition and scored for the number of phagocytosed RBC. The data were expressed as a phagocytic index defined as the number of RBC internalized per 100 FcγR expressing COS cells. Expression of receptor was estimated by rosetting activity from parallel samples incubated at 4°C. In experiments where cytochalasin D (Sigma, St Louis, MO) was used, the inhibitor was added at 1 μg/ml just prior to the RBC target. Cell viability was scored by trypan blue exclusion before and after incubation with cytochalasin D.

**Phagocytosis assay- Confocal microscopy:** Phagocytosis assays were carried out in suspension as described above. The cells were then fixed in 1% paraformaldehyde and labeled with rhodamine-conjugated wheat germ agglutinin (Pierce) to mark both the COS plasma membrane and any externally bound RBC. The cells were immobilized on coverslips using poly-L-lysine. Cells were examined by confocal microscopy (Bio-Rad MRC 600, Hercules, CA) and optical sections were taken in series every 0.5μm in two fluorescent channels through single cells. One hundred cells were examined per condition and the number of RBC internalized was scored. Three dimensional reconstructions using two color fluorescence were accomplished using a Silicon Graphics Iris workstation with Voxelview software (Vital Images, Fairfield, IA).
Measurements between internalized RBC and the COS membrane were done on three dimensional reconstructions in three axes of the cell (x,y,z) from each individual RBC internalized for 10 representative COS cells.

**F-actin staining- Confocal microscopy:** 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 varying periods of time ranging from 10 to 40 min and were then washed 2x 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 3x in PBS, and stained with FITC-phalloidin (Molecular Probes) diluted 1:20 in PBS for 45 min at room temperature. The cells were washed 3x in PBS and were mounted in Mowiol medium (Polysciences) and viewed by confocal microscopy. To determine whether F-actin polymerization was triggered by particle binding, the density of stain was quantified by measuring the pixel intensity in the area 4μm subjacent to the perimeter of the bound bead. This value was compared to background cortical F-actin staining taken at several areas of the cell devoid of particle binding to establish a baseline intensity value. Comparison of the values obtained from bead binding to baseline levels yields a numerical fold increase value which reflects the net increase in F-actin triggered by bead binding for each cell examined. Morphological criteria were also
used to judge whether bead binding triggered assembly of F-actin extensions around target beads.

**Lipid labeling:** To examine pseudopod extension in parallel with F-actin measurements, transfected COS cells were labeled with a sulfonated Dil derivative (Molecular Probes) which is soluble in aqueous solutions. Cells were incubated with IgG-coated beads for various time periods and were washed in DMEM and fixed with 3% paraformaldehyde in DMEM for 15 min at 4°C. The lipid portion of the cells membranes were then labeled by incubation with 10μM Dil in DMEM at 37°C for 20 min. Following labeling, the cells were washed 3x in DMEM and were postfixed in 1% paraformaldehyde prior to mounting in Mowiol and analysis by confocal microscopy.

**Anti-phosphotyrosine staining:** Transfected COS cells adherent to glass coverslips were challenged with IgG-opsonized RBC or IgG-coated nonfluorescent beads for 7 to 15 min at 37°C. The cells were then washed 2x in DMEM and were fixed in cold acetone for 6 min. Following fixation, the cells were washed with DMEM and incubated with 0.1% BSA in DMEM for 15 min at 4°C. Anti-phosphotyrosine antibody 4G10 (UBI. Lake Placid, NY) or PY-20 (Santa Cruz Biotechnology, SantaCruz, CA) were incubated with the cells at 2μg/ml for 1 h at 4°C. The cells were
then washed 4x and incubated with a secondary FITC-conjugated F(ab)'2 donkey anti-mouse IgG (Jackson Immunoresearch, West Grove, PA) diluted 1:100 adsorbed against human, rabbit, and sheep serum proteins to prevent cross-reactivity. After 1 h at 4°C, the cells were washed 3x and were mounted in Mowiol for viewing by confocal microscopy.

**Phagocytosis assay- Electron microscopy:** Transfected COS cells detached from plates by trypsinization, or THP-1 cells, were mixed with either IgG- or BSA-coated fluorescent latex beads at 4°C for 30 min to allow binding and to synchronize phagocytosis. In other experiments IgG opsonized RBC were used as targets and cell sorting was omitted. To test the specificity of binding, IgG or BSA-coated beads were incubated with THP-1 cells and analyzed by flow cytometry. Results indicated that 92% of the THP-1 cells bound IgG-beads, while only 4% bound BSA-beads, confirming specificity. Mock transfected COS cells bound IgG or BSA coated beads at background levels ranging from 1% to 4% and did not internalize any beads. In experiments where cytochalasin D was used, the inhibitor was added prior to addition of target beads. After 30 min, the cells were then centrifuged to a pellet and the medium containing excess beads was removed. Cells were resuspended in complete DMEM and incubated at 37°C for 1 h to allow phagocytosis to occur, and were then washed in PBS 3 times to remove serum and unbound beads. Cells were fixed with
1% glutaraldehyde (EM grade, Polysciences) in 0.1M sodium cacodylate buffer pH 7.3 at 4°C for 1 h. Fixed cells were washed twice in sodium cacodylate and in PBS, and stored at 4°C prior to sorting. The cells were then sorted based on their ability to bind or internalize the fluorescent latex beads with an Elite EPICS fluorescence activated cell sorter (Coulter, Hialeah, Fl) and collected for further processing. Sorted cells were labeled with a wheat germ agglutinin-HRP conjugate (Pierce) to mark the external membrane surface and then washed and attached with 2.5% glutaraldehyde to 3 cm culture dishes that had been coated with 0.25% poly-L-lysine for 1 h. To form an electron dense label at the external membrane surface, the wheat germ agglutinin-HRP labeled cells were reacted with a 0.5mg/ml DAB solution containing 0.01% hydrogen peroxide in Tris-HCl pH 7.6 buffer for 20 min and processed as described previously (27). The cells were post-fixed in 1% osmium tetroxide (Polysciences) in 0.1M cacodylate buffer for 1 h, and then washed in cacodylate buffer prior to dehydration in a graded ethanol series and embedding in EPON epoxy resin. Thin sections were cut on a Reichart ultramicrotome and counterstained with uranyl acetate and lead citrate (Polysciences), and then viewed on a Philips EM 300 electron microscope operated at 60kv.

**Acid phosphatase localization:** Transfected COS cells were incubated as described above with IgG-coated fluorescent latex beads for 1.5 h at 37°C. The cells were
washed twice in PBS followed by cacodylate buffer and subsequently fixed in 1% glutaraldehyde with 5% sucrose in 0.1M cacodylate buffer for 30 min at 4°C. After fixation the cells were washed in cacodylate buffer and then sorted for their ability to bind or internalize the fluorescent latex beads on a fluorescence activated cell sorter (Coulter). Sorted cells were washed twice in 0.1M sodium acetate pH 5.0 buffer with 5% sucrose and then incubated in a cerium-based reaction media to detect acid phosphatase and processed as previously described (28). Briefly, the reaction medium consisted of 2mM cerium chloride (Alfa Products, Danvers, MA), 2mM of β-glycerophosphate as substrate, 0.005% Triton X-100 to permeabilize the cells, in 0.1M sodium acetate buffer pH 5.0 with 5% sucrose. Samples were incubated without substrate to assess specificity of the reaction. The cells were post-fixed with 2% osmium tetroxide for 45 min at room temperature and then processed for electron microscopy as described above. IgG-coated bead targets were scored to determine whether they co-localized with the electron dense reaction product produced during the enzyme cytochemical reaction. Control samples in which substrate was not present showed no reaction product, confirming specificity.
2.4 RESULTS

FcyR transfected COS cells complete all steps of phagocytosis in a manner similar to professional phagocytes: Using four different microscopic approaches we found that FcyR transfected COS cells indeed phagocytose in a manner similar to professional phagocytes. FcyR-transfected COS cells were incubated with IgG-opsonized FITC-labeled sheep RBC under phagocytosing conditions, were subjected to a brief hypotonic lysis to remove external RBC, and were then fixed, stained, and viewed by phase contrast and fluorescence microscopy to assess phagocytosis. By phase contrast microscopy the RBC targets appeared internalized, compressed, and compacted suggesting that they were contained within vesicles exerting a restrictive force on the RBC shape (Fig. 1a). By fluorescence microscopy the RBC targets, resistant to hypotonic lysis, appeared inside vesicular structures within the COS FcyR transfectants (Fig. 1b).

To distinguish precisely internalized from surface bound RBC which may have survived the hypotonic lysis, a two color fluorescence confocal strategy was employed, labeling the RBC with FITC and the COS cells with wheat germ agglutinin-rhodamine
to mark the plasma membrane. The resulting sectional images in three axes show internalized RBC clearly within the COS cell borders (two axes are shown in Fig. 1c and 1d). Three dimensional reconstruction, allowing measurement of the distance between the edge of internalized RBC and the COS plasma membrane, further confirmed the intracellular location of the phagocytosed RBC. Fully 40% of the RBC targets were ≥ 1μm inside the COS plasma membrane in all three axes, clearly indicating that they were internalized. For the remaining 60%, the absence of overlap between the two fluorescence signals strongly suggested that the targets were internalized, but the distance between the RBC and COS membrane was within 0.5μm in at least one axis. Because the 0.5μm measurement approaches the limit of resolution of our technique, internalization was not unequivocally demonstrable for 60% of the targets.

To determine definitively whether internalized targets were sealed within phagosomes, we evaluated phagocytosed IgG-coated latex bead targets by electron microscopy after labeling the exposed COS cell membrane surface with a wheat germ agglutinin-HRP conjugate and DAB peroxide. Such surface labeling of external membranes differentiates between truly internalized beads and beads which are surrounded by COS membrane but are not completely sealed inside a phagosome. Although, as illustrated in Figure 2a, some beads are found inside the cell border by position, they are not
sealed inside the cell as indicated by the presence of the DAB label around the beads. However, other beads are completely internalized by the cell as indicated by the lack of the DAB staining, illustrated in Figure 2b. Thus, COS FcγR transfectants are capable of internalizing IgG opsonized targets into sealed phagosomes, much like professional phagocytes, but not all targets which appear internalized by phase, fluorescent, confocal, and electron microscopy are necessarily sealed within phagosomes.

To determine conclusively whether transfected COS cells could complete the final step of phagocytosis - fusion of phagosome with lysosome - we co-localized a lysosomal marker with target beads by electron microscopy. Transfected COS cells, after phagocytosing IgG-coated latex beads, were processed to detect acid phosphatase activity using an enzyme substrate and cerium metal to form an electron dense precipitate at sites of enzyme activity. A majority of internalized bead targets, but not all, were found co-localizing with acid phosphatase (Fig. 2d), demonstrating that FcγR COS transfectants sort targets to lysosomes to complete the process of phagocytosis.

Quantification of the differential ability of the three FcγR to mediate phagocytosis in COS cells: To examine whether each of the three classes of FcγR mediates phagocytosis, we transfected COS cells with the cDNA for each class of FcγR and
analyzed the process of phagocytosis by phase contrast, fluorescence, confocal, and electron microscopy, quantifying the process at each stage.

*FcγRIIA:* FcγRIIA COS transfectants were capable of phagocytosing IgG opsonized targets as shown by conventional fluorescence and confocal microscopy (Tab. 1). Phagocytic indices (PI) being similar by both methods, 277 ± 152 and 244 ± 81, respectively. Mock transfected cells did not bind or phagocytose opsonized RBC. Analysis of transfected cells by electron microscopy indicated that 22% (16 of 72 cells examined) of FcγRIIA expressing COS cells were capable of complete phagocytosis, i.e., capable of moving beads into sealed phagosomes showing no DAB stain.

*FcγRIIIA:* FcγRIIIA co-transfected with its associated subunit, γ-chain, supported phagocytosis as determined by fluorescence and confocal microscopy, showing phagocytic indices of 235 ± 68 and 178 ± 95, respectively (Tab. 1). These values are similar to the results obtained with FcγRIIA (Tab. 1), indicating that both receptors mediate phagocytosis to a similar extent in the COS cell system.

*FcγRIA:* On the other hand, while FcγRIA COS transfectants bound IgG-coated targets avidly both at 4°C and 37°C (not shown), they phagocytosed poorly, showing phagocytic indices of 28 ± 6 by confocal and 61 ± 39 by fluorescence microscopy (Tab.
1). clearly distinguishable from mock transfectants which did not phagocytose at all.

However, the efficiency of FcγRI-mediated phagocytosis was significantly increased when FcγRI was co-transfected with γ-chain (PI of 270 ± 35), approaching levels achieved with FcγRIIA. Analysis by electron microscopy confirmed that only 3.6% (4 of 110 cells assessed) of FcγRIA-expressing cells were capable of phagocytosis, whereas COS cells co-transfected with both FcγRI and γ-chain showed 27% (18 of 66 cells examined) of the transfectants to be phagocytosing beads. Using acid phosphatase activity as a lysosomal marker, we found that FcγRIA-γ-chain co-transfectants localized three-fold more target beads to lysosomes than FcγRIA alone (16% vs 5%).

To determine whether the cytoplasmic domain of FcγRIA collaborated with γ-chain to mediate phagocytosis, we employed a tailless FcγRIA mutant cDNA. Expressing the tailless mutant alone in COS cells we found avid binding of IgG opsonized targets but minimal phagocytosis: the phagocytic index (40 ± 25) was similar to that observed with the intact FcγRIA (Tab. 1). However, co-transfection of the tailless receptor with γ-chain greatly increased phagocytosis (PI of 400 ± 133, Tab. 1). Thus it would appear that γ-chain greatly enhances the FcγRI phagocytic signal and that the cytoplasmic domain of FcγRI is not required for this process.
Pseudopod extension is independent of the cytoplasmic domain of FcγRIIA: We analyzed by electron microscopy how FcγRIIA and FcγRIIIA direct the first three steps of phagocytosis: namely, 1) binding of ligand-coated beads to receptors on the cell surface, 2) pseudopod extension with formation of a phagocytic cup, and 3) internalization resulting in a sealed phagosome. The three phases—surface attachment, cup formation, and phagosome sealing—were defined (Tab. 2) by the position of the target beads relative to the COS cell plasma membrane identified by WGA-peroxidase and by the morphology of the surrounding structure. Using these criteria, we found that FcγRIIA and FcγRIIIA COS transfectants displayed a different distribution of cell associated targets in these three phases of phagocytosis. FcγRIIA COS cell transfectants showed minimal internalization of bound targets after 1 hour (4%), but were capable of extending pseudopods around 52% of the targets (Tab. 2). Co-transfection of γ-chain with FcγRIIA increased internalization to 26%, yet the percentage of targets in the cupped phase remained high (63%) at the expense of surface bound beads. In contrast, FcγRIIIA COS cell transfectants internalized 20% of bound targets after 1 hour, with only 5% of the targets remaining in the cupped phase, while the majority of targets (75%) were bound at the surface of the cells provoking no apparent pseudopod extension (Tab. 2). To determine if the cytoplasmic domain of FcγRIIA was involved in directing pseudopod extension, we tested the mutant tailless receptor for the ability
to induce cupping and found that it induced cupping of 48% of bound targets, similar to the 52% induced by the wildtype receptor (Tab. 2).

To study the process of pseudopod extension in a professional phagocyte that naturally expresses FcγRIA, γ-chain, and FcγRIIA, the monocytes cell line THP-1 was used. The monocytes cells were processed in the same manner as the COS cells using identical IgG-coated bead targets and were examined by electron microscopy. The THP-1 cells internalized 64% of targets bound after 1 hour, and extended pseudopods around 34% of the targets (n=70).

We also examined by electron microscopy the process of pseudopod extension using IgG-opsonized RBC targets in both THP-1 cells and COS transfectants to rule out any effects due to the nature of the IgG-coated latex beads. Both cell types were processed identically after 45 minute and 1 hour incubations to allow a direct comparison of morphological differences between monocytes cells and COS fibroblasts transfected with FcγRIA. In both cell types, morphologically similar pseudopod extensions were observed extending around the RBC target particles (Fig. 3). These extensions did not follow the contours of the RBC targets as tightly as those observed with the latex bead targets in both cell types. This slight difference may reflect the rigid nature of the beads and the possibility that the RBC targets may shrink during the electron
microscopy fixation procedures, while the solid beads do not. We did not quantify the samples in which RBC were used as targets since COS transfection efficiencies ranged from 20% to 30%, making analysis by electron microscopy very difficult. In experiments using fluorescent latex beads as targets this problem was circumvented by sorting by flow cytometry only those cells which had bound beads. Regardless of whether IgG opsonized RBC or latex beads were used as target particles, pseudopod extensions were morphologically similar in both cell types.

**The role of actin polymerization in phagocytosis:** We examined the role of actin polymerization in the first three phases of phagocytosis by incubating COS cell transfectants with cytochalasin D. Cytochalasin D blocked internalization to near zero by all three classes of FcγR in COS cells as measured by fluorescence microscopy (Tab. 1). Rosetting activity, however, which measures binding of IgG-opsonized targets, was unaffected (Tab. 1), and cells remained viable by the criterion of trypan blue exclusion (97% before, 96% after treatment). Remarkably, cytochalasin D had little or no effect on the ability of pseudopods to move around target particles as evaluated by electron microscopy (Fig. 4). Although variations in the appearance of pseudopod extensions were common in both untreated (Fig 4a, 4b) and cytochalasin D treated samples (Fig 4c, 4d), movement of the extensions around target particles was unaffected. We directly measured the quantitative extent of pseudopod formation in
both untreated and cytochalasin D treated cells expressing FcγRI and FcγRI-γ chain as illustrated in figure 4d. The extent of pseudopod formation was measured in degrees as the arc of the bead target covered by pseudopods. Measurements of 50 beads showing pseudopod formation in both untreated and cytochalasin D treated samples gave a mean and standard deviation of 270° ± 60° and 240° ± 70° respectively, indicating no significant difference in the degree of pseudopod extension.

In analyzing the qualitative effect of cytochalasin D on the phase of pseudopod extension, we determined the percentage of targets found in the cupped phase with and without treatment. COS cells transfected with FcγRI alone displayed pseudopod extension in 63% of bound targets without cytochalasin D and 54% in the presence of the inhibitor (Tab. 3). Likewise, FcγRI-γ-chain co-transfectants exhibited 59% of bound targets cupped without inhibitor and 62% with cytochalasin D treatment. Consistent with results assessed by fluorescence microscopy, electron microscopy showed that cytochalasin D treatment blocked internalization from 30% down to 3% of cell-associated beads in the FcγRI-γ-chain co-transfectants (Tab. 3). FcγRIIA transfectants also showed a blockade of internalization from 20% down to 0% with cytochalasin D treatment, with an accompanying shift of targets to the cupped phase from 4% to 17% with treatment. Thus in COS cells transfected with either the FcγRIA
complex or FcγRIIA. cytochalasin D blocked internalization but did not block pseudopod extension.

To determine whether pseudopod extension is necessarily associated with F-actin polymerization, we stained COS transfectants with FITC-phalloidin and analyzed by confocal microscopy both pseudopod extension and internalization during phagocytosis of IgG coated beads. The density of actin filaments extending around the beads was quantified by measuring the pixel intensity of staining in a 4μm area photometrically (Tab. 4). In COS cells expressing FcγRIA, only 1% of cell-associated beads showed subjacent accumulation of F-actin at 15 minutes of incubation. This same pattern was also observed at 40 minutes: only 1 bead of 241 was rimmed with polymerized F-actin. In contrast, 45% of beads associated with FcγRIIA cells were rimmed with F-actin at 15 minutes, with F-actin stain density being 9 ± 4 fold above background cortical F-actin density (Tab. 4). These differences are illustrated in Figure 5 where F-actin bundles can be seen rimming beads in FcγRIIA expressing cells (Fig. 5a) while no such F-actin extensions are seen in FcγRIA expressing cells (Fig. 5c). To monitor pseudopod extension in this experiment, parallel samples were processed and fixed identically and then stained with a DiI derivative which labels lipid in membranes (Fig. 6). We were unable technically to combine lipid and F-actin staining in the same sample. Analysis by confocal microscopy revealed that FcγRIA expressing COS cells
showed vigorous pseudopod extension around attached beads (Fig. 6c) similar to results observed by electron microscopy (Fig. 4). These data suggest that pseudopod extension mediated by FcγRIA does not require detectable F-actin polymerization.

**FcγRIA-mediated pseudopod extension is not associated with tyrosine phosphorylation:** We next asked whether pseudopod extension is necessarily associated with the accumulation of tyrosine phosphoproteins beneath bound particles. assessing transfectants presented with either IgG-coated RBC or beads. First, transfected COS cells were incubated with IgG-coated RBC. fixed, stained with anti-phosphotyrosine antibody, and then evaluated by confocal microscopy. We found no accumulation of tyrosine phosphoproteins around RBC bound to 100 COS cells expressing FcγRIA (0/100) or the FcγRIA-tailless mutant (0/100). By contrast, of 100 FcγRIIA transfectants rosetting opsonized RBC. 39 showed the accumulation of tyrosine phosphoproteins beneath bound targets (Fig. 7a). Similar results were obtained using IgG coated beads as targets. There was no accumulation of tyrosine phosphoprotein around beads bound to 25 FcγRIA-transfected cells (0%) despite our observation with the lipid stain that these beads are enveloped by pseudopods. while 9 out of 29 (31%) FcγRIIA expressing cells showed beads with tyrosine phosphoprotein accumulation. These data would indicate that pseudopod extension need not be associated with the accumulation of tyrosine phosphoproteins.
2.5 DISCUSSION

We derive three main points from the data presented in this study. First, we establish that FcγR-transfected COS cells phagocytose in a manner that appears similar both morphologically and biochemically to professional phagocytes. We have scrutinized the entire four-step process of phagocytosis using phase contrast, fluorescence, and confocal microscopy; and using electron microscopy we have applied two well-accepted criteria for phagocytosis, viz., external membrane staining to demonstrate internalization, and acid phosphatase co-localization to identify lysosome-phagosome fusion. All of these approaches converge to indicate that FcγR-transfected COS cells perform all four steps of phagocytosis: namely, receptor-ligand binding, pseudopod extension, particle internalization, and lysosome fusion in a manner that we cannot distinguish morphologically from the process observed in phagocytes. Reconstitution of the FcγR mediated phagocytic pathway in the COS cell, a simian kidney fibroblast, implies that the process in both phagocytes and fibroblasts may
utilize common cellular components. Indeed, fibroblasts are considered non-
professional phagocytes under certain conditions (29). For example, fibroblasts can
phagocytose apoptotic neutrophils through a vitronectin receptor (30), and Con-A
coated yeast by crosslinking of unidentified surface glycoproteins (31). Moreover, in
murine macrophages, talin and paxillin, two fibroblast proteins associated with focal
adhesion plaques, localize to phagocytic cups prior to internalization of IgG-opsonized
targets (32,33) suggesting the use of common pathways by the two cell types to mediate
different functions. Another fibroblast cell line, the murine 3T3, has also been shown
to mediate phagocytosis when transfected with FcγRIIA (34). However, not all
fibroblast lines are capable of reconstituting FcγR mediated phagocytosis: for instance,
CHO cells transfected with FcγRIIA do not phagocytose (21), yet are capable of sorting
the parasite Toxoplasma gondii to lysosomes after infection when expressing FcγRIIB
(35). This suggests that although basic intracellular mechanisms can support
phagocytosis, critical factors coupling FcγR with the phagocytic response may be
missing in some cells. COS fibroblasts, however, appear to possess the necessary
factors, and therefore may represent an appropriate model system for the analysis of
the proximal steps of FcγR mediated phagocytosis.

The second main point to be drawn from our data is that although previous studies of
phagocytosis by FcγR-transfected COS cells (18,19,20,36) have yielded valuable
information, they have used methods which either assumed that particles appearing intracellular by phase contrast or fluorescence microscopy were indeed internalized or failed to prove definitively that the targets were internalized. We have addressed and resolved these two issues. Quantitative measurements of COS cell phagocytosis by phase contrast, fluorescence, and confocal microscopy were similar when expressed as phagocytic indices. Confocal microscopy did not improve the results of phase and fluorescence in that 60% of the apparently internalized targets were so close to the COS plasma membrane as to be unresolvable. However, electron microscopy, with plasma membranes marked by WGA-HRP, indicated that not all but certainly the vast majority of the apparently internalized beads were truly sealed from the exterior of the transfected COS cell. Likewise, colocalization of internalized beads and acid phosphatase by electron microscopy showed that most but not all bead-containing phagosomes had fused with lysosomes during the time course of the experiment. Thus, since our studies show that phase, fluorescence, or confocal microscopy come very close to approximating a true index of phagocytosis as determined by electron microscopy, we would conclude that the methodologic assumptions in these earlier studies are valid and that the results, therefore, are well grounded.

Our data support prior studies showing that the ITAM is critical for phagocytosis (18,22,36). We show that efficient phagocytosis is mediated only by FcγR associated
with the conserved tyrosine signaling motif, ITAM, implicated in signal transduction of several other immune system receptors (14,37,38). Others have shown that mutation of critical tyrosine residues in the ITAM of both γ-chain and FcγRIIA block phagocytosis (22,23,39). Functionally, the ITAM provides a means of linking receptor clustering to tyrosine kinase activation required for FcγR mediated phagocytosis (3). We have extended this observation by showing the accumulation of tyrosine phosphoproteins around opsonized particles being phagocytosed by FcγRIIA-transfected cells. Others have demonstrated that directly linking the tyrosine kinase syk to an FcγR extracellular domain results in a chimera capable of phagocytosis (40), reinforcing the central role of tyrosine kinase activity in directing phagocytosis.

The third and most novel aspect of our study is the use of the transfected complex of FcγRIA and γ-chain to examine independently the effects of the ligand binding unit, FcγRIA, and the signaling unit, γ-chain, in driving pseudopod extension and internalization. We found that pseudopod extension and internalization could be uncoupled. COS cells expressing either FcγRIA alone or a tailless FcγRIA were capable of pseudopod extension, yet incapable of particle internalization. However, co-transfection of γ-chain with FcγRIA to form an FcγRIA-γ-chain complex increased dramatically the internalization of cell associated particles while pseudopod extension remained essentially unchanged. Kinetic studies of phagocytosis over 4 hours (not
shown) indicated that the percentage of phagocytic cells and the phagocytic index reached maximal levels at 30 minutes for FcγRIIA and FcγRIIA COS transfectants, indicating that pseudopod extension was not an artifact of early sampling. Moreover, cupping by a monocytic cell line, THP-1, indicated that the phenomenon is not peculiar to transfected COS cells. Therefore we would propose that the ITAM containing γ-chain functions largely to mediate the internalization phase of phagocytosis, while pseudopod extension is primarily driven by the FcγRII ligand binding unit. In the case of FcγRIIA where both the ITAM signaling unit and the ligand binding unit are contained in the same molecule, pseudopod extension and internalization are sequentially coupled events. At early time points, F-actin rich pseudopods are observed by confocal microscopy (Fig. 5a) extending around particles. As the process continues, these particles are internalized as observed by electron microscopy (Tab. 2) such that after 1 hour particles are either completely internalized or remain surface bound with no apparent pseudopod extension, indicating that the two steps are coupled for FcγRIIA.

The role of actin polymerization in pseudopod extension and particle internalization was examined using two approaches. First, we found that an inhibitor of actin polymerization, cytochalasin D, blocked internalization of IgG-coated targets by COS cells transfected with any of the three classes of FcγR, consistent with findings of
others (18.41). It was remarkable, however, that although cytochalasin D blocked internalization of particles, it did not block pseudopod extension mediated by either FcγRIIA or FcγRIIA (Tab. 3). The effects of cytochalasin D on COS cell transfectants were seen in THP-1 cells as well, indicating that pseudopod extension in the presence of cytochalasin D is not cell type specific. Second, we directly assayed F-actin polymerization in response to IgG-particle binding at several early time points by staining transfected cells with FITC-phalloidin and analyzing them by confocal microscopy. This approach revealed that FcγRIIA was capable of triggering F-actin polymerization, but FcγRIIA was not (Tab. 4). Pseudopod extension around the particles was confirmed in parallel samples stained with the fluorescent Dil lipid label. Consideration of both the electron microscopy data and the F-actin confocal data for FcγRIIA indicated that approximately 50-60% of bound particles show pseudopod extension which is neither blocked by cytochalasin D nor associated with circumferential actin polymerization. The role of tyrosine kinases in the process of pseudopod extension and internalization was evaluated in similar fashion. We saw that tyrosine phosphoproteins were not found in the cups of FcγRIIA transfected cells but were easily visible around particles being phagocytosed by FcγRIIA transfectants. These data suggest that pseudopods can extend in the absence of tyrosine kinase activity and subsequent F-actin polymerization.
Others have recently described the separation of pseudopod extension from internalization, underscoring the multistep nature of the phagocytic process. A recent study of macrophages has shown that the step from pseudopodia extension to fusion, resulting in particle internalization, requires phosphoinositide 3-kinase (PI 3-kinase). but pseudopod extension itself is not blocked by PI3-K inhibitors (42). Similarly, a B lymphocyte line (DT40) transfected with either FcγRIIA or an FcγRIIIA-γ chain chimera manifests a block between pseudopod extension and internalization (43). These cells are able to form pseudopods and polymerize actin in response to an IgG-opsonized particle, yet are incapable of internalization, illustrating a further complexity in the stepwise mechanism of phagocytosis.

Work in another system has suggested that the two phases of phagocytosis - pseudopod extension and internalization - are functionally distinct. Studies in which the force of contraction in single granulocytes was measured during engulfment of large yeast pathogens found that the phagocytic response occurs as a sequential two step process. During the first step, the phagocyte spreads rapidly over the yeast particle without contraction of the cell body, then when spreading has stopped, contraction force increases (44). The observed two step process may reflect a functional division of the actions of a ligand binding unit and a signaling unit of phagocytic receptors which is transmitted to the cell.
The simultaneous occurrence of F-actin accumulation and pseudopod extension in macrophages and B cells has been interpreted as indicating that F-actin polymerization drives pseudopod extension (3.43). However, our findings would suggest that pseudopod extension is not necessarily dependent on the polymerization of F-actin. The formation of a pseudopod may result initially from the sequential interactions of receptor-ligand binding events that draw the cell membrane up around the particle. This process may only proceed to a certain degree without the support of the F-actin cytoskeleton, which is required to complete closure and internalize the particle. Whether this initial process reflects simply the receptor-ligand binding event or other possible signaling events that may be triggered through the receptor extracellular or transmembrane regions is not clear.

Methodological differences between our model and other models may explain why the separation of pseudopod extension from internalization has only recently been described. We and others (42) have used high resolution techniques such as electron microscopy and DiI lipid labeling with confocal microscopy to observe fine membranous pseudopods, while earlier studies used phase contrast or fluorescence microscopy which do not easily visualize these structures. The COS transfection system also allowed us to separate the effects of the ligand binding unit from the
signaling unit without the use of inhibitors which may have undesired effects on cellular functions.

This study provides further evidence for the functional separation of pseudopod extension from internalization during the process of phagocytosis. The case of the FcγRIA-γ chain complex highlights this point and raises several possibilities as to how pseudopod extension is driven. Of interest will be to examine how the ligand binding unit of FcγRIA mediates pseudopod extension to gain insight into the various mechanisms at work during FcγR-mediated phagocytosis.

Acknowledgements: We thank Kathy Wolken and Ann Osterfeld for expert technical support with confocal and electron microscopy, and Wilson Burrows for assistance with computer imaging from the confocal microscope. This work was supported by U.S. Public Health Service Award RO1-CA44983.
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Figure 2.1. Analysis of FcγR mediated phagocytosis by phase contrast and confocal microscopy. FcγR transfected COS cells were incubated with FITC-labeled sheep RBC opsonized with IgG at 37° C for 1h to allow internalization to occur. The cells were then subjected to a brief hypotonic lysis to remove externally bound RBC and were fixed and stained to mark the COS plasma membrane. Panels A and B illustrate a single FcγRIIA expressing cell stained orange with ethidium bromide examined by both phase contrast and fluorescence microscopy which has internalized several RBC targets (arrows) into large vesicles. In panels C and D, confocal sections in two axes of the same cells show green RBC targets which appear within the COS membrane labeled with a WGA-rhodamine conjugate appearing red. Panel C shows several sections in the Z axis, while panel D shows the Y axis of the same cell. The third axis, X, is similar to Panel D and is not shown. Bar = 10μm.
Figure 2.2. FcγR transfected COS cells internalize targets into sealed phagosomes which subsequently fuse with lysosomes. Transfected COS cells were incubated with IgG coated latex beads at 37 °C to allow phagocytosis to occur. The cells were then fixed and processed for electron microscopy. In panels A and B, the plasma membrane was labeled with a WGA-HRP conjugate and reacted with a DAB peroxide solution resulting in an electron dense band at the membrane surface. Panel A illustrates target beads which have been bound but not sealed within the cell as indicated by the presence of the external membrane label around the beads (arrows). Panel B shows a target bead which has been internalized within a sealed phagosome showing no membrane label whereas the plasma membrane is densely stained. In panels C and D, the cells were processed to detect acid phosphatase activity, a lysosomal marker. Panel C shows a bead target which has not been sealed within the cell and does not localize with acid phosphatase activity. Panel D illustrates a bead target which has been internalized and does co-localize with the electron dense reaction product of acid phosphatase (arrowheads), indicating lysosomal fusion. Bar = 1µm.
Figure 2.3. THP-1 monocytes and COS transfectants extend morphologically similar pseudopods. THP-1 monocytes (A, B) and FcγRIA COS transfectants (C, D) were incubated with IgG opsonized sheep RBC at 37 °C for 45 minutes (A, C) or 1 hour (B, D). The cells were then fixed, labeled with a WGA-HRP conjugate and processed for electron microscopy as described in the legend to figure 2A and 2B. In panel A, a THP-1 monocyte has extended fine pseudopods around an RBC target in an early phase of the phagocytic process. Panel B shows a later phase of pseudopod extension in which the THP-1 cell has drawn the RBC target almost completely into the cell. In panel C, an FcγRIA COS transfectant has extended fine pseudopods around the RBC targets in an early phase of the process, while panel D shows a later terminal phase in which the RBC is surrounded by the cell but not sealed within a phagosome. Bar = 1 μm.
Figure 2.4. Electron micrographs of FcγRIA (A, B, C) and FcγRIA-γ chain (D) COS transfectants engaged in the cupping phase of phagocytosis. Cells were incubated with or without cytochalasin D for 1h at 37° C and were then processed for electron microscopy. The cupping phase is observed in the absence (A, B) and presence (C, D) of cytochalasin D. Variations in morphology of the pseudopods were common as illustrated in panels A and B in which both examples were not treated with cytochalasin D. Panel D illustrates the method used to measure the extent of pseudopod formation. The circumference of the bead covered by pseudopods is measured in degrees which is indicated by the inset white area. Bar = 1μm.
Figure 2.5. Confocal analysis of F-actin following IgG-coated particle binding to FcγR COS transfectants. Transfected COS cells were incubated with IgG-coated beads for 15 min at 37°C and were then fixed, permeabilized, and stained for F-actin using FITC-phalloidin. Panels A and C show 0.5μm thick single confocal sections of F-actin staining, while panels B and D are companion non-confocal transmitted light phase contrast sections. In panel A, an FcγRIIA transfectant shows F-actin extensions surrounding bound beads, while the parallel panel B provides the position of the beads. In panel C, an FcγRIIA transfectant does not show any F-actin extensions or increases in F-actin due to target binding above background levels, while the parallel panel D shows the location of the beads. Cells were examined in all planes of focus to detect F-actin extensions as quantified in table 4. Arrows indicate F-actin extensions in panel A, and associated beads in panel B. Bar = 5μm.
Figure 2.6. Analysis of pseudopod extension by confocal microscopy. Parallel samples of transfected COS cells from the experiment described in figure 5 were incubated with IgG-coated beads for 15 min at 37°C and were then fixed and stained with a Dil derivative to label the membranes of the cells. Panels A and C show single confocal sections of Dil lipid staining, while B and D show companion non-confocal phase contrast sections. In panels A and B, an FcyRIIA transfected cell shows two beads with associated pseudopod extensions in the process of closure, while in panels C and D an FcyRII transfected cell shows several terminal pseudopod extensions around bound beads which do not fuse. Arrowheads indicate beads with associated pseudopod extensions. Bar = 5μm.
Figure 2.7. Anti-phosphotyrosine staining of FcγR transfected COS cells following IgG-opsonized particle binding. Transfected COS cells were incubated with IgG opsonized RBC for 10 min at 37°C and were then fixed and stained with anti-phosphotyrosine mab 4G10 followed by a secondary FITC-conjugated donkey anti-mouse IgG for detection. Panels A and C show anti-phosphotyrosine staining, while panels B and D show companion non-confocal phase contrast sections. In panel A, an FcγRIIA expressing cell displays tyrosine phosphoprotein accumulation at sites of IgG-opsonized particle binding, indicating triggered tyrosine kinase activity. In panel B, several cells not expressing FcγR (nuclei indicated by n) can be seen which show only background staining for phosphotyrosine in panel A, confirming specificity. Panels C and D show an FcγRIIA expressing cell which has bound many IgG-opsonized RBC but does not show any phosphotyrosine staining above background levels. Bar = 10μm.
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<td>Location of target beads relative to the COS plasma membrane</td>
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<td>Cupping is not inhibited by cytochalasin D in COS cells</td>
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<td>2.4</td>
<td>Analysis of F-actin polymerization following IgG-coated bead binding</td>
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Table 2.1. Phagocytic activity of the three FcγR classes expressed in COS cells. COS cells were incubated in the presence or absence of cytochalasin D with FITC-labeled RBC opsonized with IgG for 1h and were subjected to a brief hypotonic lysis to remove external RBC and were then fixed. Three hundred cells were examined per condition by phase and fluorescence microscopy and were scored for the number of phagocytosed RBC. Data represent the mean and SD of three experiments. ND= not done

<table>
<thead>
<tr>
<th>COS transfectant</th>
<th>Phase and fluorescence</th>
<th>Confocal</th>
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<tr>
<td></td>
<td>R.A.*</td>
<td>P.I.†</td>
</tr>
<tr>
<td></td>
<td>Cytochalasin D</td>
<td>Cytochalasin D</td>
</tr>
<tr>
<td>-</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Mock</td>
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<td>0</td>
</tr>
<tr>
<td>FcγRIA</td>
<td>20 ± 3</td>
<td>16 ± 1</td>
</tr>
<tr>
<td>FcγRIA tailless</td>
<td>18 ± 3</td>
<td>19 ± 2</td>
</tr>
<tr>
<td>FcγRIA + γ chain</td>
<td>10 ± 2</td>
<td>11 ± 4</td>
</tr>
<tr>
<td>FcγRIA tailless + γ chain</td>
<td>16 ± 4</td>
<td>15 ± 4</td>
</tr>
<tr>
<td>FcγRII A</td>
<td>16 ± 2</td>
<td>16 ± 3</td>
</tr>
<tr>
<td>FcγRIIIA + γ chain</td>
<td>11 ± 2</td>
<td>9 ± 1</td>
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</table>

* R.A., rosetting activity was determined from cells not subjected to hypotonic lysis and is defined as the percentage of cells which bound three or more RBC.
† P.I., phagocytic index is defined as the number of RBC ingested by 100 FcγR expressing COS cells.
‡ Confocal P.I. is the phagocytic index derived from three experiments analyzed by confocal microscopy.
<table>
<thead>
<tr>
<th>COS transfectant</th>
<th>Distribution of beads by location</th>
<th>Total number of beads associated with cells</th>
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<tr>
<td></td>
<td>% of total number</td>
<td></td>
</tr>
<tr>
<td></td>
<td>surface*</td>
<td>cupped‡</td>
</tr>
<tr>
<td>FcγRIA</td>
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<td>48</td>
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<td>FcγRIA + γ chain</td>
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<td>63</td>
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<tr>
<td>FcγRIIA</td>
<td>75</td>
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Table 2.2. Location of target beads relative to the COS plasma membrane after 1 hour. COS cell transfectants were incubated with IgG-coated latex beads at 37°C for 1h and were then fixed and processed for electron microscopy. The position of individual beads was determined relative to the labeled COS cell plasma membrane. The combined data from two experiments sort into three categories which define the position of the bead, i.e.,

*surface - The bead is bound to the external membrane surface with little or no pseudopod advancement. The measured arc of contact between the COS cell membrane and bead was 90°± 30° for 30 beads examined in this category.

‡cupped - The bead is enveloped by pseudopods covering 50% or more of the bead circumference. The measured arc of contact between pseudopods and bead targets was 280°± 60° for 30 beads examined in this category.

§sealed - The bead is sealed inside the cell as shown by a lack of external membrane staining around the bead.
Table 2.3. Cupping is not inhibited by cytochalasin D in COS cells. COS transfectants were incubated with IgG-coated latex beads with or without 1 µg/ml Cytochalasin D at 37°C for 1 h. Cells were processed as described in materials and methods, and were viewed by electron microscopy. The data sort into three categories, described in the legend to Table 2.2, which define the position of the bead relative to the COS cell membrane.

<table>
<thead>
<tr>
<th>COS transfectant</th>
<th>Cytochalasin D</th>
<th>Distribution of beads by location</th>
<th>Total number of beads associated with cells</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>+ / -</td>
<td>% of total</td>
<td></td>
</tr>
<tr>
<td></td>
<td>surface</td>
<td>cupped</td>
<td>sealed</td>
</tr>
<tr>
<td>FcγRIA</td>
<td>--</td>
<td>36</td>
<td>63</td>
</tr>
<tr>
<td>FcγRIA</td>
<td>+</td>
<td>46</td>
<td>54</td>
</tr>
<tr>
<td>FcγRIA + γ chain</td>
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<td>FcγRIA + γ chain</td>
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<tr>
<td>FcγRIIA</td>
<td>+</td>
<td>83</td>
<td>17</td>
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Table 2.3. Cupping is not inhibited by cytochalasin D in COS cells. COS transfectants were incubated with IgG-coated latex beads with or without 1 µg/ml Cytochalasin D at 37°C for 1 h. Cells were processed as described in materials and methods, and were viewed by electron microscopy. The data sort into three categories, described in the legend to Table 2.2, which define the position of the bead relative to the COS cell membrane.
<table>
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<tr>
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<th>total number of beads bound</th>
<th>Fold increase in F-actin staining intensity*</th>
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<tbody>
<tr>
<td></td>
<td>number</td>
<td>%</td>
<td></td>
</tr>
<tr>
<td>FcγRIIA</td>
<td>6</td>
<td>1%</td>
<td>432</td>
</tr>
<tr>
<td>FcγRIIA</td>
<td>104</td>
<td>45%</td>
<td>230</td>
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Table 2.4. Analysis of F-actin polymerization following IgG-coated bead binding. COS cells were incubated with IgG-coated beads for 15 min at 37°C and were fixed and then stained with FITC-phalloidin to detect actin filaments. The cells were analyzed by confocal microscopy and F-actin staining subjacent to bound beads was analyzed for the presence of F-actin extensions around target beads. A four μm area beneath bound beads was also measured for F-actin staining intensity and was compared against background cortical F-actin of the same cell to determine a fold increase value. The data are derived from 35 cells for each condition from two experiments. The increase in F-actin staining intensity observed for FcγRIIA compared to FcγRIIA is significant (p = 0.02).  
* Fold increase value derived only from beads which triggered F-actin accumulation.
2.8 LIST OF REFERENCES


CHAPTER 3

CHIMERIC RECEPTORS COMPOSED OF PI3-KINASE DOMAINS AND Fcγ RECEPTOR LIGAND-BINDING DOMAINS MEDIATE PHAGOCYTOSIS IN COS FIBROBLASTS.

Malcolm B. Lowry, K. Mark Coggeshall*, John M. Robinson†, Anne-Marie Duchemin’, and Clark L. Anderson. Department of Internal Medicine, Department of Microbiology*, Department of Cell Biology, Neurobiology, and Anatomy†; and Department of Psychiatry*. The Ohio State University College of Medicine, Columbus, Ohio 43210.
3.1 SUMMARY

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 phosphoinosytide 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-SH2 portion of p85 triggered phagocytosis in transfected COS fibroblasts. These two chimeras also showed inositol kinase activity in vitro when immunoadsorbed. In contrast, a chimera containing only the carboxy terminal SH2 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.
3.2 INTRODUCTION

Fc receptors for IgG (FcγR) 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).

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 (11). 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.

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 but it 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 D3 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 (SH3) domain, a breakpoint cluster region (BCR), two SH2 domains, and an inter-SH2 (iSH2) domain (Figure 1). The two SH2 domains direct the interaction of PI3-K with activated receptors (22, 23), while the BCR 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 FcγRIα fused to regions of the p85 subunit of PI3-kinase. 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 Figure 1. The cytoplasmic portion of the first chimera is composed of the wildtype p85 which contains several known functional domains of PI3-K. The tail of the second contains only the inter-SH2 (iSH2) region that is responsible for binding the catalytic p110 subunit of PI3-K causing constitutive kinase activity (26). The third chimera includes only the carboxy terminal SH2 region (SH2-C) of p85 that 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.
3.3 MATERIALS AND METHODS

Cells: COS-7 cells (ATCC, Rockville, MD) were maintained in Dulbecco's Modified Eagle's Medium (DMEM, Life technologies, Gaithersburg, MD) supplemented with 10% fetal calf serum (Hyclone, Logan, UT), 2mM L-glutamine, 100 IU/ml penicillin and 100μg/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 (Indianopolis, IN). F(ab)’2 fragments of goat anti-mouse IgG and FITC-conjugated forms were obtained from Pierce Chemical Co. (Rockford, IL).

cDNAs and transfection of COS-7 cells: The cDNA for FcγRIIA (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 Univ. NYC, NY). The three chimeric receptors composed of the FcγRIIA extracellular and transmembrane regions fused to constructs of the p85 subunit of PI3-K were constructed by joining the constructs at a Xba-1 site introduced into FcγRIIA by PCR. Two different primers were used to introduce the Xba-1 site into human FcγRIIA to allow the reading frame to be compatible with the different p85 constructs which all contain a Xba-1 site.
near the start of the protein sequence. The first primer, 5' CGT CTA GAC ACC CAG AGA AC 3' on the antisense strand, was used to construct the FcγRIA Xba-1 segment joined to create the Ia-p85 and Ia-SH2-C chimeras. The second primer, 5' GTT CTA GAC GTA TTG TCA CCC 3' on the antisense strand, was used to construct the FcγRIα segment joined to create the Ia-iSH2 chimera. Nucleotides changed with respect to the wildtype FcγRIA sequence are underlined. The PCR modified FcγRIA was first cloned into T-vector (Invitrogen, San Diego, CA), and then shuttled into pBluescriptSK (Stratagene, La Jolla, CA). The wildtype 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 (UCSF, 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 3μg of plasmid DNA per 10cm dish of cells by the diethylaminoethyl-dextran method as described previously (30) and were used for functional assays 2 days post 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 4 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 4 times to remove excess IgG and were then used in phagocytosis assays. Non-fluorescent beads, 2μm in diameter, (Polysciences, Warrington, PA) with a carboxylate
coupled surface were coated with human IgG or BSA 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 minutes on ice, and were then warmed to 37°C for 10 minutes. A secondary F(ab')₂ goat anti-mouse IgG (Pierce) was added to crosslink the receptors for 3 minutes at 37°C followed by addition of 1% Triton lysis buffer containing PBS (145mM NaCl, 20mM phosphate buffer pH 7.4), 10mM EDTA, 1mM sodium orthovanadate, 2mM PMSF (Sigma Chemical, St. Louis, MO) to lyse the cells. The lysates were clarified by centrifugation at 13000 X g for 10 minutes, and the resulting supernatants incubated with anti-HA antibody (Boehringer Mannheim) and protein G-sepharose to immunoadsorb 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 10mM Hepes-KOH, pH 7.4. Substrate phosphatidylinositol liposomes were prepared by resuspending vacuum dried phosphatidylinositol in assay buffer (30mM Hepes-KOH, pH 7.4, 30mM MgCl₂, 1mM EDTA, 50µM ATP) and sonicating on ice for 5 minutes at 50MHz. The washed adsorbates were resuspended in 40µl of assay buffer to which 10µCi of (γ-³²P) ATP in 20µl of phosphatidylinositol liposomes was added. The reactions were allowed to proceed at RT for 30 minutes and were terminated by the addition of 100µl 1N HCl. Lipids were extracted with 300µl CHCl₃: CH₃OH (1:1), removing the organic phase.
which was then dried under vacuum. The lipids were resuspended in 50μl of CHCl₃: CH₂OH (2:1) and were spotted on Silica G-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 Rₐ values in this solvent system.

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 minutes. To study binding of the RBC targets to COS cells, the cells were incubated at 4°C for 1 hour 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 hour. The cells were then washed in PBS and subjected to a 45 second 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 1μg/ml just prior to the addition of the RBC targets. In experiments using wortmannin (Sigma), the inhibitor was added at a final concentration of 100nM 15 minutes prior to addition of the RBC targets.

Measurement of receptor expression: Transfected COS cells were removed from culture plates and incubated with anti-FcγRI mab32 at 5μg/ml for 1 hour on ice. The cells were washed three times in PBS supplemented with 0.1% BSA, and were then incubated with a FITC-labeled F(ab')2 goat anti-mouse IgG for 1 hour 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 to IgG isotype controls, as well as the mean fluorescence intensity of the expressing cells. Since 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 2.

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 minutes and were then washed 2x 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 3x in PBS, and
stained with FITC-phalloidin (Molecular Probes) diluted 1:20 in PBS for 45 min at room temperature. The cells were washed 3x in PBS and were mounted in Mowiol medium (Polysciences) and viewed by confocal microscopy.
3.4 RESULTS

The three chimeras depicted in Figure 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 wildtype 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 carboxy terminal of each chimera, and the adsorbates were subjected to a PI3-K assay using phosphatidylinositol lipid micelles and P32 labeled ATP. We found
that both the Ia-p85 and the Ia-iSH2 chimeras reconstituted enzymatic activity (Figure 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. Wildtype FcγRIa also did not show PI3-K activity (data not shown), as expected since FcγRIa in the absence of γ-chain does not mediate phagocytosis.

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 (Figure 3). Internalized RBC were counted for each transfectant and expressed as a phagocytic index (PI). The data are tabulated in Table 1. The chimeras were compared in each experiment to wildtype FcγRIa that shows little or no phagocytosis, and to FcγRIIa which shows efficient phagocytosis. The Ia-p85 chimera was capable of mediating phagocytosis at levels similar to FcγRIIa (PI 170 vs. 153). Cotransfection of the p110 subunit with the Ia-p85 chimera raised the phagocytic index to levels significantly above that of FcγRIIa (PI 268 vs. 153) indicating very efficient phagocytosis. The Ia-iSH2 chimera also triggered phagocytosis, although at levels slightly lower than that of FcγRIIa (PI 108 vs. 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 vs. 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 2. We found that wildtype 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 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 FcγRIα (Table 1). 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 since only cells capable of binding the IgG-coated RBC are examined.

To determine whether actin was polymerized during chimera-mediated phagocytosis, we
evaluated the effect of cytochalasin D, an inhibitor of F-actin polymerization which
blocks phagocytosis in professional phagocytes and in COS cells expressing functional
FcγR (32). As seen in Table 3, 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 minutes at 37°C and were then fixed and stained
to detect actin filaments. As illustrated in Figure 4, COS cells cotransfected with the Ia-
p85 chimera and p110 subunit show accumulations of F-actin in cup-like structures
beneath bound beads, while wildtype FcγRIα 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 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 all the chimeric receptors and of FcγRIIa which has previously been shown to be wortmannin sensitive. The degree of inhibition varied between a 70% to 95% decrease in the phagocytic index as compared to untreated cells (data not shown). Binding of the IgG-opsonized RBC as measured by rosetting was unaffected by wortmannin treatment (0 to 5% change).
3.5 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 inositol kinase activity in vitro when immunoadsorbed. In 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 FcRγ-chain with its integral activation motif (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 inositol kinase activity. Our data, therefore, do not rule out the contributions of other signaling molecules of the FcR 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)P3, 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)P2 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)P2 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)P2 showing the highest affinity, followed by PtdIns(3,4,5)P3 and PtdIns(4,5)P2 (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 were previously shown 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. Since 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 PDGF receptor indicate that some PKC family members are activated downstream of PI3-K (43, 44).

The pathway from PI3-K enzymatic activity to activation of these potential downstream effectors is not clear. One possible model that may link PI3-K to the Rho family GTPases involves recruitment of guanine nucleotide exchange factors containing pleckstrin homology (PH) domains. In this model, production of PtdIns(3,4,5)-P3 at the membrane leads to recruitment of an exchange factor through the binding of its PH 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 (45). These proteins contain not only PH 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.

Acknowledgments:

We would like to thank Dr. Anke Klippel and Dr. Lewis Williams for the generous gift of the p85 constructs used to create the chimeric receptors. This work was supported by US public health service award RO1-CA44983.
### 3.6 LIST OF FIGURES

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Figure 3.1. Schematic diagram of the three receptor chimeras. The diagram depicts the chimeras formed by fusing the extracellular and transmembrane domains of FcyRIa with various regions of the p85 subunit of PI3-kinase. Domains depicted: FcyRIa, extracellular and transmembrane regions of FcyRIa: SH3, src homology 3; BCR, breakpoint cluster region; SH2-N, amino terminus SH2 domain; iSH2, inter SH2 domain; and SH2-C, carboxy terminus SH2 domain.
Figure 3.2. PI3-kinase 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 crosslinking 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 $^{32}\text{P}\gamma\text{ATP}$ 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 PIP3 product. In lane 1, mock transfected COS cells were adsorbed as a control for specificity. In lane 2, wildtype p85 was immunoadsorbed from mock transfected COS as a positive control for PI3-K activity. In lane 3, 100nM wortmannin (W) was added to the adsorbed wildtype 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 la-iSH2 and the la-p85 chimeras show PI3-K activity (lanes 5 and 6). We have not identified the intermediate spots.
Figure 3.3. 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 hour 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, while 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.
Figure 3.4. Confocal microscopy of F-actin triggering following particle binding. Transfected COS cells were incubated with IgG-coated beads for 15 minutes 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 la-p85 chimera and p110 subunit displays actin filaments accumulating beneath bound beads in cup-like 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 = 5μm.
Figure 3.4
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3.1 Phagocytic capacity of the receptor chimeras expressed in COS cells
3.2 COS cells transfected with receptor chimeras analyzed in parallel for phagocytic capacity and receptor expression
3.3 Cytochalasin D blocks chimera-mediated phagocytosis
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<th>COS transfectant</th>
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<td>FcγRIa</td>
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<tr>
<td>FcγRIIa</td>
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<tr>
<td>Ia-p85</td>
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<tr>
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Table 3.1. 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 hour 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 fluorescence microscopy. The number of RBC internalized by 100 receptor expressing cells (mean ± SD) 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).
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<th>Percentage of cells stained positive</th>
<th>Mean fluorescence intensity</th>
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<tr>
<td>FcγRIa</td>
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<td>184</td>
<td>10%</td>
<td>4</td>
</tr>
<tr>
<td>Ia-p85 + p110</td>
<td>127</td>
<td>20%</td>
<td>22</td>
</tr>
<tr>
<td><strong>Experiment 2</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>FcγRIa</td>
<td>10</td>
<td>30%</td>
<td>34</td>
</tr>
<tr>
<td>Ia-p85</td>
<td>83</td>
<td>14%</td>
<td>4</td>
</tr>
<tr>
<td>Ia-p85 + p110</td>
<td>119</td>
<td>11%</td>
<td>12</td>
</tr>
<tr>
<td>Ia-SH2-C</td>
<td>19</td>
<td>18%</td>
<td>6</td>
</tr>
<tr>
<td>Ia-SH2-C + p110</td>
<td>59</td>
<td>18%</td>
<td>8</td>
</tr>
</tbody>
</table>

Table 3.2. 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 3.1. The data from two experiments are shown.
Percent of cells forming rosettes | Phagocytic index
--- | ---
COS transfectant

<table>
<thead>
<tr>
<th>Cytochalasin D</th>
<th>Cytochalasin D</th>
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<tbody>
<tr>
<td>--</td>
<td>+</td>
</tr>
<tr>
<td>FcγRIa</td>
<td>37</td>
</tr>
<tr>
<td>FcγRIIa</td>
<td>32</td>
</tr>
<tr>
<td>Ia-p85</td>
<td>25</td>
</tr>
<tr>
<td>Ia-p85 + p110</td>
<td>26</td>
</tr>
</tbody>
</table>

Table 3.3. Cytochalasin D blocks chimera-mediated phagocytosis. Cells were incubated with IgG-opsonized RBC at 37°C with or without cytochalasin D for 1 hour. 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. Rosettes were defined as cells that bound 4 or more RBC targets.
3.8 LIST OF REFERENCES


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kinase in the completion of macropinocytosis and phagocytosis by macrophages. 


CHAPTER 4
SUMMARY AND CONCLUSIONS

FcγR provide the functional link between IgG and the induction of protective cellular responses by the immune system. One of the primary cellular responses in host defense triggered by FcγR is the process of phagocytosis. In this study, I have focused on several aspects of the phagocytic process. In Chapter 2, the model system used to study phagocytosis was confirmed and the elements involved in pseudopod extension and internalization were analyzed. This lead to a revision in the model of phagocytosis in which pseudopod extension and internalization are discrete steps of the process mediated by different mechanisms. Further analysis of the signal transduction events involved in the internalization step was analyzed in Chapter 3. Localization of phosphoinositide 3-kinase at the site of particle attachment by way of chimeric receptor constructs was shown to be sufficient to trigger internalization.

The pseudopod extension paradigm.
The process of phagocytosis can be divided into four major steps: receptor-ligand binding, pseudopod extension, internalization, and sorting and fusion of the target with
lysosomes. Pseudopod extension and internalization were previously thought to be inseparable steps resulting from the same mechanism. The basis for this hypothesis derives from observations in macrophages in which F-actin accumulation appears simultaneously with pseudopod extension. The polymerization of actin in response to receptor clustering is dependent on tyrosine kinase activity (1). Inhibitors of tyrosine kinases block F-actin remodeling and subsequent internalization of particles. Thus it has been proposed that actin polymerization drives pseudopod extension. The problem with this interpretation is that in macrophages, receptor clustering cannot easily be separated from signal transduction events. Since macrophages contain a full complement of signaling molecules linked to functional FcγR, crosslinking FcγR results in signals aimed at completing all four steps of phagocytosis. Therefore it is difficult to assess the contributions of the receptor ligand binding unit of FcγR in promoting pseudopod extension only. In the COS fibroblast model however, the ligand binding unit of FcγR1a can be expressed independently of the γ-chain signaling subunit, allowing analysis of the contributions of each unit to the steps of pseudopod extension and internalization. Using this approach, the ligand binding unit of FcγR1a was found to mediate pseudopod extension that did not lead to productive internalization. Electron micrographs showed fine membranous pseudopods extending out around bound targets that did not fuse. This result was also observed by confocal microscopy in which the lipid membrane of the cells was labeled with a fluorescent compound, ruling out artifact from the electron microscopy procedure. To rule out any effects due to the nature of the latex bead
particles. Sheep red blood cells were also used as targets and examined by electron microscopy. Pseudopod extension was also observed using the RBC targets, confirming that the process depended on receptor-ligand interactions and did not result from nonspecific surface effects.

The ligand binding unit of FcγRIa does not contain any known signaling motifs in its cytoplasmic domain. To determine whether the cytoplasmic domain played any role in mediating pseudopod extension, a mutant FcγRIa lacking the cytoplasmic domain was analyzed. This "tailless" FcγRIa was capable of mediating pseudopod extension similarly to the native FcγRIa, indicating that the cytoplasmic domain was not required for this function. Co-transfection of the γ-chain subunit with either native FcγRIa or the tailless mutant restored the ability of these receptors to internalize particles. From these data, it appears that pseudopod extension can be mediated by the ligand binding unit of FcγRIa alone, while internalization is dependent on the signaling functions of the γ-chain subunit. Since pseudopod extension and internalization can be uncoupled, this suggests that the two steps may utilize different mechanisms.

One mechanism required to internalize particles is the remodeling of the actin cytoskeleton that involves actin polymerization. Actin filaments polymerize beneath bound particles during internalization, forming a phagocytic cup around the particles that appears to support the closure and fusion process of the membrane. While actin
polymerization is required for internalization, the role of actin polymerization in pseudopod extension is not clear. I examined this question in two ways. First, COS cells expressing FcγR were treated with cytochalasin D, an inhibitor of actin polymerization, and analyzed by electron microscopy. While internalization of particles was blocked in all COS cells expressing FcγR, pseudopod extension still occurred. Quantitative analysis of the extent of pseudopod extension measured in degrees of coverage around the particle showed that cytochalasin D treated cells extended pseudopods to the same level as untreated cells. Second, COS cells expressing FcγR were stained with FITC-phalloidin to directly label actin filaments. Cells were challenged with IgG-coated particles and were then fixed at various time points and stained with phalloidin. Analysis by confocal microscopy revealed that FcγRIIa expressing cells showed an increase in actin filaments accumulating beneath particles during the process of pseudopod extension and internalization. Parallel analysis to detect lipid extensions of the membrane showed that these extensions fused to complete internalization. This result was expected since FcγRIIa contains an ITAM signaling motif in its cytoplasmic domain and therefore links receptor clustering to tyrosine kinase activity and subsequent actin polymerization for internalization. In contrast, FcγRIIa expressed alone did not trigger any detectable increases in actin filaments after binding IgG coated particles. Consistent with earlier electron microscopy data, parallel analysis to detect lipid extensions of the membrane did show pseudopod extension occurring around particles. These extensions, unable to fuse, did not complete internalization. Taken together, these results demonstrate that
pseudopod extension can occur without detectable actin polymerization by a process initiated by the receptor-ligand binding unit alone. What mechanisms are at work in driving pseudopod extension are largely unknown, although there are identifiable factors.

One such factor likely to be involved in pseudopod extension is receptor-ligand affinity. Data from electron microscopy indicates that receptors with different affinities mediate varying levels of pseudopod extension under conditions where internalization is blocked. Cells expressing the high affinity FcγRIIa show the highest percentage of particles found arrested with pseudopods extending around them, usually 3 fold higher than the low affinity FcγRIIa when the cells are treated with cytochalasin D. Therefore, high ligand affinity may increase the efficiency of the process of pseudopod extension. This may result from an increased ability of high affinity receptor-ligand binding events to “zipper” up the membrane along the particle. One of the predictions of this hypothesis is that even low affinity receptors should show some pseudopod extension in the absence of internalization signals. Preliminary analysis by scanning electron microscopy using a mutant FcγRIIa lacking the ITAM and cytoplasmic domain of the receptor shows that this receptor can mediate pseudopod extension. Thus it appears that both high and low affinity receptors can mediate varying degrees of pseudopod extension in the absence of internalization signals.
A second factor involved in pseudopod extension may be that the ligand binding units of FcγR transmit specific signals to induce pseudopod extension. To analyze if any tyrosine kinase activity was triggered following binding of an IgG-coated particle, a confocal microscopy strategy was used. COS cells expressing FcγRIa or FcγRIIa were challenged with IgG-coated particles and were then fixed at various time points ranging from 5 to 20 minutes. The cells were then permeabilized and stained with an anti-phosphotyrosine antibody to detect tyrosine kinase activity. This strategy was chosen since the potential signaling interactions would have to be mediated by contacts of the transmembrane or extracellular portions of the FcγR with other molecules that may depend on an intact membrane to stabilize them. This approach allows analysis of weakly associated tyrosine kinase activity at the site of particle binding that may otherwise be lost in conventional immunoadsorption or in vitro kinase assays. Results from this approach demonstrated that although induction of tyrosine phosphoproteins was readily detected following particle binding to FcγRIIa, no induction was observed in FcγRIa expressing cells. Thus it appears likely that the potential signals involved are not dependent on tyrosine kinase activity.

Other types of signals may be involved in pseudopod extension that have not yet been identified. A related response to phagocytosis, known as receptor capping, provides some insight into potential signals. Receptor capping occurs in leukocytes following binding of soluble IgG complexes (2), or experimentally following receptor crosslinking
by anti-FcγR antibodies. The receptors move into a condensed patch at one pole of the cell by a process that requires an intact actin cytoskeleton. This suggests that the movement of receptors in the plasma membrane may be regulated by contacts with the cytoskeleton. These contacts may provide tethering of receptors to the cytoskeleton, which allows proteins associated with the cytoskeleton to react to physical changes caused by receptor clustering.

An analogy to this arrangement is found in integrin receptors that associate with several cytoskeletal scaffold proteins. Clustering of these receptors leads to formation of focal adhesion plaques and actin stress fibers by a process dependent on the small GTPase Rho (3). Inhibitors of Rho function appear to block the ability of FcγR to cap and cluster (4), suggesting that Rho may be a potential candidate that is activated during FcγR mediated pseudopod extension. This is an attractive hypothesis since several proteins that regulate cytoskeleton to membrane attachments are dependent on Rho function. A direct target downstream of Rho is Rho-associated kinase, which is a serine/threonine protein kinase. This kinase can phosphorylate members of the ezrin/radixin/moesin (ERM) family of proteins that act as cross-linkers between actin filaments and the membrane. Phosphorylation of these proteins by Rho-kinase interferes with the ability of the ERM proteins to assemble together to form the actin to membrane crosslinks (5). Therefore activation of Rho may serve to remodel the attachments of the actin cytoskeleton to the membrane, allowing the necessary flexibility of the membrane to form pseudopod.
extensions after particle binding. Further analysis in this area will be needed to determine if these or other signals are involved in the process of pseudopod extension.

**The signal transduction pathway to internalization.**

FcγR-mediated phagocytosis results in the activation of several signal cascades following the critical event of receptor clustering. The earliest identifiable signal is tyrosine kinase activity, which is required for phagocytosis to proceed (1). The cytoplasmic regions of FcγR do not contain intrinsic tyrosine kinase activity. Instead, these receptors either contain (FcγRIIa) or associate with a subunit (γ-chain) that contains a conserved amino acid motif, which acts as a docking site for SH2 domain bearing proteins. This domain is designated an ITAM, and it becomes phosphorylated on critical tyrosine residues following receptor clustering. In chapter 2, I showed that FcγR complexes that contained an ITAM mediated efficient internalization of IgG-coated targets. Furthermore, the recruitment and activation of tyrosine kinase activity following particle binding was observed by confocal microscopy in COS cells expressing ITAM containing FcγR. Therefore the COS cell model is capable of reconstituting the initial events of the signal pathway leading to internalization much the same as in professional phagocytic cells.

To understand the hypothesis underlying my extended analysis of the signal pathway leading to phagocytosis, I will first sketch out a working model of FcγR signal transduction. Following receptor clustering, the ITAM is phosphorylated by a src kinase
family member, leading to the creation of an SH2 docking site for tyrosine kinases of the Syk/ZAP70 family and others. Src kinase members are found associated with FcγR in the resting state, through interactions that are not dependent on the ITAM (6). The activity of src kinase members increases following receptor clustering, leading to phosphorylation of the ITAM. Recruitment of the non-receptor tyrosine kinase Syk then occurs through direct binding of Syk to the phosphorylated ITAM (7). The binding of Syk to the ITAM enhances the enzymatic activity of Syk (8), further amplifying the signal cascade. Phosphoinositide 3-kinase (PI3-K) then associates with the receptor complex by a mechanism requiring phosphorylation of both the ITAM and Syk (7). This suggests that Syk activation allows recruitment of PI3-K through binding to Syk or to an intermediate adaptor molecule. The end result is the activation and recruitment of PI3-K to the receptor complex subjacent to the membrane where substrates for PI3-K are readily available. Here we postulate that the production of lipid second messengers by PI3-K in the inner leaflet of the membrane is the key step to organize and activate the necessary machinery for internalization to occur.

To test directly the hypothesis that localization and activation of PI3-K at the receptor complex is sufficient to trigger internalization, I constructed chimeric receptors composed of FcγR ligand binding domains fused to regions of the p85 subunit of PI3-K. These chimeras were designed to mimic the recruitment of PI3-K to receptor clusters without the need for ITAMs, src family kinases, or Syk kinase acting upstream. As described in
chapter 3. receptor chimeras containing the entire p85 subunit or just the inter-SH2
domain of p85 were capable of triggering internalization of IgG-coated particles in COS
cells. These two chimeric receptors also reconstituted inositol kinase activity in vitro
when immunoadsorbed. In contrast, a chimera composed of only the carboxy terminal
SH2 domain of p85 that does not interact with the p110 subunit did not trigger
internalization nor did it show inositol kinase activity in vitro. These data, combined
with the observation that wortmannin inhibited phagocytosis by these chimeras, indicates
that the enzymatic activity of PI3-K is a critical mediator of phagocytosis. Furthermore,
the adaptor functions of p85 other than the binding of the p110 subunit do not appear to
be essential for the mediation of phagocytosis. These results implicate the lipid products
of PI3-K enzymatic activity as central messengers in activating the process of
internalization.

How the lipid products of PI3-K mediate internalization is largely unknown. To
complete internalization, the actin cytoskeleton must be remodeled to support closure of
apposing pseudopods. In chapter 3, the phagocytic chimeras were shown to stimulate
actin remodeling by confocal microscopy and by sensitivity to cytochalasin D, an
inhibitor of actin polymerization. Therefore the lipid products of PI3-K are likely to act
on molecules that either directly or indirectly regulate the actin cytoskeleton.

Two examples of proteins that directly regulate the actin cytoskeleton which bind PI3-K
lipid products are gelsolin and profilin. These two proteins regulate actin filament
growth and stability: additionally gelsolin is also capable of severing actin filaments to initiate remodeling of a filament network. The lipid products of PI3-K bind directly to these proteins and modify their functions. Binding of PtdIns(3,4)P2 results in removal of profilin from actin filaments and a net increase in filament growth (9). While this same lipid product can inhibit the actin severing ability of gelsolin (10). Other lipid products of PI3-K have different affinities for profilin and gelsolin, implying that the levels of these lipids may regulate the remodeling of the actin cytoskeleton by these two proteins.

The observation that direct localization of PI3-K to the site of particle attachment is sufficient to trigger the complex process of phagocytosis suggests that PI3-K can coordinately activate several signal cascades. Clustering of native FcγR leads to the activation of several signal cascades including tyrosine kinase activity, Protein kinase C activation, phospholipase C (PLC) activation, AKT activation, Rac activation and others (11-14). Of the potential signals triggered by native FcγR, which of these can be activated by PI3-K lipid products? It appears that all of these cascades downstream of tyrosine kinase activity can be activated by PI3-K activity. Recent work in several fields shows that PI3-K can act as a major integration point of signal transduction from which branching of the signal to activate several cellular functions occurs. One primary mechanism that accounts for this effect is the ability of the lipid second messengers to bind and localize proteins to discrete cellular locations. Binding of proteins to inositol phospholipids is mediated by a common structural protein module known as a pleckstrin

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homology domain. This domain is roughly 100 amino acids in size and confers specificity for various inositol lipid products and substrates of PI3-K and PLC (15).

Through the localization of effector proteins by the binding of pleckstrin homology (PH) domains to the lipid products of PI3-K, a variety of proteins can be recruited to complete a complex cellular response.

As an example, during phagocytosis several signaling molecules may need to be activated in a precise series to coordinate the cellular processes required to complete phagocytosis. Therefore the branching of the signal by PI3-K will need to be organized spatially and sequentially. The receptor complex limits PI3-K activity spatially to the area beneath the bound particle. This ensures that PH domain bearing proteins are recruited to the site where they are needed for function. Insight into the sequential organization of the signal pathway comes from recent work showing a link between PI3-K and PLC. In this pathway, production of PtdIns(3,4,5)P3 by PI3-K leads to association of PLC with the membrane through binding of the PH domain of PLC to the lipid product (16). Membrane localization of PLC then allows activation of the enzymatic activity and production of diacylglycerol (DAG) and inositol(1,4,5) trisphosphate (IP3), second messengers that lead to activation of protein kinase C. Protein kinase C (PKC) can then associate in an active state with adaptor molecules such as RACK (17) that can further associate with PH domain bearing proteins, targetting the activity to the site of initial PI3-K activity. The major theme of this pathway is that although branching of the signal occurs, the end point leads back to recruitment of effector proteins to the site of initial
PI3-K activity. In this way, PI3-K may coordinate several pathways that lead to activation of spatially restricted cellular responses such as phagocytosis.

Considering the type of cellular functions that must be coordinated during phagocytosis, a model based on available data can be constructed. If we first consider the sequence of functions needed to complete internalization, we arrive at the following chain of events. First, the actin cytoskeleton must be activated to remodel the filament network, potentially involving actin-severing proteins such as gelsolin. This initial regulation may be accomplished by direct binding of PI3-K lipid products to the protein, rapidly activating its function. Next, actin polymerization is required to support the advancing pseudopods around the particle. Two candidate molecules that regulate actin polymerization are Rac and Cdc42 that have been shown to be essential for the completion of phagocytosis in leukocytes (14). These small GTPase proteins are regulated by guanine nucleotide exchange factors that exchange GDP for GTP, which results in activation of effector function. Recruitment of a PH domain bearing exchange factor is a likely mechanism whereby PI3-K lipid products can trigger activation of Rac and Cdc42. Indeed, a family of proteins that act as exchange factors for related small GTPases has been identified that contain PH domains which bind PtdIns(3,4,5)P3 (18) the major product of PI3-K. Next, the apposing pseudopods must close to internalize the particle through membrane fusion events. One candidate molecule that may alter membrane structure to promote fusion is pleckstrin, a protein that can induce membrane projections once activated. Activation and association of pleckstrin with membranes is
dependent on two integrated signals. PH domain binding to inositol lipids and phosphorylation by PKC (19). A second candidate to mediate membrane fusion is dynamin, a protein implicated in the regulation of membrane scission events during endocytosis (20). Dynamin also contains a PH domain and is a substrate for PKC, suggesting that it may be regulated in a similar fashion to pleckstrin. Thus coordinate regulation can be achieved by the activity first of PI3-K and next of PKC which can be activated downstream through the PI3-K cascade. In this way, the sequential nature of the phagocytic process can be regulated through the integration of multiple signals.

PKC has been implicated in phagocytosis through the use of inhibitors that block phagocytosis in monocytes and is also found enriched in forming phagosomes (11). Since PI3-K can activate PKC through the signal cascade, the role of PKC during phagocytosis may be to contribute to the regulation of membrane fusion as described for pleckstrin and dynamin. An additional role in regulating sorting of newly formed phagosomes is also suggested by the enrichment of PKC in phagosomes. PKC may regulate phagosome sorting by interacting with proteins that direct intracellular trafficking. One such protein is beta-COP, a coatamer protein found surrounding endosomes that directs trafficking. Beta-COP can act as an adaptor for activated PKC (21), indicating that PKC may influence sorting by modifying beta-COP. Furthermore, assembly of beta-COP proteins into a functional coatamer complex around endosomes requires the action of a small GTPase protein known as ARF (22). The activity of ARF requires GTP, the loading of which is controlled by guanine nucleotide exchange factors.
(GEF). A family of GEFs for ARF proteins has been identified that contain PH domains specific for PtdIns(3,4,5)P3, the major PI3-K lipid product (18). Therefore the sorting mechanism requires the integration of PI3-K activity and PKC activity to produce a productive cellular response. This is likely to be a general theme in the coordinated control of phagocytosis, where specific steps of the process are dependent on signals from two or more signal pathways.

Further experiments are suggested by this hypothesis that could be addressed using the COS cell model and the chimeric receptors discussed in chapter 3. One area of interest is the recruitment of exchange factors for small GTPases that control the activation of Rac and Cdc42. Competition experiments with known sequences of PH domains may help to identify potential targets for cloning of these factors. Alternatively, immunoadsorptions of activated Rac complexes may allow purification of these factors. The role of PKC could also be examined by analyzing the potential downstream targets such as pleckstrin and beta-COP. As a starting point, the phosphorylation status of these targets could be studied following receptor clustering, to ensure the relevance to phagocytosis. Once established, strategies to block the recruitment and activation of these molecules could be used to address the functional role to particular steps of the phagocytic process. One such strategy could involve competition experiments by transfecting the binding domain of PKC adaptors such as beta-COP or RACK1 into cells expressing the chimeric receptors and assaying the effect on fusion or sorting. This strategy may be a more specific way to identify the importance of potential targets of PKC without disturbing general PKC
function in the cell. There are several future areas to examine in which the chimeric receptors can be a valuable starting point to study the pathway of signal transduction during phagocytosis.

In my study, I have examined the functional requirements for the specific steps of pseudopod extension and internalization during FcγR-mediated phagocytosis. Using a COS fibroblast model, I demonstrated that pseudopod extension and internalization are discrete steps dependent on different mechanisms. This led to a revision in the general model of phagocytosis that provided insight into the underlying mechanisms triggered by FcγR. Next, focusing on the signals required for internalization. I demonstrated that localization and activation of PI3-kinase at the site of particle attachment was sufficient to mediate internalization. The enzymatic activity of PI3-K was shown to be essential for internalization to occur, implicating the lipid products of PI3-K as central messengers in organizing and activating phagocytosis. From this work, the nature of how cell surface receptors trigger a cellular response can begin to be addressed, through the linking of signal transduction to discrete functional steps of a complex process such as phagocytosis. The major themes found in the signal pathway of phagocytosis, such as spatial localization, sequential organization, and integrated signaling networks, will certainly be applicable to the fundamental investigation of how receptors work.
4.1 LIST OF REFERENCES


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