Fibrillar β-Amyloid-stimulated Intracellular Signaling Cascades Require Vav for Induction of Respiratory Burst and Phagocytosis in Monocytes and Microglia*

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Microglial interaction with extracellular β-amylloid fibrils (Aβ) is mediated through an ensemble of cell surface receptors, including the B-class scavenger receptor CD36, the αβ1-integrin, and the integrin-associated protein/CD47. The binding of Aβ to this receptor complex has been shown to drive a tyrosine kinase-based signaling cascade leading to production of reactive oxygen species and stimulation of phagocytic activity; however, little is known about the intracellular signaling cascades governing the microglial response to Aβ. This study reports a direct mechanistic link between the Aβ cell surface receptor complex and downstream signaling events responsible for NADPH oxidase activation and phagosome formation. The Vav guanine nucleotide exchange factor is tyrosine-phosphorylated in response to Aβ peptides as a result of the engagement of the microglia Aβ cell surface receptor complex. Co-immunoprecipitation studies demonstrate an Aβ-dependent association between Vav and both Lyn and Syk kinases. The downstream target of Vav, the small GTPase Rac1, is GTP-loaded in an Aβ-dependent manner. Rac1 is both an essential component of the NADPH oxidase and a critical regulator of microglial phagocytosis. The direct role of Vav in Aβ-stimulated intracellular signaling cascades was established using primary microglia obtained from Vav−/− mice. Stimulation of Vav−/− microglia with Aβ failed to generate NADPH oxidase-derived reactive oxygen species and displayed a dramatically attenuated phagocytic response. These findings directly link Vav phosphorylation to the Aβ-receptor complex and demonstrate that Vav activity is required for Aβ-stimulated intracellular signaling events upstream of reactive oxygen species production and phagosome formation.

Many of these inflammatory events center around the senile plaques, which are primarily composed of extracellular β-amylloid (Aβ) and are surrounded by activated microglia. Although a variety of proinflammatory molecules are elaborated by activated glial cells, free radical-mediated oxidative stress has been hypothesized to be a primary source of neuronal damage found in the AD brain (3, 4). The accumulation of Aβ deposits is associated with several markers of oxidative stress, including lipid peroxidation (5, 6), nucleic acid oxidation (7), and protein oxidation (8). Oxidative damage is observed early in the progression of AD (9, 10) and can be detected prior to fibrillar Aβ (Aβ) deposition in both the human brain (11) and animal models of the disease (9). The initiating events leading to Alzheimer disease remain unknown; however, these findings suggest that oxidative damage plays an early critical role in the pathogenesis of AD.

Microglia have been postulated to be a potential source of oxidative stress in response to Aβ peptides (2, 12). Microglia are phagocytes of myeloid origin and represent the principal immune effector cells in the brain. It has recently been appreciated that “resting” or “quiescent” microglia are highly dynamic and constantly extend their processes to survey their microenvironment presumably allowing them to react quickly to local injury or invading pathogens (13, 14). Phenotypically activated microglia are found clustered adjacent to Aβ plaques (15, 16), and microglia exposed to Aβ release cytokines, neurotoxins, and both reactive oxygen (ROS) and nitrogen species (2, 12). It is hypothesized that the sustained microglial proinflammatory response results in the production of ROS that are ultimately responsible for the oxidative damage observed in both the AD brain and animal models of the disease.

We have described previously a multireceptor cell surface complex for Aβ on microglia consisting of CD36, αβ1 integrin, CD47, and the class A scavenger receptor (17). Engagement of this receptor complex initiates tyrosine kinase-based signaling cascades (17–19) and a Aβ-stimulated respiratory burst leading to release of superoxide anion (17, 19–24). Furthermore, our recent findings provided evidence that in vitro microglia are capable of mounting a phagocytic response to Aβ

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2 The abbreviations used are: Aβ, Alzheimer disease; Aβ, fibrillar β-amylloid; ROS, reactive oxygen species; GEF, guanine nucleotide exchange factor; NBT, nitro blue tetrazolium chloride; GST, glutathione S-transferase; GDI, guanine nucleotide dissociation inhibitor; SH, Src homology; PAK, p21-activated protein kinase; PMA, phorbol 12-myristate 13-acetate; PBD, p21-binding domain; DH, Dbil homology; ANOVA, analysis of variance; PIPES, 1,4-piperazinediethanesulfonic acid; DMEM, Dulbecco’s modified Eagle’s medium.
through engagement of the aforementioned ensemble of cell surface receptors (25).

Although recent findings have provided valuable insights into the role microglia play in the proinflammatory events observed in AD, the intracellular signaling molecules responsible for the initiation of these responses remain to be elucidated. Specifically, it is unknown how the fAβ receptor complex is linked to the small GTPase Rac1, which is a critical element in signaling to both the NADPH oxidase (26, 27) and the phagocytic machinery (28, 29). One candidate molecule that could potentially link the fAβ cell surface receptor complex to downstream signaling events is the proto-oncogene Vav. Vav is one of the most well characterized guanine nucleotide exchange factors (GEFs) for Rac1. We report that Vav phosphorylation is mediated through the fAβ cell surface receptor complex, and our studies further demonstrate that Vav is an essential component of the fAβ-stimulated intracellular signaling pathway that leads to NADPH oxidase activation and ROS production. We also report a critical role for Vav in the initiation of a phagocytic response to Aβ peptides.

**EXPERIMENTAL PROCEDURES**

**Materials**—Aβ peptides corresponding to the human Aβ amino acids 25–35 and 1–42 were purchased from American Peptide Co. (Sunnyvale, CA). The method used to fibrillize Aβ peptides has been well characterized (30, 31). Aβ peptides were fibrillized by reconstitution in sterile distilled water followed by incubation at 37 °C for 1 week. The initial peptide concentration reflects that of the monomeric peptides comprising the Aβ fibrils. The composition of the fibrillar solutions may include Aβ oligomers as well as fibrils.

The 4N1K and RHD peptides were purchased from Bachem (Philadelphia) and reconstituted in sterile distilled water. Fucoidan was purchased from Sigma and reconstituted in sterile distilled water. Glutathione S-transferase (GST)-CD36 peptide was a gift from Dr. Maria Febbraio (The Cleveland Clinic Foundation, Cleveland, OH). Invasin 195 was a gift from Dr. Ingo Autenrieth (University of Tubingen, Germany). PAK-PBD beads were purchased from Cytoskeleton (Denver, CO). The anti-Rac and the anti-phospho-Tyr antibody 4G10 were obtained from Upstate Biotechnology, Inc. (Waltham, MA). The anti-Vav, -Syk, and -Lyn antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Affinity-purified horseradish peroxidase-conjugated sheep anti-mouse and donkey anti-rabbit antibodies were from Amersham Biosciences. Nitro blue tetrazolium chloride (NBT) was purchased from Roche Applied Science. Piceatannol was purchased from Roche Applied Science. Nile red fluorospheres (1 μM microspheres) were purchased from Molecular Probes (Eugene, OR).

**Tissue Culture**—Human THP-1 monocytes (American Type Culture Collection, Manassas, VA) were grown in RPMI 1640 medium (Whittaker Bioproducts, Walkersville, MD) containing 10% heat-inactivated fetal bovine serum (HyClone, Logan, UT), 5 × 10⁻⁵ M 2-mercaptoethanol, 5 mM HEPES, and 15 μg/ml gentamycin in 5% CO₂. THP-1 monocytes are used in these assays as they grow in suspension and do not attach to the tissue culture substrate through integrin-based adhesive mechanisms, allowing dissection of Aβ fibril-dependent signaling mechanisms in the absence of high basal levels of tyrosine kinase-based integrin signaling. Responses of THP-1 monocytes to fAβ faithfully replicate the responses of primary microglia (17, 18, 25, 32–34). Primary microglia were derived from postnatal day 1–3 mouse brains as described previously (18, 32–34).

**Cell Stimulation and Immunoprecipitations**—THP-1 monocytes were collected and resuspended in Hanks’ balanced salt solution for 30 min at 37 °C. Cells were then stimulated with fAβ-(25–35) or fAβ-(1–42) peptides for 3 min at 37 °C. For analysis of the elements of the fAβ receptor, cells were incubated with fucoidan, 4N1K, GST-CD36, invasin 195, or RHD peptide for 30 min at 37 °C prior to stimulation with Aβ peptides. Cells were collected by centrifugation and lysed in Triton buffer (1% Triton X-100, 20 mM Tris, pH 7.5, 100 mM NaCl, 40 mM NaF, 1 mM EDTA, 1 mM EGTA, and 1 mM Na₂VO₄). The insoluble material was removed by centrifugation at 10,000 × g for 12 min at 4 °C. Protein concentration of cell lysates was determined by the Bradford method (35). Aliquots of the cellular lysates (500 μg/ml) were added to protein A-agarose (30 μl) with the anti-Vav, -Lyn, or -Syk primary antibodies (2 μg of primary antibody/ml of lysate) and incubated with rocking for 2 h at 4 °C. Immune complexes were washed three times in Triton buffer; sample buffer was added, and samples were boiled for 5 min. Samples were resolved on 9 or 12% SDS-polyacrylamide gels and Western-blotted to polyvinylidene difluoride membranes. Blots were probed with either anti-phospho-Tyr (1:1000), -Vav (1:1000), -Syk (1:1000), or -Lyn (1:1000) antibodies overnight at 4 °C. The protein was detected by enhanced chemiluminescence. Blots were stripped and reprobed with the appropriate primary antibody as a loading control. Band intensities were quantified using NIH Image 1.62 software (Bethesda, MD). All experiments were performed a minimum of three times. Values statistically different from controls were calculated using a one-way ANOVA, and the Tukey-Kramer multiple comparisons test was used to determine p values.

**Rac Activation Assay**—THP-1 cell lysates were subjected to affinity precipitation using the specific interaction of the Rac GTPase with its downstream effector, the PBD of PAK (36). The PBD-PAK is bound to glutathione-agarose beads and was used according to the manufacturer’s instructions (Cytoskeleton, Denver, CO). Following stimulation with fAβ-(25–35) (60 μM/63.6 μg/ml), cells were collected and lysed with Mg²⁺-lys buffer (25 mM Tris, pH 7.5, 5 mM MgCl₂, 0.15 mM NaCl, 1% Igepal (Nonidet P-40), 5% sucrose). PBD-PAK beads were added to 1 mg/ml cell lysate and incubated with rocking for 1 h at 4 °C. Beads were collected by centrifugation and resuspended in sample buffer. Samples were subjected to 12% SDS-PAGE immunoblot analysis using an anti-Rac antibody (1:1000). Aliquots of the cell lysates (40 μg/lane) were run as protein loading controls. The protein was detected by enhanced chemiluminescence, and band intensities were quantified using NIH Image 1.62 software (Bethesda, MD). Values statistically different from controls were calculated using a one-way ANOVA, and the Tukey-Kramer multiple comparisons test was used to determine p values.
Requirement for Vav in Aβ-stimulated Intracellular Signaling

Cellular Fractionation—For these experiments we utilized a cell fractionation protocol described previously (37). Briefly, THP-1 cells (6 x 10^6 cells) were collected and resuspended in Hanks’ balanced salt solution for 30 min at 37 °C. Cells were then stimulated for 0 or 10 min with fAβ-(25–35) (60 μM) and lysed by incubation in relaxation buffer (100 mM KCl, 3 mM NaCl, 3.5 mM MgCl₂, 1.25 mM EGTA, and 10 mM PIPES, pH 7.3) on ice for 15 min followed by 10 s of sonication. Cells were cleared by centrifugation at 500 x g for 5 min at 4 °C. The supernatant was then centrifuged for 1 h at 110,000 x g at 4 °C in a Beckman Coulter SW50.1 rotor. The resulting supernatant was removed and saved as the “cytosolic” fraction, and the membrane pellet was resuspended in relaxation buffer. Lysates were then subjected to 12% SDS-PAGE immunoblot analysis using an anti-Rac antibody (1:1000) to determine the relative amount of Rac in each fraction. The membrane marker flotillin (1:1000) was used to assess the efficacy of the fractionation procedure. These experiments were performed four times (n = 4), and the data were analyzed by Student’s t test with a confidence interval of 99%.

Measurement of Superoxide Production—Intracellular superoxide radical generation was assayed by analyzing nitro blue tetrazolium (NBT) reduction (19, 38). Superoxide generation is determined at the microscopic level by the presence of an insoluble formazan precipitant, which appear as dark purple/black granules within the cell (39). For these experiments primary microglial cells from either Vav^−/− mice or Vav^+/− mice (SV129) were harvested as described previously. The Vav^−/− mice (40) were a generous gift from Dr. Juan Rivera (NIAMS, National Institutes of Health, Bethesda) and Dr. Victor Tybulewicz (National Institute for Medical Research, London, UK). Microglia were plated in 24-well plates overnight in DMEM/F-12 without serum. The cells were then stimulated with either fibrillar Aβ-(1–42) or Aβ-(25–35) (60 μM) at 37 °C for 30 min. Phorbol 12-myristate 13-acetate (PMA; 390 nM) was used as a positive control for the stimulation of ROS production (17, 19, 21). These findings confirmed that the microglia employ a multireceptor cell surface receptor complex is required for Vav phagocytosis.

RESULTS

Stimulation of Vav-Tyr Phosphorylation in THP-1 Monocytes by Fibrillar Aβ Peptides—Vav can act as a Rac-specific GEF upon its phosphorylation by Src family tyrosine kinases. We tested whether fAβ engagement of the microglia led to Vav tyrosine phosphorylation. Here we report the Tyr phosphorylation of the Vav protein following fAβ stimulation with either fibrillar Aβ-(1–42) or Aβ-(25–35) peptides (Fig. 1A). fAβ treatment resulted in the transient phosphorylation of Vav, which was maximal after 5 min (Fig. 1, B and C). These findings are consistent with our previous findings that demonstrated that fAβ peptides, Aβ-(1–40), Aβ-(1–42), and Aβ-(25–35), stimulate tyrosine kinase-based signaling in both THP-1 monocytes and microglia (17–19, 21). These findings confirmed that the magnitude of stimulation between these different Aβ peptides was qualitatively similar, and at this concentration (60 μM) the fibrillar Aβ-(25–35) peptide most effectively drives intracellular signaling (17, 21) as it contains the biologically active C-terminal β-pleated sheet domain that is necessary for fibril formation (42).

Engagement of the Microglial Fibrillar Aβ Cell Surface Receptor Complex Is Required for Vav Phosphorylation—We have recently shown that microglia employ a multireceptor cell sur-
face complex to detect and respond to Aβ fibrils. These receptor elements act in concert to stimulate intracellular signaling cascades as well as initiate a novel type of phagocytosis in microglia (17, 25). Importantly, we have shown that perturbation of the interaction of fAβ with individual subunits abrogates the signaling and phagocytic response from the complex as a whole. To determine whether fAβ engagement of this receptor ensemble was responsible for the observed Vav tyrosine phosphorylation, we tested the effect of antagonists to each of the individual receptor subunits. The participation of the class A and B scavenger receptors was assessed using fucoidan, which is an antagonist of these receptors (43). The role of the class B scavenger CD36 was directly assessed using GST-CD36, which is composed of the 93–120-amino acid extracellular binding domain of CD36 coupled with a GST tag. This fusion protein is designed to competitively inhibit the binding of ligands to cell surface CD36 (44). We found that fucoidan and GST-CD36 both inhibited Vav phosphorylation in response to fAβ stimulation in THP-1 monocytes (Fig. 2, A and B). To evaluate the contribution of the α6β1 integrin, we employed two different antagonists specifically directed toward the β1 integrin. First, the RHD peptide, which contains an epitope that binds to β1 integrins (45), dramatically reduced Vav phosphorylation in response to fAβ (Fig. 2C). The second β1 integrin antagonist was derived from invasin protein (invasin 195), which is encoded by the enteropathogenic Yersinia (46), and acts to functionally block β1 integrin activation (47). We demonstrate that invasin 195 also inhibited fAβ-stimulated Vav phosphorylation in THP-1 monocytes (Fig. 2D). The CD47 antagonist 4N1K, which is derived from the cell-binding domain of thrombospondin, blocks intracellular signaling by directly binding to CD47 (48). Incubation of THP-1 monocytes with 4N1K inhibited Aβ fibril stimulation of Vav phosphorylation (Fig. 2E). Taken together, these results establish that fAβ engagement of the fAβ cell surface receptor complex initiates Vav phosphorylation and activation.
Vav Associates with Both Lyn and Syk Kinases in Fibrillar Aβ-stimulated THP-1 Monocytes—Microglia and THP-1 cells respond to fAβ by activating the Src family kinase Lyn as well as the tyrosine kinase Syk, and these kinases have been reported previously to be required for proximal signaling events in the microglial response to Aβ fibrils (17–19, 25). Interestingly, both Lyn and Syk have been shown to stimulate Vav phosphorylation in response to a variety of other stimuli (49, 50). Therefore, we sought to determine whether Lyn or Syk associate with Vav following stimulation with fAβ. Either Lyn or Syk was immunoprecipitated from THP-1 monocytes stimulated with or without fAβ. The immunoprecipitants were transferred to immunoblots and probed with a Vav antibody. The immunoblots revealed that Lyn (53–56-kDa protein doublet) and Syk (80-kDa) co-immunoprecipitated with Vav following fAβ stimulation (Fig. 3, A and B). To further establish a role for either Lyn or Syk kinases in Vav phosphorylation, inhibitors directed against either Src or Syk were analyzed. Pretreatment of THP-1 cells with either piceatannol, a Syk inhibitor, or PP2, an Src inhibitor, resulted in an inhibition of Aβ-stimulated Vav tyrosine phosphorylation (Fig. 4, A and B). These findings directly link Vav to proximal fAβ receptor signaling events.

Rac1 Is Activated and Translocates to the Membrane in fAβ-stimulated THP-1 Monocytes—We wanted to establish whether the downstream target of Vav, the small GTPase Rac1, also displayed a fAβ-dependent activation. Rac1, the predominant isoform in myeloid lineage cells (51), is a member of the Rho family of small monomeric GTPases and is an integral component of the active NADPH oxidase (52). Although fAβ-stimulated ROS production has been shown previously (17, 19, 24), direct evidence for activation of Rac by Aβ peptides has not been demonstrated. We demonstrate that THP-1 cells stimulated with fAβ exhibit a robust increase in Rac GTP loading. Affinity precipitation assays reveal that GTP-bound Rac is increased within 5 min of fAβ treatment, and the levels of Rac-GTP return to near base-line levels within 30 min (Fig. 5, A and B). Following GDP/GTP exchange and conversion into an active conformation, Rac must translocate to the plasma membrane

FIGURE 3. Fibrillar Aβ-stimulation leads to Vav association with both Lyn and Syk kinases. THP-1 cell were stimulated with fAβ-(25–35) (60 μM) for 3 min, and cell lysates were immunoprecipitated (IP) with either an anti-Lyn (A) or anti-Syk (B) antibody. Immunoprecipitates were resolved by 9% SDS-PAGE and analyzed by Western blot (WB) using an anti-Vav antibody. Blots were stripped and reprobed with the appropriate antibody as a protein-loading control.

FIGURE 4. Inhibition of either Src kinase or Syk kinase activity inhibits tyrosine phosphorylation of Vav. A, THP-1 cells were pretreated with either PP2 (10 μM) or piceatannol (25 μg/ml; PICE) for 30 min prior to stimulation with fAβ-(25–35) (60 μM) for 3 min. Vav was immunoprecipitated (IP) from cell lysates with an anti-Vav antibody and analyzed by Western blot (WB) analysis using the anti-phospho-Tyr antibody 4G10. Blots were stripped and reprobed with an anti-Vav antibody as a protein-loading control (Ctrl). B, analysis of band intensity of Western blots of phosphorylated Vav normalized to Vav protein levels and expressed as relative density. *, p < 0.05 compared with control.

FIGURE 5. Time course of Rac GTP loading following stimulation with fAβ. A, THP-1 cells stimulated with fAβ-(25–35) (60 μM) for 0, 1, 5, 10, 15, or 30 min were subject to a Rac affinity precipitation assay that captures only GTP-bound Rac. Cell lysates were analyzed by immunoblot analysis using an anti-Rac antibody. Cell lysate samples (40 μg/lane) were run in parallel as protein loading controls. B, analysis of the relative density ratio of Rac-GTP loading. The data shown are reflective of pooled data from three independent studies. *, p < 0.05 at 5 and 10 min as compared with 0 min.
where it interacts with other NADPH oxidase subunits to form the active oxidase complex and produce superoxide. Rac also fulfills a number of other functions related to cytoskeletal organization and presumably activated by other GEFs; thus the cells exhibit a significant basal level of active Rac associated with the membrane. Important to our studies, fAβ-exhibit a 2-fold increase in Rac membrane association (Fig. 6, A and B), which is reflective of fAβ-specific actions in stimulating NADPH oxidase activity and phagosome formation.

**Fibrillar Aβ-stimulated Reactive Oxygen Species Production Is Inhibited in Vav<sup>−/−</sup> Primary Microglia**—In light of our findings that Vav is phosphorylated in response to Aβ stimulation, we hypothesized that Vav might be a necessary upstream intermediate in the intracellular signaling pathway leading to NADPH oxidase assembly and subsequent ROS production. To examine the role of Vav in Aβ-stimulated respiratory burst, we utilized primary murine microglia obtained from Vav<sup>−/−</sup> mice. Analysis of intracellular superoxide production was monitored by the reduction of NBT. We observed that superoxide production was severely attenuated in fAβ-stimulated Vav<sup>−/−</sup> microglia when compared with fAβ-stimulated Vav<sup>+/+</sup> microglia (Fig. 7). Vav<sup>−/−</sup> microglia produced significant levels of ROS in response to PMA indicating that the deletion of Vav does not inhibit the function of the NADPH oxidase in response to an alternate stimulus. These data demonstrate an obligatory role for Vav activity in fAβ-stimulated intracellular signaling pathways leading to effective NADPH oxidase assembly and respiratory burst in primary microglia.

**The Genetic Deletion of Vav Inhibits the fAβ-induced Phagocytic Response in Primary Microglia**—In an effort to establish whether Vav plays a broad role in response to fAβ stimulation of microglia, we analyzed the role Vav might play in the intracellular signaling events that link fAβ engagement of the cell surface receptor complex to the phagocytic machinery. Both Vav and Rac1 have been implicated in both cytoskeletal alterations (53) and membrane ruffling (54) observed during phagocytosis. We sought to identify whether genetic deletion of Vav activity upstream of its effector, Rac1, would alter the fAβ-induced phagocytic response. Primary microglia from Vav knock-out (Vav<sup>−/−</sup>) and wild type (Vav<sup>+/+</sup>) mice were evaluated for their ability to phagocytose microspheres. Vav<sup>−/−</sup> microglia treated with fAβ-(25–35) failed to mount a phagocytic response when compared with Vav<sup>+/+</sup> microglia (Fig. 8). These findings establish Vav as an essential proximal component of the fAβ-stimulated signaling pathway that catalyzes Rac1-dependent phagosome formation.

**DISCUSSION**

The present study extends our previous efforts to elucidate the intracellular signaling mechanisms responsible for microglial proinflammatory responses to fibrillar Aβ peptides. The dissection of proximal intracellular signaling cascades is essential for understanding how fAβ at the cell surface is coupled to the release of proinflammatory secretory products from microglia. Fibrillar Aβ engages an ensemble of cell surface receptors that initiate intracellular signaling cascades that may utilize similar signaling molecules before diverging to assemble the NADPH oxidase and the phagosome.

A primary goal of these experiments was to evaluate potential signaling intermediates upstream of the NADPH oxidase. Of particular interest was a group of signaling molecules that act as guanine nucleotide exchange factors (GEFs) for Rac. These molecules are known to activate the NADPH oxidase subunit Rac1 by facilitating GDP/GTP exchange on Rac1. Several Rac-GEFs have been identified, including Vav (28, 55), Tiam1 (56), Dock180/Elmo (57), and Eps8/E3b1/Sos-1 (58). We chose to analyze Vav activity because it has been the most tightly linked to the activation of the NADPH oxidase (52). Vav is a multidomain protein composed of adjacent Dbl homology (DH) and pleckstrin homology domains that are common to almost all Rho family GEFs (59). The DH domain is responsible for the catalysis of GDP/GTP exchange (60). The DH and pleck-
Requirement for Vav in Aβ-stimulated Intracellular Signaling

Vav is essential for Aβ-stimulated phagocytosis in primary murine microglia. Neonatal primary microglial cells from Vav−/− mice or Vav+/− (SV129) mice were treated with Aβ(25–35) (60 μM) for 30 min before the addition of microspheres for an additional 30 min. The ingestion of fluorescent microspheres was visualized on an inverted microscope, and three random fields of cells (≥100 cells) were counted to determine the percentage of phagocytic cells. The data are reflective of pooled data from three independent experiments. **, p < 0.01 compared with control.

Strain homology domains are bordered on the C terminus by a zinc finger motif, a short proline-rich region, and an Src homology (SH) SH3-SH2-SH3 domain and bordered on the N terminus by a calponin homology domain and an acidic region. Tyrosine phosphorylation of Vav is critical for activation of its GEF activity, and recent studies have demonstrated that tyrosine phosphorylation of Tyr-174 relieves N-terminal autoinhibition between the acidic region and the DH domain (61). Notably, Crespo et al. (62) have demonstrated that Vav tyrosine phosphorylation is necessary for activation of its exchange activity toward Rac1 and subsequent activation of downstream targets. In this study, we establish that Vav displays a Aβ-dependent phosphorylation on tyrosine residues, and this tyrosine phosphorylation is contingent upon fibril engagement of the microglia Aβ cell surface receptor complex.

The SH2 domain of Vav mediates its interaction with activated protein-tyrosine kinases, presumably by binding phosphotyrosine residues. Several protein-tyrosine kinases have been shown to facilitate Vav phosphorylation (59). In Aβ-stimulated microglia, Vav phosphorylation is likely contingent on its interaction with either Src family kinases or Syk kinase. Indeed, elements of the Aβ receptor complex are found to physically associate with Src family kinases and Syk (17). We have demonstrated previously a dramatic reduction in Aβ-stimulated ROS production in THP-1 cells pretreated with the Syk inhibitor piceatannol (19). Furthermore, Bianca et al. (24) have demonstrated a similar reduction in ROS production in both monocytes and microglia treated with the Src family-selective tyrosine kinase inhibitor PP1. These findings verify the necessity for both of these kinases in the signaling pathways leading to NADPH oxidase assembly. We have also demonstrated previously that inhibition of either of these tyrosine kinases results in inhibition of the phagocytic response to Aβ (25). In this study, we demonstrate a Aβ-dependent interaction between Vav and the Src family kinase Lyn and the tyrosine kinase Syk, and inhibition of kinase activity leads to a dramatic reduction in Vav tyrosine phosphorylation. This association provides a mechanistic link between Vav and the most proximal Aβ-stimulated intracellular signaling events.

The NADPH oxidase normally plays an essential role in innate immunity. The oxidase is found associated with phagocyte membranes where it can facilitate the destruction of invading microorganisms by releasing O2− into the phagocytic vesicle (63, 64). This released O2− serves as a precursor for additional ROS that are rapidly formed, including hydrogen peroxide, hydroxyl radical, peroxynitrite, and other oxidants that aid in the killing of the invading pathogen (27). Importantly, excessive production of these ROS is cytotoxic and can damage tissue adjacent to sites of inflammatory action (65); consequently, NADPH oxidase assembly is a highly regulated process (27, 63). The NADPH oxidase is a multicomponent enzyme system that is composed of two integral membrane proteins, p22phox and gp91phox (together known as cytochrome b22, a), and three essential cytosolic components, p47phox, p67phox, and Rac1. A fourth nonessential cytosolic component, p40phox, may serve in a regulatory capacity. The activation of the PHOX subunits is regulated through signaling pathways that are independent from Rac activation and translocation (27, 66, 67). Following their phosphorylation, the cytosolic PHOX subunits translocate to the membrane where they interact to assemble the functional oxidase complex (26, 63). The importance of the NADPH oxidase in host defense is illustrated by the hereditary loss-of-function disorder, chronic granulomatous disease, which results from mutations in any of these individual subunits. Chronic granulomatous disease patients are subject to recurrent bacterial and fungal infections and a reduced life expectancy.

In the AD brain, microglial NADPH oxidase-derived ROS are believed to be a primary source of oxidative damage (19, 22–24). Previous studies have demonstrated the translocation of the p47phox and p67phox subunits from the cytosol to the membrane in both human AD brain tissue (68) and Aβ-stimulated monocytes and microglia (24). We extended these findings to document the ability of Aβ to activate parallel signaling pathways leading to Rac1 GTP loading. In resting cells, Rac is held inactive in the cytosol through an interaction between its C-terminal prenyl moiety and the effector region of the GDP dissociation inhibitor, RhoGDI. The timing of Rac translocation in relation to its activation remains unclear; however, recent findings suggest Rac moves to the membrane prior to its GTP loading (28). During activation, Rac undergoes GDP/GTP exchange facilitated by a GEF and becomes dissociated from RhoGDI. GTP-loaded Rac is then free to interact with other components of the NADPH oxidase where it is an integral and essential component for ROS production (52, 69). Our findings demonstrate that Rac1 translocates to the plasma membrane and is GTP-loaded in an Aβ-dependent manner. The temporal activation of Rac1 is also similar to the activation time course identified for Vav. We hypothesize that following Aβ stimulation Vav acts as the GEF for Rac1 in microglia, thereby facilitating the participation of Rac in ROS formation. We directly examined the role Vav plays in upstream Aβ-stimulated signaling pathways leading to NADPH oxidase assembly by ana-
lyzing ROS production in Vav-deficient primary microglia. Our data confirm that Vav is indeed required for Aβ-dependent intracellular signaling cascades leading to functional NADPH oxidase assembly. Taken together, these results provide evidence that Aβ drives assembly of the microglial NADPH oxidase through an ensemble of cell surface receptors that initiate a tyrosine kinase-Vav-Rac1-based signaling cascade (Fig. 9).

It remains plausible that in the AD brain Aβ-induced oxidative events are mediated by cell types other than microglia. Indeed, astrocytes have been shown recently to have a functional NADPH oxidase (70), and Aβ stimulation elicits activation leading to neuronal death in a co-culture model (71). However, it remains unclear what the quantitative contributions of astrocytes are in Aβ-induced ROS production.

There is compelling evidence implicating a Vav-Rac interaction in formation of the phagosome in FcyR-mediated (type I) phagocytosis (29, 72). Vav mutants lacking their catalytic domain (DH domain) responsible for GEF activity abolished the FcyR-mediated activation of Rac and consequently reduced the phagocytic response of macrophages (28). The activation of both Vav and Rac has also been implicated in the novel β1-integrin-dependent phagocytic mechanism used to internalize the bacteria Yersinia (47). In the AD brain, microglia exhibit a proinflammatory phenotype, yet fail to mount an effective phagocytic response to Aβ plaques (73); however, microglia have a well established ability to elicit a phagocytic response to both Aβ fibrils and isolated senile plaques in vitro (74–76). We have recently reported that in vitro microglia use a β1-integrin-dependent phagocytic mechanism to engulf Aβ fibrils analogous to the phagocytic response elicited by Yersinia (25). Intracellular signaling resulting in phago-
