CD36 Signals to the Actin Cytoskeleton and Regulates Microglial Migration via a p130Cas Complex

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The pattern recognition receptor CD36 initiates a signaling cascade that promotes microglial activation and recruitment to β-amyloid deposits in the brain. In the present study we identify the focal adhesion-associated proteins p130Cas, Pyk2, and paxillin as novel members of the tyrosine kinase signaling pathway downstream of CD36 and show that assembly of this complex is essential for microglial migration. In primary microglia and macrophages exposed to β-amyloid, the scaffolding protein p130Cas is rapidly tyrosine-phosphorylated and co-localizes with CD36 to membrane ruffles contemporaneous with F-actin polymerization. These β-amyloid-stimulated events are not detected in CD36 null cells and are dependent on CD36 activation of Src family tyrosine kinases. Fyn, a Src kinase known to interact with CD36, co-precipitates with p130Cas and is an essential upstream intermediate in the signaling pathways leading to phosphorylation of the p130Cas substrate domain. Furthermore, the p130Cas-interacting kinase Pyk2 and the cytoskeletal adapter protein paxillin also demonstrate CD36-dependent phosphorylation, identifying these focal adhesion molecules as additional members of this β-amyloid signaling cascade. Disruption of this p130Cas complex by small interfering RNA silencing inhibits p44/42 mitogen-activated protein kinase phosphorylation and microglial migration, illustrating the importance of this pathway in microglial activation and recruitment. Together, these data are the first to identify the signaling cascade that directly links CD36 to the actin cytoskeleton and, thus, implicates it in diverse processes such as cellular migration, adhesion, and phagocytosis.

In Alzheimer disease the extracellular deposition of fibrillar β-amyloid incites a focal recruitment of microglia that is a hallmark of senile plaques. Microglia are the primary immune effector cells of the central nervous system and, like other tissue macrophages, phagocytose exogenous or modified endogenous ligands and initiate an appropriate immune response. Activation of microglia by β-amyloid triggers the release of pro-inflammatory mediators, including cytokines, chemokines, and reactive oxygen species, which are believed to drive the chronic inflammation and neurotoxicity associated with Alzheimer disease (1). Microglia express multiple pattern recognition receptors that have been implicated in the innate immune response to β-amyloid, including three members of the scavenger receptor (SR) family, SR-A, SR-BI, and CD36 (2). Although all of these SR pathways are thought to contribute to β-amyloid clearance by microglia, CD36 has uniquely been linked to a pro-inflammatory signaling cascade that initiates microglial activation and recruitment (3–5).

CD36 is highly expressed in both neonatal and adult microglia (6) and has been shown to increase in the Alzheimer diseased brain (7). We and others have demonstrated that β-amyloid binding to CD36 on microglia, monocytes, and macrophages initiates signal transduction, cellular activation, and the elaboration of proinflammatory mediators (3–5, 8). Characterization of the tyrosine kinase signaling cascade downstream of CD36 has identified the Src family kinases Lyn and Fyn as key regulators of the mitogen-activated protein kinase (MAPK) pathways and cytokine, chemokine, and reactive oxygen species production (3–5). In vitro targeting of this signaling cascade by deletion of CD36 or Lyn dramatically reduces microglial accumulation at sites of β-amyloid deposition in the brain, highlighting the potential importance of this pathway in modulating microglial recruitment and activation in Alzheimer disease (3, 4).

Upon transfection with CD36, Bowes melanoma cells that do not normally express this scavenger receptor gain the ability to bind β-amyloid, suggesting that CD36 mediates cell surface recognition of this modified endogenous ligand (6). In addition, CD36 has been shown to bind other amyloid proteins, including fibrillar forms of apolipoprotein C-II and α1-antitrypsin (9–11). Recently, CD36 has been proposed to recognize β-amyloid as part of a receptor complex involving SR-A, β1 integrin, and the integrin-associated protein CD47 (8, 12). Although evidence of a direct interaction between these receptors is still...
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forthcoming, inhibitors of each individual receptor can abrogate the tyrosine kinase-based signaling response to β-amyloid. Cooperation of these receptors has been shown to facilitate microglial internalization of β-amyloid via a β1 integrin-specific mechanism that is distinct from those used by the classical phagocytic receptors FcγRI, FcγRIII, and the complement receptor (12).

Microglia are highly dynamic cells that undergo rapid cellular remodeling during membrane extension, migration, and phagocytosis. These processes are orchestrated by changes in the organization of the actin cytoskeleton and the assembly and disassembly of focal adhesions. Focal adhesions provide structural tethers linking the actin cytoskeleton to the extracellular matrix and also serve as a convergence point for signaling pathways regulating numerous cellular processes, including migration, proliferation, transformation, and apoptosis. The scaffolding protein p130Cas resides in focal adhesions and, through its multiple interaction domains, provides a platform for the interface of kinases and phosphatases regulating focal adhesion complex formation (13). Tyrosine phosphorylation of the p130Cas substrate domain facilitates interactions with SH2 domain containing proteins such as Crk and focal adhesion kinase, whereas its SH3 domain interacts with proline-rich tyrosine kinase 2 (Pyk2, also known as RAFTK and CAKβ) and the tyrosine phosphatases PTP1B and PTP-PEST (13). The coordinated assembly of these multiprotein p130Cas complexes serves as a “molecular switch,” inducing sequential kinase phosphorylation, rearrangement of the actin cytoskeleton, and induction of cell migration and phagocytosis (13).

We identify herein a focal adhesion signaling cascade engaged by CD36 that through phosphorylation of p130Cas links this receptor to the remodeling of the actin cytoskeleton after exposure to β-amyloid. In macrophages and microglia, β-amyloid rapidly induces tyrosine phosphorylation of p130Cas and its redistribution to membrane ruffles and podosomal rings concurrent with actin polymerization. p130Cas colocalizes with CD36 in β-amyloid-stimulated cells, and targeted deletion of this receptor abrogates p130Cas phosphorylation and mobilization. Additional members of this p130Cas complex activated by β-amyloid include the Src kinase Fyn, the kinase Pyk2, and the cytoskeletal scaffold paxillin. Targeted inhibition of this complex using p130Cas-directed small inhibitors abrogates Fyn and Lyn tyrosine kinase phosphorylation, rearrangement of the actin cytoskeleton, and induction of cell migration and phagocytosis (13).

Experimental Procedures

β-Amyloid—Ab₁₋₄₂ and reverse Ab₃₂₋₁ (revAbβ) peptides were obtained from American Peptide Co. (Sunnyvale, CA). To induce fibril formation, Ab₁₋₄₂ was resuspended in H₂O at 1 mg/ml and incubated for 1 week at 37 °C, and reverse Ab₃₂₋₁ peptide was similarly treated as we described (3, 4). For induction of signaling, peptides were used at 40 μM as we described (3).

Mice—The Cd36⁻/⁻ (Cd36tm1MGH) mice were generated in our laboratory as described and were backcrossed to C57BL/6 mice for eight generations (3). Age-matched Cd36⁺/⁺ mice generated from crosses of Cd36⁺/⁻ mice were used as controls. Fyn⁻/⁻ and wild type littermate control mice were generated from intercrosses of Fyn⁺/⁺ mice obtained from Jackson Laboratories. All mice were maintained in a pathogen-free facility with free access to rodent chow and water. Experimental procedures were approved by the Massachusetts General Hospital Subcommittee on Research Animal Care and were conducted in accordance with the United States Department of Agriculture Animal Welfare Act and the Public Health Service Policy for the Humane Care and Use of Laboratory Animals.

Microglial and Macrophage Cell Culture—Primary microglia were prepared from brain extracts of 2-day-old mice as we described (3, 6). The murine microglial cell line N9 and the human monocytic leukemia cell line THP-1 were cultured as previously described in RPMI 1640 medium (Invitrogen) supplemented with 10% fetal bovine serum, penicillin (100 units/ml), and streptomycin (100 μg/ml) (6). Peritoneal macrophages were collected from mice by peritoneal lavage 4 days after intraperitoneal injection with 3% thioglycollate as we described (9, 14). For signaling experiments, 8 × 10⁶ cells were incubated overnight at 37 °C in the appropriate medium with 0.5–2% fetal bovine serum before use.

Immunoprecipitation—Cells were washed in ice-cold PBS and lysed in radioimmunoprecipitation buffer containing protease and phosphatase inhibitors as we previously described (3, 9). For immunoprecipitation assays, 1 mg of protein lysate (in a volume of 1 ml) was incubated with 0.25 μg of chromatographically purified rabbit IgG (Zymed Laboratories Inc., 02-6102), and complexes were precipitated with protein A-Sepharose (Amersham Biosciences) for 30 min at 4 °C. Supernatants were washed 4× with 1 ml of SDS lysis buffer. The precipitated proteins were washed 3× in radioimmunoprecipitation buffer and resuspended in 40 μl of SDS lysis buffer. The samples were run on 8% denaturing SDS-polyacrylamide gels and analyzed by Western blotting for phosphotyrosine, p130Cas, and Fyn.

Western Blot Analysis—Cells were washed in ice-cold PBS and lysed in radioimmunoprecipitation buffer containing protease and phosphatase inhibitors as we previously described (3, 9). 40–60 μg of protein were run on 10% denaturing SDS-polyacrylamide gels, and proteins were transferred to a nitrocellulose membrane. Membranes probed with 4G10 anti-phosphotyrosine (Tyr(P)) mouse monoclonal antibody (Upstate Biotechnology, Inc., 05-321), rabbit anti-p130Cas IgG (Cascade Biosciences, 06-500), and Pyk2 rabbit polyclonal IgG (Upstate Biotechnology, 06-559) were blocked for 1 h at room temperature in 3% nonfat dry milk in PBS. All other blots were blocked in 5% nonfat dry milk in Tris-buffered saline with 0.1% Tween 20 (TBS-T) for 2 h at room temperature. Blots were incubated overnight at 4 °C with primary antibodies (1:1000), including p130Cas phospho-Tyr-165, phospho-paxillin Tyr-118 rabbit polyclonal IgG (2541), phospho-p44/42, and p44/42 (all from Cell Signaling Technology), Fyn-binding protein (Fyb) mouse IgG (BD Transduction Laboratories, 610945), Fyn and Lyn rabbit polyclonal IgGs (Santa Cruz, sc-16 and sc-15), phospho-
**Results**

**β-Amyloid Activates a Phosphotyrosine Signaling Cascade That Includes the Scaffolding Protein p130Cas**—Recently we showed that β-amyloid binding to CD36 initiates a tyrosine phosphorylation cascade that involves the scaffolding protein p130Cas. Immunoblotting and immunofluorescence studies demonstrated that CD36 activates p130Cas phosphorylation in macrophages and microglia. Western blotting of cellular protein lysates from wild type and Cd36-/- cells revealed that p130Cas is phosphorylated in Cd36+/+ but not in Cd36-/- macrophages. Immunofluorescence staining of Cd36+/+ macrophages with an antibody that detects tyrosine phosphorylation of p130Cas, followed by Alexa488-conjugated secondary antibody, revealed that p130Cas is phosphorylated at tyrosine residues in Cd36+/+ macrophages. These findings indicate that CD36 activation leads to the recruitment of p130Cas to the cell membrane, where it is phosphorylated on tyrosine residues.

**Chemotaxis Assays**—Chemotaxis of N9 microglia was measured by transfilter migration assay using 96-well modified Boyden chambers with a 5-μm pore size (NeuroProbe) as described (17). Untreated, control, or p130Cas siRNA-treated microglia were resuspended in RPMI 1640 medium supplemented with 0.1% bovine serum albumin, and chemotaxis was measured on triplicate samples in the absence and presence of 10 nM recombinant human monocyte chemotactic protein (MCP-1; R&D Systems, Inc., Minneapolis, MN, 279-MC), 10 nM recombinant human leukotriene B4 (LTB4), or culture supernatants from microglia stimulated with 10 μg/ml Aβ1-42 for 24 h. Chemotactic agents or cell supernatants (31 μl) were placed in the lower wells of the chemotaxis chamber, and 5 × 10⁵ cells in 50 μl were placed in the upper compartment. Cells were allowed to migrate for 1.5 h, and the number of cells migrating into the lower chamber was counted using an inverted light microscope equipped with a grid. Migration was expressed as chemotactic index per high-power field, which was calculated by dividing the number of migrating cells in the treated groups by the number of migrating cells in the lowest control well.

**Discussion**

Our findings suggest that CD36 activation leads to the recruitment of p130Cas to the cell membrane, where it is phosphorylated on tyrosine residues. This phosphorylation cascade may contribute to the recruitment of additional signaling proteins, leading to the enhanced chemotaxis of N9 microglia.

**Experimental Details**

Pyk2 Tyr-580 rabbit polyclonal IgG (BIOSOURCE, 44-634Z), and rabbit polyclonal anti-mouse CD36 antiserum (3). Blots were washed 3× in TBS-T and incubated with species-appropriate horseradish peroxidase-conjugated secondary antibody (1:10,000 dilution) for 1 h at room temperature. Blots were washed an additional 3× and exposed to ECL reagent (Amersham Biosciences, RPN3114K) or using the Alpha Innotech Fluorchem 8800 image analysis system.

**Immunohistochemistry**—Cells were grown overnight on coverslips or in chamber slides and stained with vehicle, Aβ1-42 or revAβ for the indicated times. For phospho-p130Cas staining, cells were fixed in 4% paraformaldehyde and incubated with anti-phospho-p130Cas antibody (Cell Signaling 4015) overnight as described (15, 16). Cells were washed 3× in PBS, 1% bovine serum albumin, and incubated with anti-rabbit fluorescein isothiocyanate-labeled IgG secondary antibody. For p130Cas staining, cells were fixed in methanol and incubated with mouse anti-p130Cas antibody (BD Biosciences, 1:50), washed 3× in PBS, 1% bovine serum albumin, and incubated with goat anti-mouse IgG-Alexa594 or Alexa488 conjugate alone or with hamster anti-mouse CD36 IgG Alexa488 (BioLegend) or phalloidin Alexa594 (Molecular Probes). Cells were washed again and mounted using Vectashield fluorescence mounting medium (Vector Laboratories). Immunofluorescent staining was visualized using an Axioskop 2 MOT microscope (Zeiss).

**siRNA Transfection**—To inhibit the expression of p130Cas in N9 microglia, the cells were transiently transfected with murine Cas SMARTpool siRNAs or non-targeting control SiRNAs (Dharmacon Research, Lafayette, CO) using Lipofectamine Plus as previously described (17). Three hours after transfection cells were washed and incubated in RPMI 1640 medium (Invitrogen) supplemented with 10% fetal bovine serum, penicillin (100 units/ml), and streptomycin (100 μg/ml) for 48 h before use. p130Cas silencing was confirmed by Western blotting.

**Chemotaxis Assays**—Chemotaxis of N9 microglia was measured by transfilter migration assay using 96-well modified Boyden chambers with a 5-μm pore size (NeuroProbe) as we described (18). Untreated, control, or p130Cas siRNA-treated microglia were resuspended in RPMI 1640 medium supplemented with 0.1% bovine serum albumin, and chemotaxis was measured on triplicate samples in the absence and presence of 10 nM recombinant human monocyte chemotactic protein (MCP-1; R&D Systems, Inc., Minneapolis, MN, 279-MC), 10 nM recombinant human leukotriene B4 (LTB4), or culture supernatants from microglia stimulated with 10 μg/ml Aβ1-42 for 24 h. Chemotactic agents or cell supernatants (31 μl) were placed in the lower wells of the chemotaxis chamber, and 5 × 10⁵ cells in 50 μl were placed in the upper compartment. Cells were allowed to migrate for 1.5 h, and the number of cells migrating into the lower chamber was counted using an inverted light microscope equipped with a grid. Migration was expressed as chemotactic index per high-power field, which was calculated by dividing the number of migrating cells in the treated groups by the number of migrating cells in the lowest control well.

**Results**

**β-Amyloid Activates a Phosphotyrosine Signaling Cascade That Includes the Scaffolding Protein p130Cas**—Recently we showed that β-amyloid binding to CD36 initiates a tyrosine phosphorylation cascade in macrophages that involves the scaffolding protein p130Cas. Immunoblotting and immunofluorescence studies demonstrated that CD36 activation leads to the recruitment of p130Cas to the cell membrane, where it is phosphorylated on tyrosine residues. This phosphorylation cascade may contribute to the enhanced chemotaxis of N9 microglia.

**Discussion**

Our findings suggest that CD36 activation leads to the recruitment of p130Cas to the cell membrane, where it is phosphorylated on tyrosine residues. This phosphorylation cascade may contribute to the enhanced chemotaxis of N9 microglia.
kinase-based signaling cascade in microglia and macrophages, including the phosphorylation of four unknown proteins of ~130, 109, 87, 70 kDa in size (3). As observed in microglia, THP-1 monocytes stimulated with Aβ_{1-42} but not the reverse peptide Aβ_{42-1}, demonstrate a rapid increase in tyrosine phosphorylation of a 130-kDa protein (Fig. 1A). Levels of this phosphoprotein are detectable within 2 min and peak at ~10 min post Aβ_{1-42} stimulation. To begin to identify this protein, Western blots were stripped and reprobed with antibodies directed against 3 candidate signaling molecules of 125–130 kDa: p130Cas, the Fyn-binding protein Fyb, and focal adhesion kinase. Of these proteins, only p130Cas overlaid with the prominent 130-kDa protein that was tyrosine-phosphorylated in β-amyloid-stimulated THP-1 cells (data not shown).

**CD36 Initiates Phosphorylation of the Tyr-X-X-Pro Motifs in the p130Cas Substrate Domain**—Using a phospho-p130Cas specific antibody, we demonstrate that Aβ_{1-42} induced a rapid phosphorylation of Tyr-165 in the central substrate domain of p130Cas that was greatly reduced in cells similarly treated with revAβ peptide (Fig. 1B). This phosphorylation of the p130Cas substrate domain required cellular expression of CD36, as Tyr-165 phosphorylation was no longer detectable in macrophages and microglia derived from mice with a targeted deletion of this receptor (Fig. 1, C and D). As in THP-1 monocytes, Aβ_{1-42} induced the accumulation of phospho-p130Cas in wild type murine macrophages and microglia within 5 min post-stimulation. By contrast, no significant increase in accumulation of phospho-p130Cas was detectable in similarly treated CD36^{-/-} cells by Western analysis. Characterization of the intracellular localization of phospho-p130Cas by immunohistochemical staining showed that Aβ_{1-42} induced the accumulation of phospho-p130Cas in a punctate pattern characteristic of focal adhesions (Fig. 2). Phospho-p130Cas was not detected in unstimulated macrophages and was greatly reduced in Aβ_{1-42} treated cells lacking CD36 (Fig. 2). Together, these data demonstrate a requisite and conserved role for CD36 in the β-amyloid signaling pathway leading to phosphorylation of the adapter protein p130Cas in microglia, monocytes, and macrophages.

**The CD36-interacting Kinase Fyn Regulates p130Cas Phosphorylation**—In myeloid cells CD36 association with Src protein-tyrosine kinases regulates downstream signaling events including MAPK activation and reactive oxygen species production (3, 19–21). To determine whether Src kinases phosphorylate the Tyr-X-X-Pro motifs in the p130Cas substrate domain, we treated THP-1 monocytes with a broad inhibitor of this kinase family, PP1. Inhibition of Src protein-
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**FIGURE 4. β-Amyloid activates CD36-dependent phosphorylation of Pyk2 and paxillin.** A–B, Pyk2 kinase and the cytoskeletal protein paxillin are specifically phosphorylated in Aβ1–42-treated cells. Cellular protein lysates from THP-1 monocytes stimulated for the indicated times with Aβ1–42 or the revAβ peptide were immunoblotted with antibodies directed against phospho-Pyk2 (A) or phospho-paxillin (B) (upper panels). Western blots were stripped and reprobed to detect total Pyk2 or Paxillin protein (lower panels). C–D, β-amyloid binding to CD36 initiates Pyk2 and paxillin phosphorylation. Western blotting of cellular protein lysates from wild type and CD36−/− macrophages stimulated for the indicated times with Aβ1–42 demonstrates that CD36 is essential for phosphorylation of Pyk2 kinase (C) and paxillin (D). A–D, the ratio of phosphoprotein to total protein is presented graphically for each Western blot and is representative of three-four experiments.

Tyrosine kinase activity abrogated phosphorylation of p130Cas Tyr-165 in Aβ1–42-stimulated monocytes, identifying members of this kinase family as critical intermediates in this CD36 signaling cascade (Fig. 3A). Immunoprecipitation of p130Cas revealed a constitutive association of this scaffolding protein with Fyn, a Src kinase known to associate with CD36, that was not enhanced by Aβ1–42 treatment (Fig. 3B). Protein lysates from untreated or Aβ1–42-stimulated THP-1 monocytes were immunoprecipitated with anti-p130Cas or control IgG, and the immune complexes were run on SDS-polyacrylamide gels. Immunoblotting with antibodies directed against Fyn and Lyn revealed that p130Cas co-precipitated with Fyn in both untreated and Aβ1–42-stimulated monocytes (Fig. 3B). However, p130Cas protein immunoprecipitated from Aβ1–42-treated monocytes demonstrated a 2-fold increase in tyrosine phosphorylation as measured by its immunoreactivity with the 4G10 anti-Tyr(P) antibody (Fig. 3B). No association of p130Cas with Lyn kinase was detected in untreated or Aβ1–42-stimulated monocytes (data not shown). To determine whether the observed association of p130Cas with Lyn kinase is essential for greatly reduced or absent in cells treated with revAβ1–42 peptide. Because Pyk2 and paxillin are approximately the size of two of the additional unknown phosphoproteins in the CD36 signaling cascade (116 and 68 kDa, respectively), we investigated whether phosphorylation of these focal adhesion molecules was CD36-dependent. β-Amyloid increased phosphorylation of Pyk2 and phospho-paxillin accumulation in CD36-expressing macrophages (wild type); however, this response was greatly reduced in similarly treated Cd36−/− macrophages (Fig. 4, C and D). These data position the p130Cas-interacting kinase Pyk2 and the cytoskeletal scaffold paxillin in the β-amyloid signaling cascade downstream of CD36.

**β-Amyloid Activates p130Cas Localization to Membrane Ruffles and F-actin Polymerization**—The assembly and membrane localization of p130Cas complexes has been shown to lead to cytoskeletal rearrangements and activation of the GTPases Rac and Rho (13). We characterized the spatiotemporal localization of p130Cas in wild type and Cd36−/− macrophages exposed to Aβ1–42 by immunohistochemistry. In unstimulated macrophages, p130Cas staining was present dif-
Fusely in the cytosol of the cell. However, upon exposure of wild type macrophages to Aβ1-42, p130Cas was mobilized to membrane ruffles and the leading edge of the cell, where it colocalized with CD36 (Fig. 5). This redistribution of p130Cas was dependent on CD36 signaling, as p130Cas translocation to membrane ruffles was not detected in similarly treated Cd36−/− macrophages (Fig. 5). Studies in N9 microglia demonstrated that this rapid polarization of p130Cas staining coincides with actin polymerization (Fig. 6A). By 15 min post Aβ1-42 stimulation, 90% of microglia demonstrated reorganization of both p130Cas and F-actin (Fig. 6B). As has been described in Src-transformed cells (16), we found that β-amyloid induced the polarized organization of p130Cas into peripheral belt structures and internal rings characteristic of the formation of podosomes in microglia and macrophages (Fig. 6B and C). These ring and belt structures were found in 55% of cells.
stimulated with β-amyloid but less than 10% of unstimulated cells. Reorganization of p130Cas into these structures occurred in parallel with F-actin polymerization as determined by phalloidin staining (Fig. 6B). These p130Cas structures have previously been associated with increased p130Cas phosphorylation, migration, and invasiveness (16, 22).

Gene Silencing of p130Cas Inhibits Microglial Migration and MAPK Activation—p130Cas deficiency results in embryonic lethality in mice, thus precluding traditional loss-of-function studies of p130Cas null microglia and macrophages (23). Therefore, to understand the role of CD36 assembly of p130Cas complexes, we performed siRNA-mediated knockdown of p130Cas in microglia to study its function in focal adhesion-dependent processes. N9 microglia treated with p130Cas siRNA show a specific decrease in p130Cas protein by Western analysis as compared with untreated or control siRNA-treated cells (Fig. 7A). This targeted deletion of p130Cas was specific and did not alter microglial expression of CD36 (Fig. 7A). To determine whether assembly of p130Cas complexes affects microglial motility, migration assays were performed using Boyden chambers. In several independent experiments, silencing of p130Cas expression blocked microglial migration. Untreated or control siRNA-treated microglia readily migrated in Boyden chamber assays to MCP-1, LTB4, or supernatants from microglia exposed to Aβ1–42, which we have shown are chemotactic (4). Exposure of these cells to MCP-1 or LTB4 led to a 2-fold enhancement of the migration capacity, whereas Aβ1–42-stimulated cell supernatants elicited a more potent 4-fold increase in microglial migration (Fig. 7B). By contrast, no increase in chemotactic index was observed in p130Cas siRNA-treated microglia exposed to MCP-1, LTB4, or Aβ1–42-stimulated cell supernatants over media alone. The failure of p130Cas siRNA-treated microglia to respond to these chemotactic stimuli illustrate the critical role for this scaffolding protein in the signaling pathways regulating cellular motility.

p130Cas has also been shown to be upstream of the small GTPases Ras and Rac, which can activate MAPK signaling via c-Jun N-terminal kinases and extracellular signal-related kinase p44/42 (13). Because p44/42 is known to be a primary effector of β-amyloid-CD36 signaling, we investigated whether p130Cas was required for activation of this MAPK. β-Amyloid-induced phosphorylation of p44/42 was greatly reduced in p130Cas siRNA-treated microglia (Fig. 7C). Untreated and control siRNA-treated microglia show an accumulation of phosphorylated p44/42 at 5 and 15 min post Aβ1–42 stimulation. This response was blunted in p130Cas siRNA-treated microglia, suggesting that p130Cas is a key intermediate in the Aβ1–42-induced CD36 signaling cascade leading to p44/42 MAPK activation.

**DISCUSSION**

The recruitment of microglia to β-amyloid deposits and their accumulation in senile plaques are believed to fuel a chronic inflammatory response that promotes neurodegeneration in Alzheimer disease (1). The molecular mechanisms regulating microglial migration and activation in this disease are incompletely understood. Like other tissue macrophages, microglia are equipped with pattern recognition receptors that facilitate innate immune recognition and phagocytosis of foreign or harmful ligands, including modified host proteins. Work from our group and others has shown that the scavenger
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rearrangements leading to cellular polarization in response to β-amyloid and microglial migration.

The initiation of phagocytosis, adhesion, and cell migration are complex events that require morphological polarization of the cell, coordinated by assembly and disassembly of focal adhesions and reorganization of the actin cytoskeleton. The assembly of p130Cas complexes serves a convergence point for signaling networks regulating these events. p130Cas is a versatile scaffolding protein that orchestrates the formation of multiprotein complexes that regulate kinase phosphorylation and dephosphorylation, intracellular localization, and focal adhesion assembly (13). For these reasons, p130Cas is a major regulator of the actin cytoskeleton and plays critical roles in processes as diverse as migration, adhesion, phagocytosis, and tumor invasion. We now demonstrate that p130Cas, activated downstream of CD36, is also important in regulating microglial responses to β-amyloid, including cell migration (summarized in Fig. 8). The involvement of p130Cas in cell migration depends on the tyrosine phosphorylation of its substrate domain and the assembly of p130Cas scaffolds at adhesion sites that drive localized Rac activation, actin polymerization, and the recruitment of integrin receptors necessary for lamellipodia extension (24–26). We find that upon stimulation with β-amyloid, p130Cas becomes redistributed to membrane ruffles where it colocalizes with CD36 concurrent with the polymerization of F-actin. In addition, in β-amyloid-treated microglia, p130Cas organizes into ring- and belt-like structures, characteristic of p130Cas hyperphosphorylation seen in v-Src-transformed cells (16, 22). Consistent with these observations, β-amyloid rapidly activates phosphorylation of the Tyr-X-X-Pro motifs in the p130Cas substrate domain and induces the cellular accumulation of this phosphoprotein in a pattern characteristic of focal adhesions. These signaling events are dependent upon CD36, as targeted deletion of this receptor abrogates phosphorylation of p130Cas and polymerization of F-actin. In microglia, the silencing of p130Cas by siRNA blocks these cytoskeletal rearrangements, abrogating microglial migration. Together, these data implicate CD36-mediated assembly of p130Cas complexes in the transfer of signals from the cell surface to the cytoskeleton.

p130Cas was originally isolated as a major phosphotyrosine protein associated with v-Src in transformed cells, where hyperphosphorylation of p130Cas promotes tumor metastasis (16, 22, 27). Members of the Src family of phosphotyrosine kinases have previously been shown to initiate signaling downstream of CD36 and are instrumental in the activation of MAP kinases and the production of inflammatory mediators (3, 5, 20,
21, 28). Therefore, to understand how CD36 signals to regulate the assembly and activation of p130Cas complexes in response to β-amyloid, we investigated the contribution of Src kinases. We find that in myeloid cells, p130Cas is associated with the Src kinase Fyn that is recruited to CD36 upon ligand binding (20). Moreover, CD36-mediated phosphorylation of p130Cas is abrogated by inhibitors of Src kinase activity or by targeted deletion of Fyn. Together, these data directly implicate CD36 activation of Fyn upstream of the phosphorylation of the p130Cas substrate domain.

For other systems the migratory and cytoskeletal changes downstream of p130Cas are well defined. In cooperation with paxillin, phosphorylated p130Cas activates the Rac1 exchange factor complex Dock180/ELMO that induces membrane ruffling and lamellipodia formation (29, 30). These events are necessary for diverse processes such as phagocytosis, cell adhesion, and migration. In support of a role for this pathway in the cellular response to β-amyloid, a Vav-Rac1-dependent signaling pathway, activated in part via CD36, was recently shown to contribute to reactive oxygen species production and enhanced phagocytosis in monocytes and microglia exposed to β-amyloid (8). Consistent with a previous report, we observed that β-amyloid rapidly activates phosphorylation of the protein-tyrosine kinase Pyk2 and its substrate paxillin (31). These Cas-binding proteins act upstream of Rac1 and play essential roles in the regulation of morphology and migration by macrophages as well as other cell types (32). Importantly, phosphorylation of these proteins is not seen in the absence of CD36, confirming that CD36 is an essential receptor for this response to β-amyloid. Furthermore, targeted silencing of Cas, the scaffolding protein to which Pyk2 and paxillin bind, blocks microglial migration, supporting an essential role for the formation of this complex in effecting changes in the organization of the actin cytoskeleton. These data are the first to definitively place these focal adhesions molecules and cytoskeletal regulators downstream of CD36. However, whether CD36 acts alone or as part of a receptor complex, potentially including β1-integrin, SR-A, or CD47, molecules also implicated in β-amyloid recognition (12), remains to be defined.

Although our work has focused on β-amyloid as a ligand, the ability of CD36 signaling to directly regulate p130Cas and the cytoskeleton has far-reaching implications. As an example, tyrosine phosphorylation of the p130Cas substrate binding domain has previously been shown to play a role in the induction of migration and invasiveness of tumor cells, which demonstrate cellular changes similar to those we observed in microglia and macrophages exposed to β-amyloid; that is, increased phosphorylation of p130Cas, paxillin, and the Pyk2-related focal adhesion kinase as well as the formation of actin-rich podosomal aggregates appearing as ring and belt structures (16, 22). These data raise the intriguing possibility that CD36 signaling in transformed cells, potentially triggered by endogenous matrix components or other known CD36 ligands such as thrombospondin-1, may play a role not only in microglial activation but also contribute to tumor cell invasion.

Another potential role for CD36-mediated assembly of p130Cas complexes is in the regulation of microglial and macrophage inflammatory responses. Activation of CD36 signaling has previously been linked to the production of proinflammatory mediators in response to β-amyloid, including cytokines and reactive oxygen species, in part via activation of MAPK signaling (3–5). p130Cas, acting upstream of the small GTPases Ras and Rac, can activate MAPK signaling via c-Jun N-terminal kinases 1/2 and p44/42 (13). Consistent with a role for p130Cas in regulating inflammation, we find that β-amyloid activation of p44/42 MAPK is reduced in p130Cas-depleted microglia. CD36 activation of MAP kinase signaling has also been implicated in 1) the inhibition of angiogenesis by thrombospondin-1, 2) the regulation of the immune response to Plasmodium falciparum-infected erythrocytes, and 3) the response to oxidized low density lipoprotein, an inflammatory ligand believed to promote cellular cholesterol accumulation and atherosclerosis (20, 21, 28, 33). Subtle differences in the CD36-signaling cascades activated by these various ligands are apparent, including the involvement of different members of the Src and MAP kinase family, and these distinctions are likely determined by cell type and/or ligand. The role of p130Cas in MAPK activation in these CD36 signaling pathways is not known.

Recently, we and others have also demonstrated that CD36 cooperates with the Toll-like receptors (TLR) to direct the signaling response to the Gram-positive bacterium Staphylococcus aureus (34, 35). This family of mammalian pattern recognition receptors has evolved to identify highly conserved pathogen-associated molecular patterns, including lipopolysaccharide, peptidoglycan, lipoteichoic acid, CpG-DNA, and double-stranded RNA (36). We showed that CD36, through its C-terminal cytoplasmic domain, activates bacterial phagocytosis and initiates TLR2/TLR6 signaling leading to the production of cytokines essential for host defense against S. aureus (34). It will be of interest to determine whether components of the CD36 signaling pathway described in this work, such as p130Cas, paxillin, and Pyk2, are also required for CD36-mediated augmentation of TLR signaling and whether CD36 might engage similar TLR pathways to respond to modified endogenous ligands such as β-amyloid and oxidized low density lipoprotein. Taken together, these studies emphasize the continued importance of dissecting the molecular mechanisms of CD36 signaling as it may contribute to a number of pathological processes.

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