γ-Secretase Is a Functional Component of Phagosomes*

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γ-Secretase is a high molecular mass complex that catalyzes the intramembrane cleavage of its protein substrates. Two proteins involved in phagocytosis, CD44 and the low density lipoprotein receptor-related protein, are γ-secretase substrates, suggesting that this complex might regulate some aspects of phagocytosis. Our results indicate that the four components of γ-secretase, viz. presenilin, nicastrin, APH-1, and PEN-2, are present and enriched on phagosome membranes from both murine macrophages and Drosophila S2 phagocytes. The γ-secretase components form high molecular mass complexes in lipid microdomains of the phagosome membrane with the topology expected for the functional enzyme. In contrast to the majority of the phagosome proteins studied so far, which appear to associate transiently with this organelle, γ-secretase resides on newly formed phagosomes and remains associated throughout their maturation into phagolysosomes. Finally, our results indicate that interferon-γ stimulates γ-secretase-dependent cleavages on phagosomes and that γ-secretase activity may be involved in the phagocytic response of macrophages to inflammatory cytokines.

γ-Secretase catalyzes the cleavage of selected type I transmembrane protein substrates within their membrane-spanning domains, an atypical hydrolytic event considering that it occurs in the hydrophobic conditions of the lipid bilayer (1). This type of intramembrane proteolysis was discovered as an essential step in the release of β-amyloid peptides from the amyloid precursor protein (APP), a pivotal process in the development of Alzheimer disease pathology (2). γ-Secretase activity was later found to function in Notch signaling by releasing the Notch intracellular domain, which translocates to the nucleus and activates the transcription of target genes (2). Several other γ-secretase substrates, including CD44, Notch ligands, and the low density lipoprotein receptor-related protein (LRP; also known as CD91), have been identified, and all share a common type I transmembrane protein topology (3).

γ-Secretase cleavage of these substrates is preceded by proteolysis within their extracellular domains (3). Although the biological function of γ-secretase cleavage remains to be deciphered for many substrates, recent evidence indicates that the role of γ-secretase in signal transduction may not be limited to the Notch pathway. Other γ-secretase substrates, including APP, CD44, and LRP, have indeed been shown to generate transcriptionally active C-terminal fragments (4–6). Conversely, cleavage by γ-secretase terminates intracellular signaling mediated by the netrin receptor DCC (deleted in colorectal cancer) (7). Taken together, these studies suggest that γ-secretase-mediated cleavage may be generally involved in cell signaling.

Significant research efforts have recently been invested in characterizing the biochemistry of γ-secretase. Cumulative evidence indicates that γ-secretase consists of a high molecular mass complex composed of at least four different proteins, viz. presenilin (PS)-1 or the less abundant homolog PS2, nicastrin, APH-1, and PEN-2, all of which are integral membrane proteins (8). Current models propose that PS1 contains the catalytic site of γ-secretase, an unusual aspartyl protease with two aspartates located in transmembrane domains 6 and 7 of PS1, respectively (9). Nicastrin and APH-1 are suggested to function in stabilizing and trafficking full-length PS1 through the secretory pathway (10). PS1 undergoes endoproteolysis in its cytoplasmic loop, located between transmembrane domains 6 and 7, generating the N-terminal (NTF) and C-terminal (CTF) fragments, which remain associated in the complex (11, 12). PEN-2 apparently promotes this cleavage event, a key step in the activation of γ-secretase (13). Several reports have demonstrated that the coexpression of all four proteins is required to produce functional γ-secretase (13–17). Although the assembly of a functional γ-secretase complex can occur in the endoplasmic reticulum (18), γ-secretase probably meets its substrates in late compartments of the secretory pathway and in compartments of the endosomal/lysosomal pathway (19, 20). It remains unclear whether γ-secretase cleavage events occur at the plasma membrane, given that the endocytosis of Notch has recently been described as a prerequisite for its processing by γ-secretase (20). Phagocytosis is a process used by a wide range of organisms, from amoeba to vertebrates, to internalize large particles, typically the size of 1 μm or more (21). Newly formed phagosomes engage in a maturation process that involves fusion with endosomes of increasing age and ultimately with lysosomes. The killing of phagocytosed microbes in mammalian professional phagocytes such as macrophages forms the first line of defense against infectious diseases. Microbe degradation in the phagosome produces protein antigens, which are presented at the cell surface to activate specific lymphocytes and elicit appropriate immune responses. Phagosome-associated functions are thus important participants in the immune system.

At least two acknowledged substrates of γ-secretase, CD44 and LRP (also known as CD91) (3), are involved in phagocytosis and are known residents of phagosome membranes (22, 23). These findings prompted us to investigate if the γ-secretase complex localizes to phagosomes and hence if γ-secretase cleavage events occur on this compartment. In this...
study, we show that all of the components of the γ-secretase reside on phagosomes of both murine macrophages and Drosophila phagocytic cells. The biochemical characterization of γ-secretase on phagosomes demonstrates that γ-secretase associates with lipid rafts on phagosome membranes, is present predominantly as a high molecular mass complex of 440 kDa, and catalyzes γ-secretase–specific cleavages on phagosomes. Our results show that the γ-secretase complex is an abundant, stable, and conserved component of phagosomes, suggesting that γ-secretase actively regulates some of the functions of this highly specialized organelle.

EXPERIMENTAL PROCEDURES

Antibodies—The rat anti-Lamp1 luminal monoclonal antibody ID4B was from the Developmental Studies Hybridoma Bank. The rabbit anti-Rab5a and Rab7 polyclonal antibodies (pAbs) and the goat anti-LRP pAb were from Santa Cruz Biotechnology, Inc. The mouse monoclonal antibody raised against the nicastrin N terminus and anti-flotillin monoclonal antibody were from BD Biosciences. The rabbit anti-PS1 antibody raised against the nicastrin N terminus and anti-flotillin monoclonal antibody were from BioMol. The rabbit anti-nicastrin pAb was raised against residues 62–93 of nicastrin (25). The rabbit anti-SNARE pAb was raised against residues 369–407, respectively, of PS1 (11, 24). The rabbit anti-nicastrin pAb was raised against residues 1–26 of PEN-2 (26). Rabbit pAb 369 was raised against the C-terminal domain of APP (27).

Cell Culture and Organelle Isolation—J774 murine macrophages were cultured in Dulbecco’s modified Eagle’s medium (Sigma) supplemented with 10% heat-inactivated fetal bovine serum, 1% glutamine, 100 units/ml penicillin, and 100 μg/ml streptomycin at 37 °C in 5% CO₂. Drosophila melanogaster S2 cells were cultured in Schneider’s medium (Sigma) supplemented with 10% heat-inactivated fetal bovine serum, 100 units/ml penicillin, and 100 μg/ml streptomycin at 26 °C. For caspase inhibition, J774 macrophages were incubated overnight in the presence of benzoylcarbonyl-Val-Asp-cholesteryl-fluoromethyl ketone (Calbiochem). For cytotoxic-treated cells, 200 units/ml interferon-γ (IFN-γ) alone or with 5 ng/ml tumor necrosis factor-α (TNF-α) (Calbiochem) was added in the medium 24 h prior to the isolation of phagosomes. For treatment with γ-secretase inhibitors, N-(N-(3,5-difluorophenacetyl-L-alanyl))–S-phenylglycine t-butyl ester (DAPT) or L-685,458 (γ-secretase inhibitor IX and X, respectively; Calbiochem), was added in the medium 24 h prior to the isolation of phagosomes. Phagosomes were formed by the internalization of 0.8-μm blue-dyed latex beads (Estapor® Microspheres) (28). Cells were allowed to internalize the beads for various periods as indicated in the figure legends. When the internalization was followed by a chase period, the cells were washed with ice-cold phosphate-buffered saline (PBS), and new medium was added. The cells were then further incubated for various periods as indicated. For observations under a electron microscope, cells were fixed and processed as described previously (28). To isolate phagosomes, cells were washed and scraped in ice-cold PBS. The cells were lysed in ice-cold homogenization buffer containing 250 mM sucrose, 3 mM imidazole (pH 7.4), and protease inhibitors (Roche Applied Science) using a 22-gauge 1½-inch needle. The post-nuclear supernatant was adjusted to 40% sucrose; placed on a cushion of 62% sucrose; and layered with a sucrose gradient composed of 35, 25, and 10% layers. After centrifugation at 24,000 rpm for 1 h, the isolated phagosomes were collected at the 10–25% sucrose interface. To isolate the total cell membranes, post-nuclear supernatants were centrifuged at 120,000 × g for 1 h onto a 62% sucrose cushion.

Mass Spectrometry Analysis—Phagosome proteins were separated by SDS-PAGE, and 1-mm-wide bands were excised and trypsin-digested. The resulting tryptic peptides were extracted with 0.2 M urea in 50% aqueous acetonitrile and analyzed by nanoscale liquid chromatography/mass spectrometry using a Waters capillary liquid chromatograph coupled to a Q-Tof Ultima mass spectrometer.

SDS-PAGE and Western Blotting—Purified phagosomes or total cell membranes were resuspended in Laemmli lysis buffer prior to SDS-PAGE and Western blotting. The amount of proteins and latex beads in the samples was quantified using an EZQ™ protein quantification kit (Molecular Probes, Inc.) and by spectrophotometry, respectively.

Deglycosylation Assay—The glycosylation pattern of nicastrin was examined by treating phagosome protein extracts with endoglycosidase H or peptide N-glycosidase F (New England Biolabs Inc.) according to the manufacturer’s protocol.

Immunofluorescence—Phagosomes were formed by the internalization of 3-μm latex beads (Estapor® microspheres) for 1 h, and purified phagosomes were incubated with the appropriate primary antibody in PBS containing 0.5% bovine serum albumin (PBS/bovine serum albumin) for 1 h at 4 °C. The phagosomes were washed with PBS/bovine serum albumin and further incubated with Alexa Fluor® 488-conjugated anti-rabbit or anti-rat antibody (Molecular Probes, Inc.) for 30 min at 4 °C. The immunolabeled phagosomes were washed with PBS/bovine serum albumin and observed using a Leica SP2 confocal microscope.

Pronase Assay—To determine the sensitivity of phagosome-associated proteins to Pronase, purified phagosomes were incubated for 1 h at 37 °C with Pronase (Sigma), which consists of a mixture of proteases, as described previously (29). Equivalent amounts of phagosomes were treated in parallel with or without Pronase and subjected to SDS-PAGE prior to Western blotting.

Lipid Raft Isolation—Lipid rafts were isolated from purified phagosomes as described previously (29). Briefly, phagosome membranes were incubated in 0.5% Triton X-100 or 0.5% CHAPS for 30 min on ice. Lipid rafts were separated from the solubilized membranes by floatation on an OptiPrep™ gradient, from which seven equivalent fractions were collected. The proteins in each fraction were precipitated with methanol/chloroform and resuspended in Laemmli lysis buffer.

Blue Native (BN) PAGE—For BN-PAGE, the γ-secretase protein complexes were isolated from purified phagosomes by detergent solubilization in 0.5% n-dodecyl β-d-maltoside. The solubilized fractions were subjected to BN-PAGE as described (30). After electrophoresis, either the gel was transferred directly to a polyvinylidene difluoride membrane for Western blotting, or a lane of the gel was subjected to Tricine/SDS-PAGE prior to Western blotting.

γ-Secretase Activity—To assess the generation of the APP CTFγ (the C-terminal product of APP cleavage by γ-secretase (43)), purified phagosomes were resuspended in PBS containing 5 mM EDTA and 0.5 mM 1,10-phenanthroline, and aliquots were incubated at either 4 °C or 37 °C for 2 h. The reactions were terminated by the addition of Laemmli lysis buffer, and proteins were separated by SDS-PAGE. The presence of APP CTFs was analyzed by Western blotting. γ-Secretase enzyme activity was also monitored with a fluorescent polymer superquenching-based assay using a peptide containing the sequence GVVIAVTVK flanked by biotin and a fluorescence quencher (QTL Lightspeed™ γ-secretase assay, QTL Biosystems) according to the manufacturer’s protocol. The protein contents in total cell membranes or in purified 1-h-old phagosomes were quantified in purified phagosomes or in total cell membranes. Increasing amounts of membranes or of purified phagosomes were incubated with the peptide substrate for 3 h at 37 °C in the absence or presence of 1 μM L-685,458 (γ-secretase inhibitor X), and the fluorescence was measured using a SpectraMax GeminiEM instrument (Molecular Devices Corp.).
**RESULTS**

In a recent proteomic analysis of purified latex bead-containing phagosomes, we identified >500 proteins on this organelle by mass spectrometry (31, 32). This analysis revealed the presence of four of the components of the γ-secretase complex, viz. PS1, PS2, nicastrin, and APH-1 (TABLE ONE). To assess the significance of these observations, the relative abundance of γ-secretase components in phagosomes compared with a total cell lysate and a total cell membrane preparation was determined. As demonstrated in Fig. 1A, PS1, nicastrin, and PEN-2 were highly enriched on phagosome membranes. The PS1 NTF and CTF were clearly the predominant forms of PS1 compared with full-length PS1 (Fig. 1A), indicating that mainly the processed form of PS1 localizes to phagosomes. Likewise, the deglycosylation profile of nicastrin indicated that nicastrin was present in its mature glycosylated form (Fig. 1B). The slight shift in molecular mass observed after treatment with endoglycosidase H is expected of highly glycosylated proteins such as nicastrin (33). The PS1 CTF on phagosomes was present in three distinct bands, possibly corresponding to a previously characterized phosphorylated form of the PS1 CTF (highest band) and a product of PS1 CTF cleavage by caspase-3 (lowest band) (see Fig. 6B) (34). Nicastrin and PS1 were also detected by immunofluorescent labeling of isolated phagosomes (Fig. 1C). In contrast to the uniform Lamp1 labeling of the phagosome surface, PS1 and nicastrin labeling was often observed in phagosomes (Fig. 1C). In contrast to Rab5 and Lamp1, the proteins of the γ-secretase complex remained abundant and stable on phagosomes throughout their maturation into phagolysosomes (Fig. 2). The dynamics of γ-secretase association with phagosomes also demonstrate that the complex is not targeted to phagosomes simply to be degraded. These results indicate an enrichment of the γ-secretase complex on late phagosome membranes. In contrast, nicastrin displayed a much greater enrichment than PS1 on purified lysosomes (19). As late phagosomes and lysosomes may interact with different intracellular compartments, the cellular origin of the γ-secretase complex found on these distinct organelles could differ, and γ-secretase components may thus be present in different proportions.

Purified latex bead-containing phagosomes constitute a unique tool to determine the membrane topology of resident proteins. These purified phagosomes have indeed been shown to be free of membrane contaminants from other organelles (28). Through the isolation protocol, the phagosome membrane surrounding the latex bead remains intact, and the protein segments located in the phagosome lumen are inaccessible from the exterior milieu. When purified phagosomes are incubated...
with mild concentrations of proteases, protein segments exposed on the cytoplasmic side of the phagosome are thus degraded, whereas the luminal segments remain intact. The topology of γ-secretase proteins was examined by incubating purified phagosomes with Pronase, and the extent of protein degradation was evaluated by Western blotting (Fig. 3A). As depicted in Fig. 3B, the observed degradation patterns of PS1 indicate that the N terminus and cytoplasmic loop of PS1 are located on the cytoplasmic side of the phagosome, with the antibodies used in Fig. 3B, are in agreement with the membrane topologies predicted from their hydrophathy plots and from previous observations (33, 37–40).

The γ-secretase complex has been reported to associate with cholesterol-enriched lipid domains, also termed lipid rafts (41). In addition, cholesterol is suspected to play a role in the processing of γ-secretase substrates, particularly of APP (42). Detergent-resistant membrane domains were thus isolated from purified phagosomes, and the distribution of γ-secretase components in a flotation gradient was determined. Fig. 4 shows that the solubilization of phagosome membranes in Triton X-100 resulted in little partitioning of γ-secretase components with lipid rafts, whereas solubilization in CHAPS resulted in the flotation of almost half of the total γ-secretase phagosome content. These results are in agreement with recent observations indicating that the solubility of γ-secretase is greater in Triton X-100 than in other non-ionic detergents such as CHAPSO and Lubrol WX (41). The γ-secretase components are thus associated with CHAPS-resistant lipid rafts on phagosomes.

Current models suggest that γ-secretase enzyme activity is generated within a highly molecular mass complex comprising the γ-secretase components (3). To investigate the oligomeric state of the γ-secretase components on the phagosome, native electrophoresis of phagosome proteins was performed. The PS1 NTF and CTF were part of multiple high

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**FIGURE 1.** Components of the γ-secretase complex are enriched on phagosomes. A, the presence of γ-secretase components was assessed in J774 macrophages using total cell lysate (TCL), a total cell membrane preparation (TM), and 1-h-old phagosomes (Phago). Equal amounts of proteins were loaded in each lane. Western blotting was performed for the indicated proteins. Lamp1 is shown as a control for a highly enriched phagosome protein. B, protein extract from 1-h-old phagosomes was incubated in the absence (Control) or presence of endoglycosidase H (Endo H) or peptide N-glycosidase F (PNGase). Equal amounts of proteins were loaded in each lane, and the presence of nicastrin was revealed by Western blotting. C, purified phagosomes from J774 cells were immunofluorescently labeled with the appropriate antibody and observed under a confocal microscope. Identical fields are shown for immunofluorescence (left panels) and phase-contrast (right panels) imaging. Enlargements of a single phagosome are shown in insets. D, the presence of γ-secretase components was assessed in Drosophila S2 cells using a total cell membrane preparation (TM) and phagosomes purified after the internalization of latex beads for 1 h (Phago). Equal amounts of proteins were loaded in each lane. Western blotting was performed for the indicated proteins. Rab7 is shown as a control.

**FIGURE 2.** γ-Secretase components remain associated with phagosomes throughout phagolysosome biogenesis. J774 macrophages were fed latex beads for 30 min and chased for the indicated periods (minutes and hours) prior to phagosome isolation. Equivalent amounts of phagosomes from each time point were separated by SDS-PAGE. Western blotting was performed for the indicated proteins. Lamp1 and Rab5 are shown as markers, which increase and decrease during phagolysosome biogenesis, respectively.

**FIGURE 3.** Membrane topology of PS1, nicastrin, and Pen-2 on phagosomes. A, purified 1-h-old phagosomes from J774 cells were incubated in the absence (−) or presence (+) of Pronase. Equal amounts of phagosomes were loaded in each lane, and Western blotting was performed for the indicated proteins. Rab5, which is associated with the cytoplasmic side of the phagosome, is shown as a control for degradation. B, shown is schematic representation of the predicted membrane topology of PS1 (red), nicastrin (green), and Pen-2 (yellow) on the phagosome, with the antibodies used in A.
molecular mass complexes on purified phagosomes (Fig. 5A). Three distinct complexes of ~300, 440, and 660 kDa were visible, although the 440-kDa complex was by far the predominant oligomer (Fig. 5A). Nicas- trin and PEN-2 could not be detected when the native gel was directly transferred for Western blotting, probably due to the reduced exposure of nicastrin and PEN-2 epitopes in the native complexes. However, fur- ther separation of the phagosome proteins present in the native gel by SDS-PAGE revealed that nicastrin and PEN-2 were indeed part of these high molecular mass complexes (Fig. 5A). Mass spectrometry analysis of the proteins in the native gel allowed the identification of APH-1 only in bands corresponding to the 440-kDa complex (Fig. 5A), although the low abundance of the 300- and 660-kDa complexes may have impaired the identification of APH-1 in these complexes. All of the γ-secretase components thus remain assembled in a high molecular mass complex on the phagosome.

To determine whether the complex is enzymatically functional, γ-secretase cleavage of endogenous APP was assessed on isolated phagosomes. As shown in Fig. 5B, the APP CTFγ, the C-terminal product of APP cleavage by γ-secretase (43), was specifically generated when purified phagosomes were incubated at 37 °C. γ-Secretase activity was also measured on purified phagosomes using a peptide substrate (Fig. 5C). Substrate processing was observed to rise with increasing amounts of phagosomes, and this activity was specifically inhibited by the γ-secretase inhibitor L-685,458 (Fig. 5C). Purified phagosomes also displayed a significant increase in γ-secretase-specific activity compared with total cell membranes (Fig. 5C), consistent with the enrichment of mature γ-secretase on phagosomes. These results demonstrate that γ-secretase-dependent processing of phagosome-associated substrates occurs on this organelle.

Phagosomes play key roles in macrophage immune responses, e.g. in the killing of microbes and the processing of protein antigens (21). These immune functions are regulated by cytokines such as IFN-γ, an inflammatory cytokine that stimulates the bactericidal activity of phagocytes and the presentation of antigens at the cell surface (44). Because inflammatory cytokines have recently been shown to stimulate γ-secretase activity (45), we tested whether IFN-γ could influence the activity of this enzyme complex on phagosomes. Accordingly, macrophages were treated with IFN-γ prior to phagosome formation, and the generation of a γ-secretase-specific cleavage product on isolated phagosomes, the 12-kDa CTF of LRP, was assessed (46). As shown in Fig. 6, the processing of LRP by γ-secretase was up-regulated upon IFN-γ stimulation, whereas the abundance of the various γ-secretase components remained stable on phagosomes. We noted, however, that phagosomes from IFN-γ-treated cells displayed a significant decrease in the lowest band of the
The cleavage product of the PS1 CTF by caspase-3 is indicated by the presence of the 12-kDa C-terminal fragment of LRP, a product of γ-secretase processing, and of the γ-secretase components was assessed in J774 macrophages incubated with IFN-γ for 24 h prior to phagosome formation. 1-h-old phagosomes were purified, and equal amounts of phagosomes from control (−) or IFN-γ-treated (+) cells were loaded in each lane. Western blotting was performed for the indicated proteins. The cleavage product of the PS1 CTF by caspase-3 is indicated by the asterisk. B, J774 cells were incubated overnight in the absence (Control) or presence of a caspase inhibitor (benzyloxycarbonyl-VAD-fluoromethyl ketone (Z-VAD)) prior to the internalization of latex beads for 1 h and phagosome isolation. Equal amounts of phagosomes were separated, and the PS1 CTF was revealed by Western blotting. The cleavage product of the PS1 CTF by caspase-3 is indicated by the asterisk.

Inflammatory cytokines are known to alter phagocytosis in macrophages; these cytokines may either inhibit or stimulate phagocytosis depending on the activation and differentiation states of the macrophages (44, 47). In J774 macrophages, long-term incubation with IFN-γ results in a diminished capacity to phagocytose latex beads, an effect potentiated by the combined incubation of IFN-γ and TNF-α (Ref. 44 and Fig. 7A). Because γ-secretase activity appears to respond to inflammatory cytokines, we tested whether γ-secretase inhibition could impair the effects of IFN-γ and TNF-α. As shown in Fig. 7A, both DAPT and L-685,458, two inhibitors of γ-secretase, could stimulate phagocytosis in cytokine-treated cells. Moreover, γ-secretase inhibition with DAPT stimulated phagocytosis in a dose-dependent manner (Fig. 7B). These results were confirmed at the electron microscopic level by counting the number of internalized latex beads in individual cells observed in a thin section. Fig. 7C demonstrates that γ-secretase inhibition resulted in a general increase in the percentage of cells containing six beads or more. γ-Secretase activity may thus take part in the pathway leading to the inhibition of phagocytosis in J774 macrophages treated with inflammatory cytokines.

DISCUSSION

The identification of several γ-secretase substrates has recently revealed the presence among them of two known components of phagosomes, CD44 and LRP. To examine the possibility that these and other yet to be identified γ-secretase substrates could be processed on phagosomes, we sought to determine whether γ-secretase activity is associated with purified phagosomes. In this study, we have demonstrated that γ-secretase is a stable and evolutionarily conserved component of phagosomes, organelles dedicated to essential immune func-

![Image](http://www.jbc.org/)

**FIGURE 6.** IFN-γ increases the γ-secretase cleavage of LRP on phagosomes. A, the presence of the 12-kDa C-terminal (C-term) fragment of LRP, a product of γ-secretase processing, and of the γ-secretase components was assessed in J774 macrophages incubated with IFN-γ for 24 h prior to phagosome formation. 1-h-old phagosomes were purified, and equal amounts of phagosomes from control (−) or IFN-γ-treated (+) cells were loaded in each lane. Western blotting was performed for the indicated proteins. The cleavage product of the PS1 CTF by caspase-3 is indicated by the asterisk. B, J774 cells were incubated overnight in the absence (Control) or presence of a caspase inhibitor (benzyloxycarbonyl-VAD-fluoromethyl ketone (Z-VAD)) prior to the internalization of latex beads for 1 h and phagosome isolation. Equal amounts of phagosomes were separated, and the PS1 CTF was revealed by Western blotting. The cleavage product of the PS1 CTF by caspase-3 is indicated by the asterisk.

**FIGURE 7.** γ-Secretase inhibition stimulates phagocytosis in J774 macrophages treated with IFN-γ and TNF-α. A, J774 macrophages were incubated for 24 h with or without IFN-γ and TNF-α and with or without a γ-secretase inhibitor (20 μM DAPT or 1 μM L-685,458) prior to phagosome formation. 1-h-old phagosomes were purified from an equal number of cells under each condition, and the total amount of isolated phagosomes was quantified for each condition by spectrophotometry. The results shown are the number of phagocytosed beads relative to the control (means ± S.E.) from five separate experiments. B, J774 cells were incubated for 24 h with IFN-γ and TNF-α in the absence or presence of increasing concentrations of DAPT. 1-h-old phagosomes were purified from an equal number of cells, and the total amount of isolated phagosomes was quantified for each condition by spectrophotometry. The results shown are the number of phagocytosed beads relative to the control (means ± S.E.) from four separate experiments. C, J774 cells were incubated for 24 h with IFN-γ and TNF-α in the absence or presence of 20 μM DAPT. Cells were incubated with latex beads for 1 h, washed, fixed, and processed for electron microscopy to quantify the number of beads/cell under each condition. The results shown represent at least 500 cells for each condition observed in electron microscopic sections from three separate experiments (means ± S.E.).
γ-Secretase on Phagosomes

tions. First, phagosomes represent a major residing compartment for γ-secretase in macrophages, as the enrichment of γ-secretase was found to be comparable with that of bona fide phagosomal markers. Second, the γ-secretase components were found to be enriched on Drosophila phagosomes, suggesting conserved functions for γ-secretase on this compartment. Third, the dynamics of γ-secretase association with phagosomes indicate that the level of γ-secretase remains stable throughout phagolysosome biogenesis. Our results further indicate that phagosome-associated γ-secretase components display the characteristics of the active enzyme, such as the formation of high molecular mass complexes and the association with lipid rafts. Finally, purified phagosomes were shown to contain γ-secretase activity.

A number of recent studies have focused on characterizing the complexes of various molecular masses formed by the γ-secretase components. In agreement with our results, most reports have demonstrated an abundant complex of ~400 kDa (43, 48–50). In addition to the major 400-kDa complex, γ-secretase complexes ranging from 150 kDa to 2 MDa have also been described (51, 52). The variations reported for the size of γ-secretase may depend on the detergent used in the extraction of the complex and the method used to evaluate the size of the complex. Our results demonstrate the presence of minor complexes of ~300 and 600 kDa on the phagosome. The 600-kDa complex was recently described as a low abundance complex, exhibiting the highest γ-secretase-specific activity compared with the abundant 400-kDa complex (53). The stoichiometry of the γ-secretase components in these various complexes remains to be characterized; however, active γ-secretase complexes clearly reside on phagosomes.

Phagosome-associated γ-secretase may originate from different cellular compartments. The engulfment of large particles by phagocytic cells involves the contribution of substantial amounts of membranes. Although the plasma membrane clearly participates in the formation of phagosomes, an important body of evidence indicates that the exocytosis of intracellular membranes at the site of nascent phagosomes is required for phagocytosis (36). Recycling endosomes and lysosomes have, for instance, been shown to contribute membranes during phagosome formation (54, 55). Recently, we demonstrated that endoplasmic reticulum membranes are also recruited to nascent phagosomes (56), a process that contributes important immune functions to phagosomes (57). Phagosomes thus contain proteins of diverse origins, including the plasma membrane, endosomal/lysosomal compartments, and the endoplasmic reticulum. In agreement with this view, phagosome-associated PS1 was observed as both full-length PS1, an abundant form in the endoplasmic reticulum (58), and processed PS1, a predominant form in post-Golgi compartments (41). Processed PS1 was markedly more abundant than full-length PS1 on phagosomes, in agreement with the enrichment of mature γ-secretase complexes on phagosomes. Further studies will be required to characterize the trafficking pathway accounting for the presence of γ-secretase on phagosomes.

Our results suggest that the cellular functions of phagosomes could be segregated into distinct membrane microdomains such as flotillin- and γ-secretase-containing lipid rafts. Flotillin has been shown to be absent from nascent phagosomes and to be acquired during phagolysosome biogenesis (29). In contrast, γ-secretase components are present on newly formed phagosomes, indicating that the γ-secretase complex and flotillin associate with distinct lipid rafts. In line with this proposal, our results show that γ-secretase and flotilllin on phagosome membranes display different susceptibilities to solubilization in Triton X-100 and CHAPS. These results are in agreement with the recent finding that γ-secretase and three SNARE (soluble N-ethylmaleimide-sensitive factor attachment protein receptor) proteins reside in lipid rafts that exhibit similar solubility characteristics in Triton X-100 (41). We have previously shown that the intracellular parasite Leishmania can inhibit the recruitment of flotillin-containing lipid rafts, a process coupled to the capacity of the parasite to survive inside phagosomes (29). Given their distinct biochemical characteristics and dynamics of association with phagosomes, flotillin- and γ-secretase-containing lipid rafts may thus play different roles in pathogen survival strategies.

A pivotal function of phagosomes is in processing proteins, both to degrade the phagosome content and to generate peptides for antigen presentation. In this context, γ-secretase represents an additional proteolytic activity, which likely contributes to the processing of membrane proteins in phagosomes. Rather than simply degrading proteins, however, increasing evidence indicates that the general function of substrate processing by γ-secretase may be to generate active CTFs from these membrane proteins. Upon γ-secretase cleavage, several membrane proteins, including Notch, CD44, and LRP, have indeed been demonstrated to release CTFs, which are capable of activating transcription (3–5). Hence, phagosome-associated γ-secretase activity may be involved in modulating the different gene expression programs that are induced during phagocytosis of various particles. For instance, LRP at the surface of phagocytic cells has recently been shown to stimulate the expression of inflammatory cytokines during phagocytosis of foreign particles (59). As γ-secretase has been localized to many intracellular compartments (19, 49, 60), it is likely that organelle-specific substrates of γ-secretase exist. Our study provides the first indication that phagosome-specific γ-secretase cleavage events may contribute to phagosome functions. In line with this proposition, our results show that IFN-γ increases the γ-secretase cleavage of LRP on phagosomes. In addition, our findings indicate that γ-secretase is necessary for the phagocytic response of macrophages to IFN-γ and TNF-α, suggesting that γ-secretase cleavage events are part of the pathway triggered by inflammatory cytokines. Further studies should decipher the role that γ-secretase substrates play in this process.

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