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Phagocytic signaling molecules in lipid rafts of COS-1 cells transfected with FcγRIIA ☆,☆☆

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Abstract

COS-1 cells bearing FcγRIIA were used as a model to demonstrate co-localization of several enzymes previously shown to regulate neutrophil phagocytosis. In COS-1 cells, phospholipase D (PLD) in the membrane fraction was activated during phagocytosis. PLD was found almost exclusively in lipid rafts, along with RhoA and ARF1. Protein kinase C-δ (PKCδ) and Raf-1 translocated to lipid rafts. In neutrophils, ceramide levels increase during phagocytosis, indicating that FcγRIIA engagement initiates ceramide generation. Applying this model, we transfected COS-1 cells with FcγRIIA that had been mutated in the ITAM region, rendering them unable to ingest particles. When the mutant receptors were engaged, ceramide was generated and MAPK was activated normally, thus these processes did not require actual ingestion of particles. These results indicate that signaling proteins for phagocytosis are either constitutively present in, or are recruited to, lipid rafts where they are readily available to activate one another.

Keywords: Fc receptors; Phagocytosis; Cell activation; Signal transduction

Neutrophil ingestion of antibody-tagged microbes through FcγRIIA ligation is an important event in the immune response. Upon receptor engagement, the immunoreceptor tyrosine-based activation motif (ITAM) region of FcγRIIA is tyrosine-phosphorylated by *src* kinases, leading to recruitment of syk and phosphoinositide 3-kinase (PI3K) [1,2]. Previously we developed a model wherein FcγRIIA engagement in neutrophils resulted in activation of phospholipase D (PLD) [3], producing phosphatidic acid, which is hydrolyzed by phosphatidate phosphohydrolase to diglyceride, an activator of protein kinase C (PKC). During phagocytosis, PKCδ and Raf-1 translocate to the plasma membrane of neutrophils [4]. Raf-1 then activates MAP kinase kinase (MEK), an effector of mitogen-activated protein kinase (MAPK), which in turn activates myosin to allow pseudopodia formation [5].

FcγRIIA activation involves signaling proteins and lipids which increasingly are viewed as localizing subcellularly in plasma membrane microdomains providing a framework for their interaction. One such domain is the lipid raft, consisting of sphingolipids, cholesterol, and associated proteins [6]. Proteins that may reside in rafts, or be transferred in and out of them, include receptors for epidermal growth factor and tumor necrosis factor-α, *src* kinases, PI3K, and FcR [6–8]. One type of raft contains the scaffolding protein caveolin-1, which interacts

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☆☆ *Abbreviations:* EIgG, erythrocytes opsonized with IgG; ERK, extracellular signal-regulated kinase; ITAM, immunoreceptor tyrosine-based activation motif; MAPK, mitogen-activated protein kinase; MEK, MAP kinase kinase; PC, phosphatidylcholine; PI3K, phosphoinositide 3-kinase; PKC, protein kinase C; PLD, phospholipase D.
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with many other proteins. Platelet activation via FcγRIIA is likely to begin in rafts [9]. Ceramide generation from sphingomyelin may occur primarily in lipid rafts [10,11] and may play a role in receptor capping [12]. Ceramide is a regulator of phagocytosis [3] and may facilitate clustering of FcγRIIA and its association with rafts [13], suggesting that much of early phagocytic signaling occurs in rafts. Since PLD is activated by Fc ligation and ceramide accumulation also occurs, the close physical association of FcγRIIA and ceramide led us to examine the possibility that PLD and its cofactors are also present in rafts, along with other molecules involved in phagocytosis such as the MAP kinases, Raf, and PKCδ.

COS-1 cells stably transfected with FcγRIIA have been used to demonstrate tyrosine phosphorylation of the receptor and the effect on phagocytosis of co-transfection with syk [14–16]. In this system, no other FcRs are present. Thus, signaling pathways that follow ligand binding can be assigned directly to FcγRIIA. These cells were used in this study as a model to examine co-localization in lipid rafts of several enzymes shown to be important in neutrophil phagocytic signaling. Use of COS-1 cells also allowed us to transfect mutant FcγRIIA in order to investigate the role of the ITAM region in ceramide generation.

Materials and methods

Materials. COS-1 cells stably expressing FcγRIIA were a gift from Dr. Alan Schreiber, University of Pennsylvania, Philadelphia, PA. Antibody against ARF1 was kindly provided by Dr. Sylvain Bourgoin, Université Laval, Québec, Canada. Antibodies against RhoA, extracellular signal-regulated kinase (ERK) 2, PLD1, Raf-1, and FcγRIIA were purchased from Santa Cruz Biotechnology, Santa Cruz, CA. Antibodies against paxillin and specific isozymes of PKCδ were obtained from BD Transduction Laboratories, San Diego, CA. BAPTA-AM was purchased from Molecular Probes (Eugene, OR) and PD098059 was from Calbiochem, San Diego, CA. Caveolin-1 antibody was obtained from Chemicon (Temecula, CA), and antibody against phosphorylated MAPKs p42/44 (ERK1 and ERK2) was from New England Biolabs (Beverly, MA). [^{3}H]Phosphatidylcholine (PC) was purchased from Amersham, Arlington Heights, IL.

Phagocytosis. Sheep erythrocytes were opsonized with antibody as previously described [17] (EIgG). COS-1 cells were incubated with EIgG for various times to stimulate FcγRIIA. EIgG not ingested were lysed with distilled water as previously described [17]. COS-1 cells were harvested with trypsin unless otherwise noted. Phagocytosis rates were quantified microscopically.

PLD activity in membrane fraction. FcγRIIA-bearing COS-1 cells were stimulated with EIgG, harvested, and suspended in assay buffer (PBS with 2.5 mM EDTA, 2 mM $CaCl_2$, 3 mM $MgCl_2$, 1 mM PMSF, 1 mM Na_3VO_4, and 10 μg/ml each of aprotinin, leupeptin, pepstatin, and soybean trypsin inhibitor). Cells were probe sonicated twice for 12–15 s ($\sim 20 \times 10^6$ in 350 μl) and then spun 500*g* 15 min. The supernatant was spun 200,000*g* 30 min to obtain a membrane pellet, which was then suspended in 100 μl assay buffer. Liposomes of 8.3 μM PC, 138 μM phosphatidylethanolamine, and 0.15 μCi [^{3}H]PC per sample were prepared by evaporation under nitrogen and then sonication in assay buffer [18]. Cell samples were added to tubes with liposomes and bath sonicated for 1 h at 37 °C. A blank was prepared with no protein and was subtracted from all samples. The assay was stopped with methanol:chloroform 2:1. The aqueous phase of the samples was partitioned using a toluene scintillation cocktail to separate [^{3}H]choline as described by Singh et al. [19]. Counts were corrected for the amount of protein in each sample.

Lipid raft isolation. After phagocytosis, COS-1 cells were harvested by scraping in buffer containing 20 mM Tricine (pH 7.8), 0.25 M sucrose, and 1 mM EDTA. Cells were washed and then disrupted in the same buffer with 30 strokes in a Wheaton tissue grinder. The postnuclear supernatant fraction was obtained by centrifugation of the cell lysate at 400*g* for 10 min. The plasma membrane fraction was removed from 30% Percoll in 0.25 M sucrose buffer after centrifugation at 84,000*g* for 30 min. Raft membrane fractions were isolated from the purified plasma membrane fractions using Opti-Prep (Invitrogen, Grand Island, NY) gradients as described by Smart et al. [8]. Equal amounts of protein from membrane fractions and lipid raft fractions were analyzed by SDS–PAGE. Proteins were transferred and subjected to Western blotting as described previously [20].

Construction of human FcγRIIA expression plasmid and mutant. A FcγRIIA expression vector, pcDNA3-FcγRIIA-HA carrying the human FcγRIIA gene tagged with c-myc at the carboxy-terminus, was generously provided by Dr. Alan Schreiber (University of Pennsylvania). FcγRIIA has three cytoplasmic tyrosines, Y275, Y282, and Y298. Y282 and Y298 are located in the immunoreceptor tyrosine-based activation motif (ITAM). The FcγRIIA mutant vector was constructed by the two-step overlap extension polymerase chain reaction (PCR) method. The mutant vector replaced the tyrosine residues at 282 and 298 amino acid with phenylalanine. The primers used for PCR were FcγRIIA-*Hin*dIII (5′-CCCAAGCTTGGGACTATGGAGACCCAAATG-3′), FcγRIIA-*Xba*I (5′-AGCGCTCTAGACTGTTATTACTGTTGAC-3′), Y282F-R (5′-TTCAGAGTCATGAAGCCGCCGTCAGCTGT-3′), and Y298F-F (5′-ATGACTCTGAACCCCAGGGCACCTACTGACGATGATAAAAACATCTTCCTGACTCTTCCTCCCAACGACCATGTCAACAGTAAT-3′). The resulting DNAs were transfected into wild-type COS-1 cells using Lipofectamine (Invitrogen, Carlsbad, CA), and cell lines stably expressing FcγRIIA and the double tyrosine mutant were selected using G-418 sulfate (Invitrogen).

MAPK activation. The cell lines with mutant and wild-type receptors were treated with low-serum media (0.25%) for 24 h before stimulation with EIgG to reduce basal MAPK phosphorylation. After detergent solubilization and centrifugation to remove non-soluble factors, samples were subjected to SDS–PAGE, Western blotting, and probed with antibody against phosphorylated p42/p44 MAPK. Membranes were stripped of protein and reprobed with antibody against total p42/p44 MAPK to show equal loading.

Ceramide formation. Lipids were extracted by the method of Van Veldhoven and Bell [21]. Total cellular ceramide was assayed by the method of Preiss et al. [22].

Results

PLD activation is essential to phagocytosis in neutrophils; therefore, we evaluated COS-1 cells as a model system by measuring PLD activation. COS-1 cells stably transfected with FcγRIIA were allowed to ingest EIgG, after which the membrane fraction was isolated and then added to liposomes containing [^{3}H]PC. During phagocytosis, PLD activity in membranes more than doubled (Fig. 1). PLD activity was highest at 5–10 min after phagocytosis was initiated and remained elevated through 30 min.

After preparing rafts, we tested the cell fractions for marker proteins to confirm that rafts had been

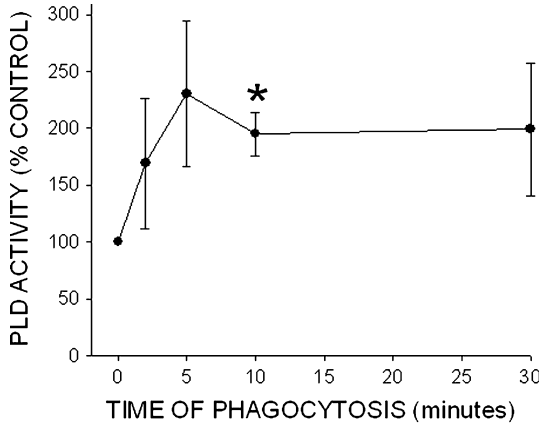

Fig. 1. PLD activity during phagocytosis in FcγRIIA-bearing COS-1 cells. Cells were stimulated with EIgG for the indicated times and disrupted by probe sonication. Membrane fractions were collected by centrifugation, suspended, and added to liposomes containing [^{3}H]PC. Samples were incubated for 1 h at 37 °C and then 2:1 methanol:chloroform was added. [^{3}H]Choline was separated from the aqueous phase using a toluene scintillation cocktail. A blank sample with no protein was subtracted from all samples. Results are means ± SEM of five experiments. *Significantly different from time 0.

successfully isolated. The fraction isolated from COS-1 cells as lipid rafts contained caveolin-1, a scaffolding protein and a marker for rafts (Fig. 2). Paxillin, which is excluded from rafts [8], was present in very small amounts. Conversely, the membrane fraction from which the rafts had been removed contained little or no caveolin-1 and was enriched in paxillin (Fig. 2), indicating separation of rafts from the rest of the membrane. Phagocytosing cells were not noticeably different from unstimulated cells with respect to the presence of these two proteins.

FcγRIIA is known to be recruited to lipid rafts upon receptor cross-linking [7], and we confirmed the presence of FcγRIIA in our raft preparations (data not shown). Because PLD activation occurs during phagocytosis, we investigated whether PLD1 and its cofactors were also present in rafts where PLD could interact with

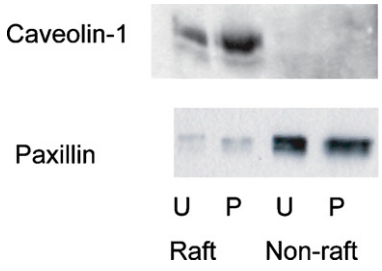

Fig. 2. Presence of marker proteins in raft and non-raft fractions from FcγRIIA-bearing COS-1 cells. Cells were stimulated with EIgG for 30 min, then scraped, homogenized, and fractionated using Percoll and then Opti-Prep. The non-raft fractions represent plasma membrane after the rafts were removed. Samples were Western blotted with equal protein in each lane to detect caveolin-1, a raft marker, or paxillin, which is excluded from rafts. U, unstimulated; P, phagocytosing cells. Results are representative of four experiments.

FcγRIIA or other molecules involved in early phagocytic signaling. PLD1 was not detectable in samples containing equal amounts of protein taken from the post-nuclear supernatant or the cytosol, but it appeared in membrane fractions and was notably enriched in the raft fraction (Fig. 3). Amounts of PLD1 in rafts did not change appreciably during phagocytosis. ARF1 and RhoA were present in lipid rafts as well as non-raft membranes. ARF1 occurred in approximately the same amounts in rafts and non-raft membranes, while RhoA was slightly enriched in rafts; neither changed during phagocytosis (Fig. 3).

PLD generates phosphatidic acid which is hydrolyzed by phosphatidate phosphohydrolase to diglyceride, a cofactor for PKC. In neutrophils we have previously shown that PKCδ and Raf-1 translocate to the plasma membrane during phagocytosis. Similarly, in COS-1 cells these molecules translocated to the membrane fraction. More PKCδ was present in membranes at 5 and 10 min of phagocytosis than at time 0 (Fig. 4A). Raf-1 was undetectable in the membrane before phagocytosis was initiated, appearing at 2 min and increasing by 10 min. A similar pattern was seen when lipid raft fractions were evaluated, PKCδ increasing with phagocytosis and Raf-1 strongly increasing (Fig. 4B). Evidence for PKCδ regulation of phagocytosis in the COS-1 cell system was also provided by using rottlerin, a PKC inhibitor which shows specificity for PKCδ when used at low micromolar concentrations [23]. Phagocytosis was inhibited almost completely by incubation with 3–10 μM rottlerin (Fig. 5). Similarly, using BAPTA to chelate intracellular calcium inhibited phagocytosis by only 25% (significantly different from controls at $p < 0.05$), suggesting that calcium-dependent PKCs are not as important to phagocytosis as the calcium-independent PKCδ.

The MAPK pathway, activated by Raf-1 and PKC, is activated during neutrophil and COS-1 phagocytosis [17,20]. Activation of ERK1 and ERK2 was prevented using a MEK inhibitor, however, phagocytosis was inhibited only by about 35% (significantly different from controls at $p < 0.05$), indicating that this pathway is involved in, but not the key factor for, phagocytosis in these cells. Lipid rafts contained ERK2 in about the same amount as non-raft membranes (data not shown).

In neutrophils, FcγRIIA engagement is followed by activation of sphingomyelinase and accumulation of ceramide; however, the region of the FcR molecule regulating ceramide generation is unknown. The abundance of sphingolipids in rafts, along with the presence of FcγRIIA, suggests that rafts are the location for phagocytosis-mediated ceramide production. Additionally, the downstream activation of ERK1 and ERK2 may be dependent on the interactions of ceramide and PLD in rafts. The ITAM region of FcγRIIA, site of tyrosine phosphorylation and recruitment of syk and PI3K, seemed a likely candidate for signaling ceramide production and

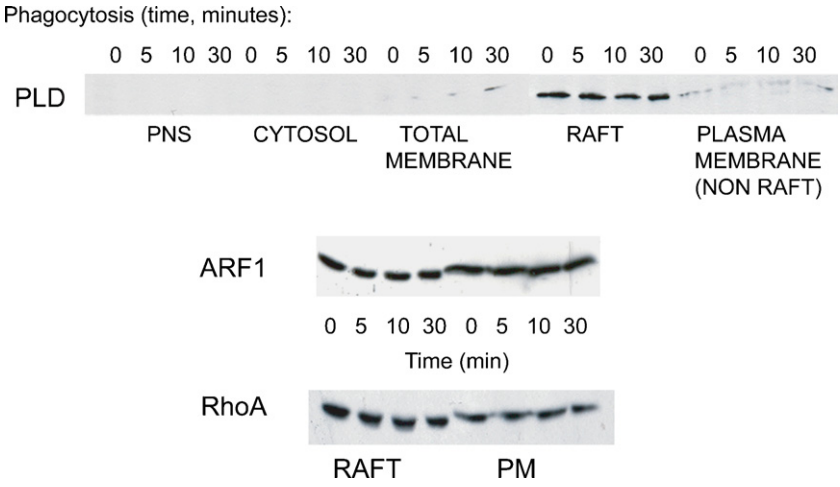

Fig. 3. PLD and cofactors present in lipid rafts during phagocytosis. FcγRIIA-bearing COS-1 cells were stimulated with EIgG for the indicated times, and cell fractions were prepared as in Fig. 2. Equal amounts of protein were loaded for Western blotting and probed for PLD, ARF1, and RhoA. PNS, post-nuclear supernatant. PM, plasma membrane. Results are representative of four experiments.

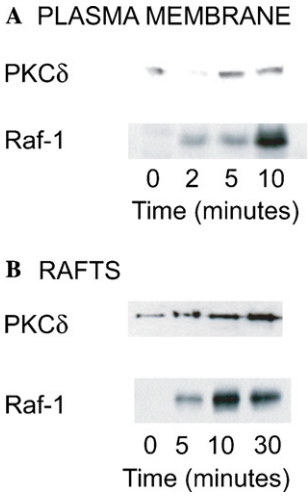

Fig. 4. PKCδ and Raf-1 translocation to the membrane during phagocytosis. FcγRIIA-bearing COS-1 cells were stimulated with EIgG for the indicated times. (A) Membrane samples were taken as in Fig. 1. Results are representative of three experiments. (B) Rafts were isolated as in Fig. 2. Results are representative of five experiments. (A,B) Equal amounts of protein were loaded for Western blotting, and the membrane was probed for PKCδ and Raf-1.

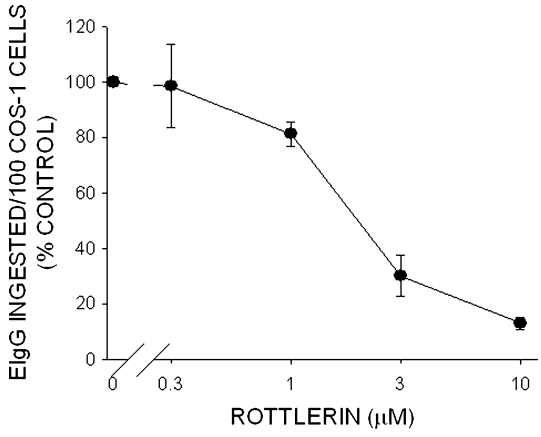

Fig. 5. Phagocytosis inhibition by rottlerin, a PKCδ inhibitor. FcγRIIA-bearing COS-1 cells were incubated with rottlerin for 1 h and then stimulated with EIgG for 30 min. Phagocytosis was evaluated microscopically. Results are means ± SEM of three experiments.

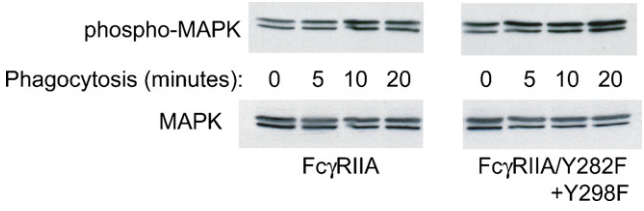

Fig. 6. ERK1 and ERK2 activation by engagement of mutant FcγRIIA. COS-1 cells were transfected with FcγRIIA or mutated FcγRIIA with tyrosines 282 and 298 changed to phenylalanine. Cells were stimulated with EIgG for the indicated times and then solubilized with detergent. Samples (equal protein) were subjected to Western blotting and probed for phosphorylated ERK1/ERK2 (upper panel). Blots were stripped and reprobed for total ERK1/ERK2 to show equal loading (lower panel). Results are representative of three experiments.

further kinase activation. We investigated whether the ITAM region was involved in regulation of ceramide production and ERK1/ERK2 activation by mutating tyrosines 282 and 298 to phenylalanine and stably transfecting COS-1 cells with the mutant FcγRIIA, as well as control COS-1 transfected with the wild-type FcγRIIA. Cells transfected with the wild-type receptor accumulated ceramide after 30 min of stimulation with EIgG, a 73 ± 31% increase over unstimulated controls. Although the mutant receptor was unable to initiate phagocytosis, 30 min of binding of EIgG also resulted in increased ceramide production in the cells transfected with mutant FcγRIIA (90 ± 49%, no significant difference from wild-type). ERK1/ERK2 activation subsequent to stimulation of mutant FcγRIIA also was similar to cells transfected with the wild-type receptor (Fig. 6). Scanning densitome-

try showed that phosphorylation of ERK1/ERK2 increased two- to threefold by 5–10 min after phagocytosis began in both cell types.

Discussion

In this study, we determined that lipid rafts play an important role in phagocytic signaling of phagocytosing COS-1 cells bearing FcγRIIA. We first wished to determine whether PLD was a mediator of phagocytosis in these cells, as we observed previously in PMNs. PLD activity in membranes of COS-1 cells more than doubled during phagocytosis, reaching a plateau in 5–10 min. This finding was similar to our observations in phagocytosing FMLP-primed neutrophils, where PLD activity increases two- to threefold, but reaches peak activity within 2–3 min [3]. We then determined that PLD1 was almost entirely found in the raft fractions of COS-1 cells and did not increase appreciably during phagocytosis. Similarly, PLD is also found in lipid rafts of endothelial cells and appears to mediate MEK/ERK activation [24]. Additionally, COS-7 rafts as well as plasma membrane contain PLD1, but phosphorylation of PLD1 is only observed in rafts [25]. Although a recent study found no detectable PLD1 in COS-7 cells [26], the samples were whole-cell lysates and we similarly could not detect PLD1 in Western blots of whole-cell COS-1 lysates, only when PLD1 was concentrated in rafts. The membrane PLD activity we observed (Fig. 1) was not determined to be specifically PLD1 or PLD2, the latter of which is also present in COS-7 cells [26]. We also demonstrated the presence in rafts of the PLD1 cofactors ARF1 and RhoA; the latter being slightly enriched in rafts. In neutrophils, ARF1 and RhoA translocate from cytosol to plasma membrane during cell activation [27]; in COS-1 cells these GTPases are also present in the cytosol (data not shown), but we were unable to detect translocation upon cell stimulation. Another cofactor of PLD, phosphatidylinositol-bisphosphate (PIP_2), previously has been shown to be enriched in lipid rafts [28]. Thus, PLD and its activating mechanisms are present in rafts.

The detergent-free raft isolation method we used [8,29] yielded membrane fractions containing proteins that were consistent with previous studies [6,8]. Specifically, caveolin-1 was present in the raft fraction but not the plasma membrane fraction, while conversely paxillin was present in plasma membrane but not rafts. We chose not to use a detergent method due to concerns about changes in lipid composition and protein solubilization. For example, the presence of FcγRIIA in rafts isolated as detergent-resistant membranes depends on whether detergent is included in all gradients during fractionation [30].

As we observed in neutrophils [4], PKCδ and Raf-1 strongly translocated to the plasma membrane of COS-1 cells during phagocytosis. Additionally, both proteins migrated to the isolated raft fraction, a novel observation. PKCδ also is implicated in FcεRI signaling in mast cells [31]. Our results using BAPTA and the PKCδ inhibitor rottlerin suggest that PKCδ, and not another, calcium-dependent PKC regulates phagocytosis.

Using COS-1 cells as a phagocytic model allowed us to create a mutant of FcγRIIA with two tyrosines in the ITAM changed to phenylalanines. COS-1 cells stably transfected with the double mutant were unable to phagocytose EIgG. In a previous study, substituting Y282 or Y298 individually reduced phagocytosis by 65%, while the combination of the two substitutions reduced phagocytosis by 96% [32], similar to our observations. The cells were non-phagocytic most likely because the syk–PI3K pathway was rendered inactive by the tyrosine substitutions. However, phosphorylation of these tyrosines was not required for the ceramide or MAPK pathways, as binding of EIgG to the mutant receptors initiated ceramide generation and MAPK activation as we observed for the wild-type receptor. This interpretation is consistent with our previous studies showing that in neutrophils PI3K is not directly involved in the MAPK cascade [4]. These results indicate that a domain of FcγRIIA other than the ITAM is responsible for subsequent ceramide generation and MAPK activity.

FcγRIIA-bearing COS-1 cells generate ceramide during phagocytosis by a de novo pathway as previously demonstrated [20]. However, it is not known whether the de novo pathway is activated during neutrophil phagocytosis. In the neutrophil, ceramide is generated by membrane-bound neutral sphingomyelinase [33]. Ceramide accumulates during FcγRIIA-mediated phagocytosis, and its peak accumulation coincides with the time that phagocytosis rate declines [3], suggesting negative regulation. Activation of neutrophils by FMLP enhances the stimulatory effect of EIgG on sphingomyelinase activity and ceramide production [33]. Sphingomyelinase activity in COS-1 cells increases slightly during phagocytosis (unpublished data). Other studies also indicate that FcγRIIA stimulation brings about ceramide generation. At the onset of FcγRIIA cross-linking, ceramide is produced by acid sphingomyelinase at the cell surface in U937 cells [13]. Inhibition of acid sphingomyelinase diminished clustering and tyrosine phosphorylation of FcγRIIA. Exogenous C_{16}-ceramide facilitates clustering of FcγRIIA and its association with rafts [13]. Taken together, these data suggest ceramide is closely involved with early FcγRIIA signaling events, most likely in rafts.

In neutrophils, the MAPKs ERK1 and ERK2 are activated by FMLP and achieve about twofold greater activation when subsequently stimulated with EIgG [17], similar to the increase observed in this study. Basal ERK phosphorylation is somewhat higher in COS-1 cells compared to resting neutrophils, as seen in this

study and our previous work [20]. However, in both cell types the MEK inhibitor PD098059 only partially inhibited phagocytosis; in neutrophils, we observed a 50% inhibition [17] and in COS-1 cells a 35% inhibition of phagocytosis (this study). This, considered along with the ability of FcγRIIA ITAM mutants to activate ERK1 and ERK2, suggests that ERK1 and ERK2 contribute to, but are not critical factors for, particle ingestion.

Lipid rafts provide a platform for interaction of regulatory molecules in the early stages of phagocytosis. Some enzymes are constitutively present in rafts, such as PLD and its cofactors, while others translocate from the cytosol, such as PKCδ and Raf-1. Sphingolipid enrichment in rafts permits generation of ceramide and its interaction with regulatory components of phagocytosis.

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