Amyloid Precursor Protein Mediates Proinflammatory Activation of Monocytic Lineage Cells*

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Alzheimer’s disease is a progressive neurodegenerative disorder characterized by extracellular deposition of β-amyloid (Aβ) peptide containing neuritic plaques. Aβ peptides are proteolytically derived from the membrane-bound amyloid precursor protein (APP). Although the function of APP is not entirely clear, previous studies demonstrate that neuronal APP colocalizes with β1 integrin receptors at sites of focal adhesion, suggesting that APP is involved in mediating neuronal process adhesion. Integrin-dependent adhesion is also a well-characterized component of immune cell proinflammatory activation. Using primary mouse microglia and the human monocytic cell line, THP-1, we have begun investigating the role of APP in integrin-dependent activation. Co-immunoprecipitation studies demonstrate that APP is recruited into a multi-receptor signaling complex during β1 integrin-mediated adhesion of monocytes. Stimulation induces a subsequent, specific recruitment of tyrosine phosphorylated proteins to APP, including Lyn and Syk. Antibody cross-linking of cell surface APP leads to a similar response characterized by activation and recruitment of tyrosine kinases to APP as well as subsequent activation of mitogen-activated protein kinases and increased proinflammatory protein levels. These data demonstrate that APP can act as a proinflammatory receptor in monocytic lineage cells and provide insight into the contribution of this protein to the inflammatory conditions described in Alzheimer’s disease.

Amyloid precursor protein (APP) is a ubiquitously expressed integral membrane protein from which the 1–40 and 1–42 residue β-amyloid (Aβ) peptides are proteolytically cleaved (1). The longer, more insoluble peptide, Aβ1–42, is a marked component of the extracellular neuritic plaques characteristic of Alzheimer’s disease (2). The physiologic role for APP independent of the production of the Aβ peptides remains to be elucidated.

Although only a fraction of the pool of cellular APP localizes to the plasma membrane, the overall structure of the protein suggests that APP may function as a receptor or growth factor (3). APP is an integral transmembrane glycoprotein that exists as one of three major splice variants consisting of either 695, 751, or 770 amino acid residues (4). These isoforms are composed of a large glycosylated extracellular region, a single membrane-spanning domain, and a short, highly conserved cytoplasmic tail (1). Interestingly, the cytoplasmic domain of APP contains a well-defined consensus motif found in tyrosine kinase receptors, NPXY, implicating a role in signal transduction that is reinforced by the ability of multiple adaptor proteins including Fe65 and X11 to interact with this specific motif on neuronal APP (5–7). The phosphotyrosine-binding domains of Fe65 and X11 bind the phosphotyrosine binding motif (Y682ENPTY687) on the intracellular domain of APP. However, there is no evidence that phosphorylation of this domain is required for the interaction to occur (8, 9). The adaptor protein Shc has also been identified as an APP-interacting protein; however, this interaction is promoted by tyrosine phosphorylation of the APP phosphotyrosine-binding motif (10, 11). These data suggest that APP is capable of serving as a docking molecule in membrane proximal signaling events. It has also been demonstrated that neuronal APP colocalizes with β1 integrins at point contacts, suggesting a possible role in adhesion (12). Furthermore, it has been demonstrated that APP binds directly to extracellular matrix molecules, particularly collagen type I (13).

Integrins are a family of heterodimeric cell surface receptors composed of an α and β subunit that are expressed in a diverse group of cell types. There are 16 identified α and 8 β subunits that combine to form at least 22 different αβ heterodimeric integrin receptors. The specific combination of subunits dictates the binding specificity as well as overall function of the integrin receptor (14, 15). Integrins function to modulate cellular adhesion and use a characteristic tyrosine kinase-mediated activation pathway to alter cellular response (16–18). A common feature of integrin behavior is their ability to form macromolecular signaling complexes with other cell surface receptors (19–21). β1 integrin-dependent adhesion stimulates a well-characterized tyrosine kinase-based activation response in monocytic lineage cells, inducing increased expression of several inflammatory mediator genes (22–24). Interestingly, inflammatory activation of these cells increases localization of APP to the plasma membrane, suggesting APP participates in adhesion-mediated activation of this cell type (25, 26). To answer this question, we investigated the role of APP in β1 integrin-dependent adhesion and activation of the human monocytic cell line, THP-1, and primary mouse microglia.

MATERIALS AND METHODS
Materials—The 4G10 monoclonal anti-phosphotyrosine antibody was purchased from Upstate Biotechnology (Lake Placid, NY). Anti-β-

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† The abbreviations used are: APP, amyloid precursor protein; Aβ, β-amyloid; JNK, c-Jun N-terminal kinase; PPI, 4-amino-5-(4-methyl-phenyl)-7-(4-butylypyrazolo[3,4-d] pyrimidine; siRNA, small interference RNA; MAPK, mitogen-activated protein kinase; ERK, extracellular signal-related kinase; IL, interleukin; COX, cyclooxygenase.
growth medium into serum-free RPMI 1640 and incubated at 37 °C in vitro, microglia were harvested by rapid shaking for 30 min on a collagen at a density of 3 × 10^5 cells/ml. Cells were either unstimulated (control) or stimulated with anti-APP antibody, 22C11 (1 µg/ml), or mouse IgG1, isotype control (1 µg/ml), monovalent Fab APP antibody fragment (1 µg/ml), or mouse IgG1, isotype control (1 µg/ml). Cells were stimulated for 5 min or 24 h at 37 °C. Total cell lysates and immunoprecipitates were prepared as described below.

**Western Blotting**—Ice-cold RIPA buffer (20 mM Tris, pH 7.4, 150 mM NaCl, 1 mM Na3VO4, 10 mM NaF, 1 mM EDTA, 1 mM EGTA, 0.2 mM phenylmethylsulfonyl fluoride, 1% Triton, 0.1% SDS, and 0.5% deoxycholate) was used to lyse cells. Cell lysates were sonicated and centrifuged (14,000 rpm, 4 °C, 10 min) to remove insoluble material. Protein A

**APP Cross-linking**—To cross-link cell surface APP, THP-1 cells or microglia were removed from normal growth medium into serum-free RPMI 1640 and incubated at 37 °C for 15 min before stimulation. Tissue culture wells were coated with type I collagen for the indicated times (in minutes). Cells were lysed in RIPA buffer, separated by 7 or 10% SDS-PAGE, and Western blotted using anti-phosphotyrosine antibody, 4G10 (A), anti-phospho-p38 antibody, anti-p38 antibody (B), anti-phospho-JNK antibody, anti-JNK antibody (C), and anti-phospho-ERK antibody, anti-ERK2 antibody (D). Antibody binding was visualized by chemiluminescence.

**Cell Stimulation**—THP-1 monocytes were plated for 30 min on tissue culture plastic alone or on type I collagen for the indicated times (in minutes). Cells were lysed in RIPA buffer, separated by 7 or 10% SDS-PAGE, and Western blotted using anti-phosphotyrosine antibody, 4G10 (A), anti-phospho-p38 antibody, anti-p38 antibody (B), anti-phospho-JNK antibody, anti-JNK antibody (C), and anti-phospho-ERK antibody, anti-ERK2 antibody (D). Antibody binding was visualized by chemiluminescence.

**Tissue Culture**—THP-1 cells are a monocytic cell line derived from peripheral blood of a human with acute monocytic leukemia commercially available from the American Type Culture Collection (Manassas, VA). THP-1 cells were grown in RPMI 1640 (Life Technologies, Inc., Rockville, MD) containing 10% heat-inactivated fetal bovine serum and 2% penicillin/streptomycin/neomycin. Microglia were derived from the brains of postnatal day 2 C57Bl/6j mice. Briefly, cerebral cortices were isolated and trypanized for 15 min. Digestion was terminated by adding tissue to DMEM/F12 media containing 10% fetal bovine serum and 5% horse serum (U.S. Biotechnologies, Inc.). Cells were trituated to obtain a single-cell suspension and plated into 75-cm² flasks. Media were replaced the next day, and cells were fed every 5 days. At ~14 days in vitro, microglia were harvested by rapid shaking for 30 min on a reciprocal shaker.

**Generation of Fab Antibody Fragments**—The monoclonal 22C11 antibody was digested at 37 °C for 24 h in the presence of 0.02 mg/ml laminin (lam), or 0.05 mg/ml poly-L-lysine (lys). Cells were lysed in 1% Triton X-100 buffer, and APP was immunoprecipitated. A, cell lysates were resolved by 7% SDS-PAGE and Western blotted with anti-phosphotyrosine antibody, 4G10, and anti-ERK2 antibody (loading control). B, immunoprecipitates were resolved by 7% SDS-PAGE and Western blotted with anti-phosphotyrosine antibody, 4G10, and anti-APP antibody. C, THP-1 cells were plated for 30 min on tissue culture plastic alone (c) or on type I collagen (col). Cells were lysed in 1% Triton X-100 buffer, and either APP or β1 integrin was immunoprecipitated. Immunoprecipitates were resolved by 7% SDS-PAGE and Western blotted using anti-β1 integrin antibody or anti-APP antibody. Antibody binding was visualized by chemiluminescence.

**APP polyclonal antibody** was from Zymed Laboratories (San Francisco, CA). Anti-phospho-p38, anti-p38, anti-phospho-JNK, anti-JNK were from Cell Signaling (Beverly, MA). Anti-Lyn, anti-Syk, anti-phospho-1-integrin, anti-c-myc, anti-COX-2, anti-CD36, anti-NOS2, anti-phospho-extracellular signal-regulated kinase (ERK), anti-ERK2 antibody, and protein A/G PLUS-Agarose beads were from Santa Cruz Biotechnology (Santa Cruz, CA). The 22C11 anti-APP monoclonal antibody was from Chemicon (Temecula, CA). Anti-mouse (goat), anti-rabbit (donkey), and anti-goat (donkey) horseradish peroxidase-conjugated secondary antibodies were purchased from Santa Cruz Biotechnology. 4-Amino-5-(4-methylphenyl)-7-(4-buty1)pyrazolo(3,4-d)pyrimidine (PP1) was purchased from Alexis Biochemicals (San Diego, CA). APP siRNA (siGene, SMARTpool Plus) was obtained from Dharmacon (Lafayette, CO). Type I collagen was derived from rat tail connective tissue to DMEM/F12 media containing 10% fetal bovine serum and 0.1 M papain. Digestion was terminated with the addition of 0.03 M NaCl, 1 mM Na3VO4, 10 mM NaF, 1 mM EDTA, 0.2 mM phenylmethylsulfonyl fluoride, 1% Triton, 0.1% SDS, and 0.5% deoxycholate) was used to lyse cells. Cell lysates were sonicated and centrifuged (14,000 rpm, 4 °C, 10 min) to remove insoluble material. Protein band was removed from the gel, and electro-elution was performed to recover the Fab fragments. The identity of these fragments was confirmed by Western blotting for APP.

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concentrations were quantitated by the method of Bradford (27). Proteins were resolved by 7 or 10% SDS-PAGE and transferred to polyvinylidene difluoride membranes for Western blotting. Western blots were blocked in TBS-T (10 mM Tris, pH 7.4, 100 mM NaCl, and 0.1% Tween 20) containing 3% BSA for 15 min and then incubated overnight at 4 °C in primary antibodies. Blots were washed three times in TBS-T, followed by incubation for 1 h with horseradish peroxidase-conjugated secondary antibodies in TBS-T containing 5% nonfat dried milk. The blots were washed three times in TBS-T, followed by detection with enhanced chemiluminescence (Pierce). In some instances, blots were stripped in 0.2 × NaOH, 5 min, 25 °C.

**Immunoprecipitation**—For co-immunoprecipitation, cells were lysed in ice-cold Triton lysis buffer (20 mM Tris, pH 7.4, 150 mM NaCl, 1 mM NaN3, 10 mM NaF, 1 mM EDTA, 1 mM EGTA, 0.2 mM phenylmethylsulfonyl fluoride, and 1% Triton). Lysates were vortexed and then incubated on ice for 15 min, followed by pulse sonication. Cells were centrifuged 10 min at 4 °C to remove insoluble material. Primary antibody (1 μg/mg protein) was added and incubated 4 h at 4 °C. Protein A/G beads (35 μl) were added and incubated overnight at 4 °C. Beads were washed three times with lysis buffer, and immunoprecipitates were resolved and Western blotted as described above.

**Small Interference RNA (siRNA) Transfection**—THP-1 cells were transfected with APP siRNA (2 × 106 cells; 2 μg of siRNA) using the appropriate Nucleofector program as described by the manufacturer (Amaxa Inc., Gaithersburg, MD). Cells were transfected with either individual siRNA duplexes or the combined pool of duplexes. Cells were stimulated by adhesion to collagen (as described above) 24 h after transfection or lysed to determine APP expression.

### RESULTS

Collagen type I is the preferred substrate for a subset of β1 subunit containing integrin heterodimers (28–30). β1 integrin ligation reportedly stimulates activation of cytoplasmic tyrosine kinases and regulates gene expression in mononuclear lineage cells (22, 24, 31). To characterize a tyrosine kinase activation response in our system, we determined that adhesion of THP-1 monocytes to type I collagen stimulated a time-dependent increase in protein phosphoryrosine levels with a maximal stimulation at 30 min (Fig. 1A). Collagen adhesion stimulated increased phosphorylation, indicative of activation, of the p38 mitogen-activated protein kinase (MAPK) (Fig. 1B). However, adhesion-mediated activation did not stimulate activation of the ERKs nor JNKs (Fig. 1, A and C). It is important to point out that control cells display a slight increase in tyrosine phosphorylated protein levels and increased phosphorylation of p38, which is likely attributable to the progressive settling of the cells on the tissue culture plastic (Fig. 1, A and B). The profile of increased protein phosphoryrosine levels stimulated by collagen adhesion was different from that stimulated by other β1 ligands, fibronectin, and laminin, as well as the nonspecific cationic ligand poly-L-lysine (Fig. 2A) (32). Additionally, only collagen-dependent adhesion stimulated recruitment of tyrosine phosphorylated proteins to APP (Fig. 2B). Moreover, collagen adhesion stimulated formation of a multi-receptor complex including cell surface APP and β1 integrin subunits (Fig. 2C). These data suggest that APP participates in the tyrosine kinase-dependent activation response of β1 integrin receptors in monocytes, particularly those using type I collagen as a ligand. Reportedly, the potential αβ combinations that can function as collagen receptors include the receptors composed of the β1 subunit noncovalently linked to either α1, α2, α3, α10, or α12 subunit (29).

To further characterize the contribution of APP to β1 integrin-dependent activation, we identified tyrosine phosphorylated proteins recruited to APP during stimulation (Fig. 3A). Previous studies demonstrated that the non-receptor tyrosine kinases Lyn and Syk are active components of integrin-mediated signaling responses in monocytes cells (24, 33). Co-immunoprecipitation experiments verified that Lyn and Syk were recruited to APP upon collagen binding and displayed increased levels of tyrosine phosphorylation, indicative of enzyme activation (Fig. 3, A–C). Protein tyrosine kinases are often receptor proximal factors involved in the initial stage of a series of events leading to activation of cytoplasmic serine/threonine kinases and subsequent transcriptional regulation (34). The tyrosine kinase-initiated activation response mediated by β1 integrin-dependent adhesion of monocytes cells is a well-described event in the induction of immediate-early genes characteristic of monocyte differentiation including interleukin-1β (IL-1β), interleukin-8 (IL-8), and tumor necrosis factor-α (35). As expected, persistent exposure of monocytes to collagen resulted in the acquisition of a reactive phenotype including an increase in cyclooxygenase-2 (COX-2), inducible nitric oxide synthase (α-iNOS) antibody, anti-COX-2 antibody, anti-IL-1β antibody, and anti-ERK2 antibody (loading control). Antibody binding was visualized by chemiluminescence.
specific inhibitor of Src family kinases, PP1, and evaluated the downstream effects (36). Pretreatment of THP-1 cells with PP1 before plating on collagen prevented the increased activation and recruitment of the tyrosine kinases to APP and subsequent activation of p38 MAPK (Fig. 5A). Importantly, kinase inhibition also prevented the stimulated increase in COX-2 protein levels (Fig. 5B), which served as a representative indicator of reactivity, demonstrating that Src family kinases such as Lyn are critical initiating factors in acquisition of a reactive phenotype. To further define a role for APP in integrin-mediated proinflammatory activation of monocytic lineage cells, we used siRNA to significantly reduce APP expression (Fig. 5C). We determined that APP is a critical component of adhesion-mediated increased p38 MAPK activity and increased COX-2 protein levels, indicating that APP is involved in acquisition of a reactive phenotype (Fig. 5, D and E).

Our findings demonstrate that APP has receptor-like properties upon β1 integrin engagement; however, we predicted that APP may function as a receptor independent of β1 integrin ligation. We tested this hypothesis by stimulating THP-1 cells with an antibody directed against the extracellular domain of APP, clone 22C11, to simulate ligand binding (37). Direct cross-linking of cell surface APP on THP-1 cells stimulated a rapid increase in tyrosine phosphorylated proteins qualitatively similar to that induced by collagen adhesion (Fig. 6A). In addition, co-immunoprecipitations verified that Lyn and Syk were activated and recruited to APP upon antibody cross-linking similar to adhesion-dependent activation (Fig. 6B). However unlike collagen-dependent activation, antibody-dependent APP dimerization stimulated increased activation of not only p38 MAPK but also ERKs and JNKs (Fig. 6A). These data demonstrate fundamental differences in APP contribution to monocytic activation, depending upon its recruitment to β1 integrin macromolecular complexes or its ability to act as an independent receptor. Importantly, we demonstrated that proinflammatory activation was dependent on APP dimerization because the monovalent Fab antibody fragment was not sufficient to stimulate increased tyrosine phosphorylated proteins or increased MAPK activity (Fig. 6C).

We next determined whether APP dimerization stimulated...
acquisition of a reactive phenotype similar to that induced by β1 integrin engagement. Dimerization stimulated an increase in COX-2 and IL-1β protein levels in the monocytes after 24-h treatment (Fig. 7). However, in contrast to collagen-stimulated activation, direct APP cross-linking did not stimulate increased inducible nitric oxide synthase or CD36 protein levels. These data again illustrate a difference between adhesion-dependent and APP dimerization-dependent activation of monocytic cells and define the specificity of APP independent-mediated changes in a cellular proinflammatory phenotype.

To confirm that initial tyrosine kinase activity was critical for subsequent activation of the MAPK pathways and the phenotypic changes, THP-1 cells were pretreated with PP1 to inhibit Lyn activation before cross-linking. Kinase inhibition prevented the stimulated increase in tyrosine phosphorylated proteins and recruitment of Lyn and Syk to APP as well as the increase in p38 MAP kinase activity (Fig. 8A). Surprisingly, ERK and JNK activation were unaffected by kinase inhibition, demonstrating a divergence in the signaling response downstream of APP (Fig. 8A). Similar to adhesion-mediated activation, tyrosine kinase inhibition also prevented the acquisition of a reactive phenotype represented by the lack of a stimulated increase in COX-2 protein levels (Fig. 8B).

**FIG. 7.** APP cross-linking stimulates increased COX-2 and IL-1β protein levels. THP-1 cells were unstimulated (control) or stimulated for indicated times (in hours) with anti-APP, 22C11 clone (1 μg/ml), or mouse IgG1 (negative control, 1 μg/ml). Cells were lysed with RIPA buffer, separated by 7% SDS-PAGE, and Western blotted with anti-COX-2 antibody, anti-IL-1β antibody, anti-inducible nitric oxide synthase (iNOS) antibody, anti-CD36 antibody, and anti-ERK2 antibody (loading control). Antibody binding was visualized by chemiluminescence.
Although the primary focus of this report is characterizing the contribution of APP to monocyte activation, we presumed that APP participates in specific microglial activation paradigms as well. For comparison, we demonstrate that cross-linking cell surface APP on microglial cells also stimulated a rapid increase in protein phosphotyrosine levels, activation of Lyn, activation of ERKs (Fig. 9, A and B), and increased COX-2 protein levels (Fig. 9C). Importantly, APP dimerization did not induce activation of JNKs or p38 MAPK as observed in monocytes (Fig. 9A). These data demonstrate that APP is capable of acting as an independent receptor responsible for tyrosine kinase-mediated proinflammatory activation of microglia. However, the specific activation response diverges from that observed in THP-1 monocytes, suggesting differences in the resultant phenotype.

**DISCUSSION**

These data define a novel role for APP in the proinflammatory activation of monocyctic lineage cells. We demonstrate that
β₁, integrin ligation stimulates increased tyrosine kinase activity, recruitment of tyrosine kinases to APP, and a resultant reactive phenotype, indicating that APP is an important signaling molecule in adhesion-mediated activation of THP-1 monocytes. We confirmed the role for APP in adhesion-mediated proinflammatory activation through the use of APP siRNA. Decreasing APP expression does not affect initial tyrosine phosphorylation of cytoplasmic proteins, consistent with previously demonstrated data in which integrin-mediated activation is sufficient to stimulate tyrosine kinase activity (24, 33, 38). However, downstream activation of p38 MAPK and subsequent increased COX-2 protein levels are dependent on APP expression. This suggests that APP does not have a role in regulating adhesion-mediated membrane proximal tyrosine kinase activity, but it does have a role in the subsequent signaling and proinflammatory phenotype. Presumably, recruitment of these initial activated tyrosine kinases to APP is critical for the propagation of the proinflammatory signaling response, therefore decreasing APP expression is sufficient to attenuate the proinflammatory signaling cascade.

Importantly, we also demonstrate that cell surface APP dimerization stimulates a collectively similar activation of monocyteic lineage cells. Previous work has identified a hydrophobic region on the ectodomain of cell surface APP that is proposed to play an important functional role, such as dimerization or ligand binding (3). Our data support the idea that there is a biological ligand for APP and indicate that receptor ligation stimulates a series of signaling events, ultimately leading to transcriptional changes and an inflammatory state in monocyteic lineage cells. Interestingly, the resultant phenotype differs slightly between APP dimerization and adhesion-mediated activation, which further supports the proposal that APP has a specific ligand and consequently an independent function as a cell surface receptor.

We report the non-receptor tyrosine kinases Lyn and Syk as novel proteins that interact with APP during activation of this putative receptor. It remains to be determined if the interaction between APP and these tyrosine kinases is a phosphorylation-independent event as described by the interaction between neuronal APP and the cytoplasmic proteins Fe65, X11, and murine DAB-1 (6, 8, 39). It may be more probable that the interaction between neuronal APP and the cytoplasmic proteins Fe65, X11, and DAB-1 (6, 8, 39). It may be more probable that the interaction between neuronal APP and these tyrosine kinases is a phosphorylation-independent event as described by the interaction between APP and these tyrosine kinases in brain and peripheral blood cells. Presumably, recruitment of these initial activated tyrosine kinases to APP is critical for the propagation of the proinflammatory signaling response, therefore decreasing APP expression is sufficient to attenuate the proinflammatory signaling cascade.

In conclusion, these data demonstrate a novel function for APP as a cell surface receptor that mediates proinflammatory activation of monocyteic lineage cells. Moreover, these data support the idea that there is a yet unidentified APP ligand and demonstrate the significance of this ligand in stimulating a tyrosine kinase-dependent signaling cascade in monocytes and microglia that incites the production of inflammatory mediators such as COX-2 and IL-1β. The physiological consequences of this inflammatory state in vivo have yet to be studied, although it is probable that in vivo the release of these inflammatory mediators may lead to activation of cells in the immediate environment, such as endothelial cells or astrocytes. Importantly, tyrosine kinase inhibition prevents the increased COX-2 protein levels and could possibly serve as a critical regulatory point in this inflammatory pathway.

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