Published in final edited form as:

Glia. 2012 September ; 60(9): 1366–1377. doi:10.1002/glia.22355.

β -Arrestin 2-dependent activation of ERK1/2 is required for ADP-induced paxillin phosphorylation at Ser⁸³ and microglia chemotaxis

Sang-Hyun Lee¹, Ryan Hollingsworth¹, Hyeok-Yil Kwon², Narae Lee¹, and Chang Y. Chung^{1,*}

¹Department of Pharmacology, Vanderbilt University Medical Center, Nashville, TN 37232-6600

²Department of Physiology, Hallym University, College of Medicine, Chuncheon, South Korea

Abstract

Microglia play crucial roles in increased inflammation in the CNS upon brain injuries and diseases. Extracellular ADP has been reported to induce microglia chemotaxis and membrane ruffle formation through P2Y₁₂ receptor. In this study, we examined the role of ERK1/2 activation in ADP-induced microglia chemotaxis. ADP stimulation increases the phosphorylation of ERK1/2 and paxillin phosphorylation at Tyr³¹ and Ser⁸³. Inhibition of ERK1/2 significantly inhibited paxillin phosphorylation at Ser⁸³ and the retraction of membrane ruffles, causing inefficient chemotaxis. Close examination of dynamics of focal adhesion formation with GFP-paxillin revealed that the disassembly of focal adhesions in U0126-treated cells was significantly impaired. Depletion of β -Arr2 with shRNA markedly reduced the phosphorylation of ERK1/2 and Pax/Ser⁸³, indicating that β -Arr2 is required for ERK1/2 activation upon ADP stimulation. A large fraction of phosphorylated ERK1/2 and β -Arr2 were translocated and co-localized at focal contacts in the newly forming lamellipodia. Examination of kinetics and rate constant of paxillin formation and disassembly revealed that the phosphorylation of paxillin at Tyr³¹ by c-Src appears to be involved in adhesion formation upon ADP stimulation while Ser⁸³ required for adhesion disassembly.

Keywords

ERK1/2; paxillin phosphorylation; focal adhesion; chemotaxis; microglia

Introduction

Microglia are the immune effector cells that are rapidly activated in response to even minor pathological changes in the central nervous system (CNS) (Gonzalez-Scarano and Baltuch 1999). They play a crucial role in recognition and phagocytic removal of degenerating neurons (Hickey 2001; Streit 2002). Under normal conditions, microglia exist as nonmigratory ramified cells. The ramified morphology of resting microglia is rapidly

*Address correspondence to: Chang Y. Chung, 468 Robinson Research Building (MRB I), 1215 21st Ave. South @ Pierce, Nashville, TN 37232-6600; Phone: 615-322-4956; Fax: 615-343-6532; chang.chung@vanderbilt.edu.

transformed into a motile ameboid form after pathological stimuli and migrates toward lesion sites, where they become activated and exert their neuroprotective effects such as secreting a variety of substances and clearance of cell debris (Hanisch 2002; Kreutzberg 1996; Stence et al. 2001; Thomas 1999). Microglia may play a dual role, amplifying the effects of inflammation and mediating cellular degeneration as well as protecting the CNS (Gonzalez-Scarano and Baltuch 1999). Microglia chemotaxis might be important for either neuroprotective or inflammatory role. Previous studies showed that extracellular ATP or ADP induces chemotaxis of microglia via the Gi/o-coupled P2Y₁₂ receptor (P2Y₁₂R) (Honda et al. 2001; Inoue 2002; Ohsawa et al. 2007). It has been shown that activation of both PI3K and PLC signaling pathways downstream of P2Y₁₂R is required for the ATP-induced process extension by microglia (Ohsawa et al.). ADP also increases the number of focal adhesions, which plays an important role in lamellipodia protrusion and microglia chemotaxis (Lee and Chung 2009).

Elevation of ERK1/2 activity in activated microglia has been reported and ERK1/2 plays an important role in increasing expression of inducible nitric oxide synthase, cyclooxygenase-2, and proinflammatory cytokines (Choi et al. 2003). ERK1/2 activation via P2Y₁₂R upon ADP stimulation in glioma cells has also been reported (Shankar et al. 2006). However, molecular details of signaling events leading to ERK1/2 activation upon ADP stimulation and regulatory roles of ERK1/2 in the formation/disassembly of focal adhesions in microglia remain unknown.

Paxillin is a major component of focal complex (FX) and focal adhesion (FA) (Schaller 2001; Zimerman et al. 2004). FXs are transient adhesions that were disassembled and reassembled at the leading edge of migrating cells and they are involved in protrusion formation. FXs mature into FAs as a result of Rho GTPase activity, actin crosslinking by α -actinin, and tension-dependent actin-myosin contractility (Zaidel-Bar et al. 2003). FAs are involved in the contractile actomyosin system to pull the cell body and restrain the migration process. Paxillin is a key scaffolding protein bringing together signaling molecules, structural components, and regulatory proteins that play important roles in the regulation of focal adhesions and actin cytoskeleton (Deakin and Turner 2008).

Multiple sites of phosphorylation on paxillin by a variety of kinases have been reported (Webb et al. 2005). Phosphorylation of paxillin at serine/tyrosine through focal adhesion kinase (FAK) activation was reported to be involved in controlling of focal adhesion turnover in the fibroblasts and epithelial cells (Ilic et al. 1995; Ishibe et al. 2004). Paxillin is phosphorylated by FAK-Src on Tyr³¹ and Tyr¹¹⁸ (Mitra et al. 2005). Phosphorylation of paxillin at serines 126 and 130 leads to the relocation of paxillin from focal adhesions to the cytosol (Cai et al. 2006; Woodrow et al. 2003). Formation of a complex that contains active paxillin, FAK, and active ERK has been reported and a possibility that paxillin may be a direct substrate of ERK has been suggested (Monami et al. 2006). Previous reports have shown that ERK1/2 directly binds to paxillin and also induces the phosphorylation of paxillin in the epithelial cells and *in vitro* assay (Ishibe et al. 2003; Liu et al. 2002).

To analyze the molecular mechanisms underlying changes in focal adhesions in microglia upon ADP stimulation, in this study, we examined the role of ERK1/2 in the regulation of

paxillin phosphorylation and focal adhesions by performing time-lapse confocal imaging of microglia expressing EGFP-paxillin and kymographic analysis of membrane ruffles. The results demonstrate that P2Y₁₂R induces the activation of ERK1/2, which is dependent upon β -arrestin 2. Activation of ERK1/2 leads to an increase in the phosphorylation of paxillin at Ser83 that is required for adhesion disassembly. Inhibition of ERK1/2 results in decreased focal adhesion turnover, which impairs the retraction of lamellipodia and chemotaxis of microglia.

Materials and Methods

Cell culture and Transfection

BV2 microglia cells were maintained in Dulbecco's modified Eagle's medium (DMEM) MEM supplemented with 10% FBS and penicillin-streptomycin (Gibco BRL, Grand Island, NY). MISSION shRNA clones from Sigma (NM_133915.1-1455s1c1 for paxillin; NM_007783.2-1317s1c1 for c-Src; NM_145429.1-529s1c1 for β -arrestin2) containing hairpin sequences were used for knockdown. Cells were transfected with Lipofectamin 2000 (Invitrogen) according to the manufacturer's instructions, with paxillin-GFP, S83A-paxillin-GFP, Y31F-paxillin-GFP, FLAG- β -Arresin 1, and 2, and cultured for 12 h. All experiments were performed 32 h after transfection. Pharmacological inhibitors, LY294002 (20 μ M; Promega, Madison, WI); PP2 (10 μ M; Alexis, San Diego, CA); H-89 (30 μ M; Sigma, St. Louis, MO); 2MeSAMP (50 μ M; Sigma), were added to serum-free DMEM.

Chemotaxis Assay

Transwell chemotaxis assays were performed as previously described (Lee and Chung 2009; O'Connor et al. 1998). Briefly, Transwell chamber membranes (6.5-mm diameter, 8 mM pore size; Corning, Corning, NY) were coated with 3 μ g/ml of fibronectin. For chemotaxis assay, 100 μ M ADP in DMEM was added to the lower chamber. Cells suspended in serum-free DMEM were added to the upper chamber. After incubating for 6 h, non-migrating cells were removed from the upper chamber with a cotton swab and cells that had migrated to the lower surface of the membrane were fixed with 3.7% formaldehyde for 10 min and stained with 0.2% crystal violet. Cells were imaged and the intensity of staining was measured using *image quant* software.

Immunofluorescence stainings

Cells were attached to glass coverslips coated with 3 μ g/ml of fibronectin. After starvation for 4 h in serum-free DMEM, cells were pretreated with pharmacological inhibitors and stimulated with 100 μ M ADP. Cells were washed once in PBS, fixed in 3.7% formaldehyde for 10 min at 37°C, permeabilized with 0.2% Triton X-100 for 10 min at room temperature, washed in PBS, and then blocked in 1% bovine serum albumin (BSA) in PBS for 20 min at room temperature. After 1 hr of incubation with primary antibodies, cells were washed in PBS and incubated with FITC-conjugated anti-rabbit antibodies or Texas-Red conjugated anti-mouse antibodies (Santa Cruz) in PBS for 1 h. Confocal images were captured with Roper Cascade 1K digital camera and Yokogawa CSU-22, and processed using the program Metamorph 6.1 (Universal Imaging, Media, PA).

Live Cell Fluorescence Microscopy

Cells transfected with GFP constructs were attached to 35-mm glass-bottom dishes coated with 3 µg/ml of fibronectin. When cells reach 60–80% confluency, cells were starved for 4 hr in serum-free DMEM. Dishes were transferred to a heated (37°C) chamber and imaged using a spinning disc confocal microscope with a 60X oil-immersion objective. Cells were monitored over a 30 min period by capturing images every 30 s. Acquisition was performed using Metamorph.

Membrane ruffling—Membrane ruffling was examined as previously described (Lee and Chung 2009). For phase-contrast microscopy, cells were attached to glass coverslips coated with 3 µg/ml fibronectin. After starvation for 4 hr in serum-free DMEM, images of cells were taken with a 10X Ph1 objective. Lamella dynamics and cell migration velocity were analyzed by the kymographic assay (Bear et al. 2002). For each group, at least 10 individual cells were monitored over a 15 min period by capturing digital images every 6 sec. Subsequently, three areas of interest were marked on each image with lines that cross the cell lamella.

Kinetics of adhesion turnover—Quantification of focal adhesion dynamics was performed as previously described (Webb et al. 2004). To quantify adhesion turnover, the rates of formation and disassembly were determined by measuring the background fluorescent subtracted changes in fluorescent intensity of individual adhesions as a function of time from cells expressing green fluorescent protein (GFP) tagged wild type-, S83A-, or Y31F-paxillin. The incorporation of paxillin into adhesions was linear on a semilogarithmic plot of the fluorescent intensity as a function of time. The apparent rate constants for formation and disassembly were determined from the slope of these graphs.

Coimmunoprecipitation and western blotting

Microglia cells were serum-starved and stimulated with ADP. Prior to lysis, the cells were treated with 1 mM cross-linking reagent dithiobis(succinimidyl propionate) (DSP; Pierce) for 10 min. Cells were then lysed with RIPA buffer containing protease inhibitor cocktail (Sigma) on ice for 20 min. Cell debris were pelleted by centrifugation for 10 min at 3000g. Lysates were precleared with 30 µL of protein A agarose, followed by incubation with rabbit anti-Arr2 antibody for 2 h and by the addition of 30 µL of protein A agarose beads for 2 h. The beads were then washed three times with RIPA buffer, and bound proteins were eluted with Laemmli SDS sample buffer. The proteins were separated by SDS PAGE (10%) and transferred to poly(vinylidene difluoride) membrane (Millipore). Blot was incubated with primary antibodies from Cell Signaling (mouse anti-p44/42 ERK1/2 (L34F12), followed by anti-mouse horseradish peroxidase-conjugated secondary antibodies from Santa Cruz. Protein bands were visualized by enhanced chemiluminescence (ECL, Pierce) followed by exposure to X-ray film. The bands were quantified using Image J software.

Results

Inhibition of ERK1/2 activity significantly impairs the retraction of membrane ruffles and the increase of focal adhesion number upon ADP stimulation

The ruffling of the plasma membrane is the formation of motile cell surface protrusions containing a meshwork of newly polymerized actin filaments and is a characteristic feature of many actively migrating cells. To elucidate how dynamic regulation of ruffle formation is altered by ERK1/2 inhibition, we examined membrane ruffles using a video microscopy over 30 min period after stimulation with ADP (a chemoattractant of microglia chemotaxis) followed by kymography analysis. Both the frequency of membrane ruffle formation and the distance of lamellipodia extension are greatly enhanced after ADP treatment (Fig. 1A). Inhibition of ERK1/2 activation with U0126 (a MEK1/2 inhibitor) results in a modest reduction in the frequency of ruffle protrusion but a marked reduction in the frequency of ruffle retraction, leading longer distance of lamellipodia extension upon ADP stimulation. This result suggests that ERK1/2 activity might play an important role in the regulation of the retraction of membrane ruffles. To examine the effect of ERK1/2 inhibition on chemotaxis, microglia chemotaxis was assessed using a transwell assay. Microglia cells showed a robust and directional migration toward the lower chamber containing 100 μ M ADP, whereas cells treated with U0126 showed a significant defect in ADP-induced chemotaxis (Fig. 1B). However, U0126 did not show a significant effect on random (chemokinetic) migration (data not shown). These results indicate that ERK1/2 activity plays an important role in the regulation of ruffle formation and chemotaxis, presumably via the control of focal adhesion formation.

We examined changes of focal adhesions in cells treated with U0126 using immunofluorescence stainings of paxillin and live cell imaging of GFP-paxillin. In both control and U0126-treated cells, paxillin stainings are mainly localized to the focal complexes at the plasma membrane in the absence of ADP stimulation. The number of focal complexes did not show a significant difference, suggesting that ERK1/2 activity might not be required for the initiation of FX formation. After ADP stimulation for 15 min, the number and size of FAs were markedly increased up to three times in control cells (Fig. 1C). The size of FAs also increases at a similar level in U0126-treated cells upon ADP stimulation, suggesting that ERK1/2 activity might not be essential for the increase of FA size. However, U0126 showed a strong inhibitory effect on the increase of focal adhesion numbers upon ADP stimulation. This result is consistent with the lack of the retraction of membrane ruffles. Since the increase of focal adhesion number requires turnover of existing FAs, ERK1/2 activity might be required for the regulation of FA disassembly.

ADP induces paxillin phosphorylation via ERK1/2 activation

Previous studies reported that two kinases, p38 mitogen-activated protein kinase and ERK1/2, phosphorylate serine 83 in paxillin (Huang et al. 2004; Ishibe et al. 2004) as well as tyrosine residues 31 and 118 are the major tyrosine phosphorylation sites phosphorylated in a FAK-dependent manner (Bellis et al. 1995). We examined if ADP could induce ERK1/2 activation and paxillin phosphorylation through P2Y₁₂R. ADP stimulation significantly increased the level of ERK1/2 phosphorylation and paxillin phosphorylation at Ser⁸³ and

Tyr³¹ (Fig. 2A). 2-methylthio-AMP (2MeS, a P2Y₁₂R antagonist) or Pertussis Toxin (Ptx) effectively blocked ERK1/2 and paxillin phosphorylation, indicating that phosphorylation of ERK1/2 and paxillin upon ADP stimulation is mediated by P2Y₁₂R. U0126 significantly inhibited ADP-induced phosphorylation of ERK1/2 and paxillin at Ser⁸³ but not at Tyr³¹ (Fig. 2B), consistent with previous reports. Interestingly, analysis of the kinetics of ERK1/2 and paxillin phosphorylations upon ADP stimulation demonstrated that phosphorylation of paxillin at Tyr³¹ reach the peak prior to phosphorylation of ERK1/2 and paxillin at Ser⁸³ (Fig. 2C), suggesting that Tyr³¹ phosphorylation might be an early event in FA formation. These results suggest that the membrane retraction and the increase of FA number are impaired in U0126-treated cells, presumably due to the inhibition of paxillin phosphorylation at Ser⁸³ by ERK1/2.

Inhibition of ERK1/2 activity attenuates the disassembly of FA

To determine whether the phosphorylation of paxillin by ERK1/2 have any impact on assembly or disassembly of focal adhesion, we examined the assembly and disassembly of chicken paxillin-GFP in BV2 microglia cells (Pax-KD cells), whose endogenous paxillin expression was knocked down by the expression of short hairpin RNA (shRNA). Similar to U0126-treated cells, cells expressing S83A-paxillin-GFP showed significantly less number of FA, but larger in size, than FAs in cells expressing wild type paxillin-GFP (Fig. 3A). Cells expressing Y31F-paxillin-GFP also exhibited less number of FA, but they are smaller in size. We then analyzed focal adhesion dynamics using a time-lapse video microscopy for 30 min after ADP stimulation (Fig. 3B). We observed that membrane ruffles began to form within 5 min after ADP stimulation and the formation of paxillin-GFP-containing focal contacts in the newly protruding regions or the cell edge in the cells transfected with wild type paxillin-GFP. Paxillin disassembly was also found in the cell edge or the newly protruding areas after the formation of FXs. The rates of formation and disassembly of paxillin-GFP were determined by integrating the fluorescence intensity in individual adhesions over time as shown before (Webb et al. 2004). The kinetics of paxillin formation and disassembly are shown in Fig. 3C, and the rate constants for paxillin mutants are summarized in Table 1. The apparent rate constants for paxillin-GFP formation in the absence or presence of U0126 in Pax-KD cells were $(1.9 \pm 0.5) \times 10^{-1} \text{ min}^{-1}$ and $(1.37 \pm 0.3) \times 10^{-1} \text{ min}^{-1}$ (Fig. 3C and Table 1), respectively, indicating that the rate of paxillin formation was not significantly changed by the inhibition of ERK1/2 with U0126. In contrast, U0126 has a strongly negative effect on paxillin disassembly upon ADP stimulation. The rate constant for the disassembly of paxillin $((3.4 \pm 0.21) \times 10^{-2} \text{ min}^{-1})$ was about 4.82-fold less in U0126-treated cells than in wild-type Pax-KD cells (Fig. 3C and Table 1). These results suggest that the inhibition of ERK1/2 activity attenuates the disassembly of paxillin and FA. Slow turnover of focal adhesions would cause impairment of the formation of membrane ruffle and the increase of FA number by ADP. To address the role of paxillin phosphorylation at specific sites, we examined formation and disassembly of S83A-paxillin-GFP and Y31F-paxillin-GFP mutants in Pax-KD cells. The formation of FX containing S83A-paxillin-GFP was found in the newly protruding regions or the cell edge. The kinetics and rate constants revealed no significant change of paxillin formation in the S83A mutant (Fig. 3). However, the rate constant of S83A-paxillin-GFP disassembly was significantly decreased compared to wild-type paxillin. Paxillin or FA disassembly was

scarcely found in these cells. Interestingly, Y31F-paxillin-GFP showed an opposite response. The formation of Y31F-paxillin-GFP was much slower than wild-type paxillin-GFP. The rate constant for the formation of FX containing Y31F-paxillin-GFP ($(2 \pm 0.3) \times 10^{-2} \text{ min}^{-1}$) was about 9.26-fold less than that of wild-type. However, the disassembly of Y31F-paxillin-GFP did not show any significant change. These results indicate that serine and tyrosine phosphorylation sites have a specific role, respectively in focal adhesion formation and turnover upon ADP stimulation. The phosphorylation of paxillin at Tyr31 appears to be involved in adhesion formation upon ADP stimulation, while Ser83 is required for adhesion disassembly.

Phosphorylation of paxillin at Serine⁸³ and Tyrosine³¹ is regulated by β -Arrestin 2 and c-Src, respectively

To determine signaling pathway(s) involved in ERK1/2 activation via P2Y₁₂R, we first examined PI3K and PKA activation. PI3K has been demonstrated to stimulate ERK1/2 activation via activation of Ras/Raf (Hu et al. 1995). PKA also activates ERK1/2 by activating the small GTPase, Rap1, which can activate ERK1/2 through B-Raf (Bos 2005; Stork 2003). Furthermore, we have previously shown that ADP could induce PI3K and PKA activation via the P2Y₁₂R (Lee and Chung 2009). Inhibition of PI3K and PKA activity by inhibitors (LY294002, a PI3K inhibitor and H-89, a PKA inhibitor) did not show a significant effect on ADP-induced phosphorylation of ERK1/2 and paxillin at Ser⁸³/Tyr³¹ (Fig. 4A). In addition, a pull-down assay with GST-RBD/Raf did not show any significant activation of Ras upon ADP stimulation (Fig. 4B). Activation of PKA using the 8-Br-cAMP (PKA activator) or forskolin (adenylyl cyclase activator) did not have any effects on ERK1/2 activation (data not shown). These results indicate that PI3K and PKA signaling pathways are not involved in ERK1/2 activation. Recent studies reported that Src family tyrosine kinases are required for ERK1/2 and paxillin phosphorylation at Ser⁸³/Tyr³¹ (Ishibe et al. 2004; Ishibe et al. 2003; Liu et al. 2002; Sen et al. 2010). These reports demonstrated that growth factor-stimulated association of paxillin and ERK requires phosphorylation of tyrosines on paxillin by Src activation, which is then required for subsequent ERK-mediated phosphorylation of paxillin at Ser⁸³. We tested if Src kinase activity is required for ADP-induced ERK1/2 and paxillin phosphorylation by ADP. A pharmacological inhibitor of the Src family tyrosine kinases, PP2, did not show a significant effect on ADP-induced phosphorylation of ERK1/2 and paxillin at Ser⁸³. However, phosphorylation of paxillin at Tyr³¹ following ADP stimulation is markedly decreased by pretreatment with the PP2 (Fig. 4A). These results indicate that ADP-induced ERK1/2 and paxillin phosphorylation at Ser⁸³ were not dependent on Src family tyrosine kinases and ADP-stimulated phosphorylation of paxillin at Ser⁸³ and Tyr³¹ are regulated by ERK1/2 and Src kinase, respectively.

Previous studies showed that β -Arrestins (β -Arrests) could serve as scaffolds for GPCR-mediated activation of ERK1/2 (DeFea et al. 2000; Lefkowitz and Shenoy 2005). We determined if β -Arrests contribute to ADP-induced ERK1/2 activation by examining ERK1/2 phosphorylation upon overexpression of FLAG-tagged β -Arrestin 1 (β -Arr1) and 2 (β -Arr2) in microglia (Fig. 4C). Overexpression of β -Arr2 significantly increased the phosphorylation of the ERK1/2 and Pax/Ser⁸³ even in the absence of ADP stimulation, while β -Arr1 overexpression did not cause a significant increase of the phosphorylation of ERK1/2 and

Pax/Ser⁸³. In addition, depletion of endogenous β -Arr2 with shRNA markedly reduced the phosphorylation of ERK1/2 and Pax/Ser⁸³ upon ADP stimulation (Fig. 4D). These results indicate that β -Arr2 is likely to be involved in ERK1/2 activation upon ADP stimulation in microglia. To examine if there is a physical interaction between β -Arr2 and ERK1/2 upon ADP stimulation, we performed a co-IP experiment and showed the increased binding of β -Arr2 to ERK1/2 upon ADP stimulation (Fig. 4E). Confocal microscopy of microglia cells transiently transfected with Cherry- β -Arr2 and stained with pERK1/2 antibody confirmed that ADP stimulation results in the distinct colocalization of β -Arr2 and pERK1/2 in lamellapodia. β -Arr2 and pERK1/2 are mainly distributed in the cytosol without ADP stimulation. Upon ADP stimulation, a large fraction of pERK1/2 and β -Arr2 were translocated and co-localized at focal contacts in the newly forming lamellipodia near the edge of the cell (Fig. 4F).

We next examined ADP-stimulated phosphorylation of ERK1/2, Pax/Ser⁸³ and Pax/Tyr³¹ in wild type (WT), β -Arr2-KD and c-Src-KD cells. Knockdown of β -Arr2 expression with shRNA significantly reduced ERK1/2 and Pax/Ser⁸³ phosphorylation, while c-Src knockdown had no impact on ERK1/2 and Pax/Ser⁸³ phosphorylation upon ADP stimulation. In contrast, β -Arr2-KD cells did not show the significant decrease on ADP-induced Pax/Tyr³¹ phosphorylation. However, Tyr³¹ phosphorylation was markedly decreased in the c-Src-KD cells (Fig. 5A). These results demonstrated that β -Arr2 plays a key role in the activation of ERK1/2 and Pax/Ser⁸³ phosphorylation while c-Src is required for the Pax/Tyr³¹ phosphorylation upon ADP stimulation. To examine the role of β -Arr2 and c-Src in membrane ruffle formation upon ADP stimulation, we analyzed time-lapse movies of control, β -Arr2-KD, and c-Src-KD cells using kymography. β -Arr2- and c-Src-KD cells exhibited significant decreases of membrane ruffle formation and lamellipodia extension compared to control cells (Fig. 5B). Ratio between the frequency of membrane protrusion and retraction was equal after ADP stimulation in control cells. Overall, β -Arr2-KD cells make less protrusion and retraction than the control cells, but they make protrusion more often than retraction, resulting in a higher ratio (2.2 fold) and longer extension of lamellipodia. In contrast, c-Src-KD cells showed higher frequency of retraction, resulting in lower ratio and no lamellipodia extension. These results indicate that β -Arr2 and c-Src have a different role in ADP-induced membrane ruffle formation, presumably via controlling specific phosphorylation sites of paxillin. Defects of β -Arr2-KD and c-Src-KD cells in membrane ruffle formation lead to inefficient chemotaxis as shown in Fig. 5C. We again examined dynamics of FA formation and disassembly in these cells using the immunofluorescence staining and live cell imaging. Immunofluorescence staining of paxillin revealed that FA numbers are markedly reduced upon ADP stimulation in c-Src-KD cells while β -Arr2-KD cells showed only a moderate reduction. Marked reduction of FA number in c-Src-KD cells is similar to cells expressing Y31F-paxillin-GFP. The size of FA in β -Arr2-KD is about 1.4-fold bigger than FAs in control cells, which is similar to cells expressing S83A-paxillin-GFP (Fig 6A). To observe the turnover of FA, we examined dynamic changes of paxillin-GFP in the control, β -Arr2-KD, and c-Src-KD cells. The apparent rate constants for paxillin formation in WT and β -Arr2-KD cells were $(1.5 \pm 0.5) \times 10^{-1} \text{ min}^{-1}$ and $(1.18 \pm 0.44) \times 10^{-1} \text{ min}^{-1}$, respectively, indicating that the depletion of β -Arr2 did not have a significant effect on ADP-induced paxillin formation. However, ADP-

induced paxillin disassembly rate, $(3.43 \pm 0.24) \times 10^{-2} \text{ min}^{-1}$, was markedly slower than control cells (Fig 6B and Table 1). In contrast, c-Src-KD cells have an opposite response, showing that paxillin formation was slower than in control cells. The rate constant for paxillin formation $((3.1 \pm 0.5) \times 10^{-2} \text{ min}^{-1})$ in c-Src-KD was about a 4.98-fold slower than in control cells (Fig 6B and Table 1). However, the disassembly of paxillin did not show a significant change in c-Src-KD cells (Table 1). These results indicate that paxillin formation and disassembly involved in ruffle protrusion and retraction in microglia are regulated differentially by c-Src and β -Arrestin 2 upon ADP stimulation.

Results from our study strongly suggest that β -Arr2 is involved in the regulation of membrane retraction via ERK1/2-mediated phosphorylation of paxillin at Ser⁸³. In addition, c-Src activity has an important role in ADP-induced membrane protrusion through the paxillin phosphorylation at Tyr³¹.

Discussion

Cellular adhesions to the surrounding extracellular matrix (ECM) are critical determinants of cell migration. ERK1/2 has been suggested to be an important regulator of cell adhesion and migration since ERK1/2 are known to be rapidly activated by phosphorylation immediately after cell contact with the ECM (Klemke et al. 1997). ERK1/2 are the kinases responsible for Raf-induced paxillin phosphorylation at Ser¹²⁶ (Woodrow et al. 2003). In this study, we investigated molecular details of ERK1/2 activation upon P2Y₁₂R stimulation by ADP. Our results demonstrate that ERK1/2 activation in microglia upon ADP stimulation requires P2Y₁₂R and β -arrestin 2. Receptor-bound arrestins have been reported to recruit and activate a variety of signaling proteins, including the components of the endocytic machinery, small GTPases and their regulators, ubiquitin ligase, c-Src, and related kinases, etc (Shenoy and Lefkowitz 2011). Receptor-bound β -arrestins act as signaling scaffolds for mitogen-activated protein kinase (MAPK) cascades, leading to the activation of ERK1/2 and p38 kinases (Shenoy and Lefkowitz 2005). Our study showed that ERK1/2 activation upon ADP stimulation was insensitive to H-89 and sensitive to depletion of β -Arr-2 by shRNA, which is consistent with a previous study showing ERK1/2 activation via β -Arr-1 and 2 (Shenoy et al. 2006). Our study showed that inhibition of ERK1/2 prevented microglia chemotaxis by attenuating membrane ruffle retraction and the increase of FA numbers upon ADP stimulation. Multiple evidences indicated significant roles of MAPK activation via arrestins in the regulation of cell motility and chemotaxis. Suppression of β -arrestin2 endogenous expression attenuated stromal cell-derived factor 1 α -induced cell migration (Sun et al. 2002). p38 MAPK appears to play an important role in this case since inhibition of p38 MAPK activation (but not ERK activation) effectively blocked the chemotactic effect of β -arrestin 2. PAR-2 activation promotes ERK1/2- and β -arrestin-dependent reorganization of the actin cytoskeleton, polarized pseudopodia extension, and chemotaxis (Ge et al. 2003). Both T and B cells from β -arrestin 2-deficient animals were strikingly impaired in their ability to respond to CXCL12, both in transwell migration assays and in transendothelial migration assays (Fong et al. 2002). Our study clearly demonstrated that ERK1/2 activation via β -arrestin2 plays a significant role in the regulation of FA during chemotaxis of microglia.

We, in this study, showed that phosphorylation of paxillin at Ser⁸³ and Tyr³¹ upon ADP stimulation are independently regulated by ERK1/2 and c-Src kinase, respectively. A previous study also reported that inhibition of ERK1/2 phosphorylation had no effect on the calcitonin-induced tyrosine phosphorylation of paxillin (Zhang et al. 2000). Examination of kinetics and rate constant of paxillin formation and disassembly revealed that the phosphorylation of paxillin at Tyr³¹ appears to be involved in adhesion formation upon ADP stimulation, while Ser⁸³ is required for adhesion disassembly. A previous study has shown that invadopodia ring expansion is controlled by paxillin phosphorylations on Tyr³¹ and Tyr¹¹⁸ as paxillin mutant Y31F–Y118F impairs their self-organization (Badowski et al. 2008). A recent study also demonstrated that phosphomimetic mutations (Y31E–Y118E) on paxillin not only enhance lamellipodial protrusion and formation of FXs and FAs (Zaidel-Bar et al. 2007), but increase the size of the complex and the assembly rate of nascent adhesions (Choi et al. 2011). These results suggest that tyrosine phosphorylation of paxillin regulates adhesion formation via controlling the physical interactions among adhesion proteins including paxillin and FAK. The precise function of phosphorylation at Ser⁸³ in the regulation of focal adhesions has been unclear. Our results clearly indicate that phosphorylation at Ser⁸³ is required for the disassembly of focal adhesion. Our study demonstrated that the kinetics of ERK1/2 activation by P2Y₁₂R was relatively slow in the onset (peak 5–10 min) and sustained after 10 min, which was similar to that of paxillin phosphorylation at Ser⁸³. Slow activation kinetics and sustained activation of ERK1/2 is consistent with their role in FA disassembly. It is plausible that phosphorylation at Ser⁸³ might regulate the interaction of paxillin with other proteins. It has been reported that paxillin phosphorylation at Ser⁸³ by p38MAPK in NGF-induced PC-12 cells modulates focal adhesions and neurite extension by facilitating the interaction of paxillin with Pyk2 (Huang et al. 2004). In summary, our results demonstrate that ADP binding to P2Y₁₂R induces the activation of ERK1/2, which is dependent upon β -arrestin 2. Activation of ERK1/2 leads to an increase in the phosphorylation of paxillin at Ser⁸³ that is required for adhesion disassembly. Inhibition of ERK1/2 results in decreased focal adhesion turnover, which impairs the retraction of lamellipodia and chemotaxis of microglia.

Acknowledgments

We thank members of the Chung lab for useful discussions and critical reading of the manuscript. We are indebted to Dr. Donna Webb for the paxillin-GFP construct and Dr. Seva Gurevich for FLAG- β -Arrestin 1 and 2 constructs and antibodies. This work was supported, in part, by a grant from National Institute of Health (GM68097 to C.Y.C.).

References

- Badowski C, Pawlak G, Grichine A, Chabadel A, Oddou C, Jurdic P, Pfaff M, Albiges-Rizo C, Block MR. Paxillin phosphorylation controls invadopodia/podosomes spatiotemporal organization. *Mol Biol Cell*. 2008; 19(2):633–45. [PubMed: 18045996]
- Bear JE, Svitkina TM, Krause M, Schafer DA, Loureiro JJ, Strasser GA, Maly IV, Chaga OY, Cooper JA, Borisy GG, et al. Antagonism between Ena/VASP proteins and actin filament capping regulates fibroblast motility. *Cell*. 2002; 109(4):509–21. [PubMed: 12086607]
- Bellis SL, Miller JT, Turner CE. Characterization of tyrosine phosphorylation of paxillin in vitro by focal adhesion kinase. *J Biol Chem*. 1995; 270(29):17437–41. [PubMed: 7615549]
- Bos JL. Linking Rap to cell adhesion. *Curr Opin Cell Biol*. 2005; 17(2):123–8. [PubMed: 15780587]

- Cai X, Li M, Vrana J, Schaller MD. Glycogen synthase kinase 3- and extracellular signal-regulated kinase-dependent phosphorylation of paxillin regulates cytoskeletal rearrangement. *Mol Cell Biol*. 2006; 26(7):2857–68. [PubMed: 16537926]
- Choi CK, Zareno J, Digman MA, Gratton E, Horwitz AR. Cross-Correlated Fluctuation Analysis Reveals Phosphorylation-Regulated Paxillin-FAK Complexes in Nascent Adhesions. *Biophysical journal*. 2011; 100(3):583–592. [PubMed: 21281572]
- Choi HB, Hong SH, Ryu JK, Kim SU, McLarnon JG. Differential activation of subtype purinergic receptors modulates Ca(2+) mobilization and COX-2 in human microglia. *Glia*. 2003; 43(2):95–103. [PubMed: 12838502]
- Deakin NO, Turner CE. Paxillin comes of age. *J Cell Sci*. 2008; 121(15):2435–2444. [PubMed: 18650496]
- DeFea KA, Vaughn ZD, O'Bryan EM, Nishijima D, Dery O, Bunnett NW. The proliferative and antiapoptotic effects of substance P are facilitated by formation of a beta -arrestin-dependent scaffolding complex. *Proc Natl Acad Sci U S A*. 2000; 97(20):11086–91. [PubMed: 10995467]
- Fong AM, Premont RT, Richardson RM, Yu YR, Lefkowitz RJ, Patel DD. Defective lymphocyte chemotaxis in beta-arrestin2- and GRK6-deficient mice. *Proc Natl Acad Sci U S A*. 2002; 99(11):7478–83. [PubMed: 12032308]
- Ge L, Ly Y, Hollenberg M, DeFea K. A beta-arrestin-dependent scaffold is associated with prolonged MAPK activation in pseudopodia during protease-activated receptor-2-induced chemotaxis. *J Biol Chem*. 2003; 278(36):34418–26. [PubMed: 12821670]
- Gonzalez-Scarano F, Baltuch G. Microglia as mediators of inflammatory and degenerative diseases. *Annu Rev Neurosci*. 1999; 22:219–40. [PubMed: 10202538]
- Hanisch UK. Microglia as a source and target of cytokines. *Glia*. 2002; 40(2):140–55. [PubMed: 12379902]
- Hickey WF. Basic principles of immunological surveillance of the normal central nervous system. *Glia*. 2001; 36(2):118–24. [PubMed: 11596120]
- Honda S, Sasaki Y, Ohsawa K, Imai Y, Nakamura Y, Inoue K, Kohsaka S. Extracellular ATP or ADP induce chemotaxis of cultured microglia through Gi/o-coupled P2Y receptors. *J Neurosci*. 2001; 21(6):1975–82. [PubMed: 11245682]
- Hu Q, Klippel A, Muslin AJ, Fantl WJ, Williams LT. Ras-dependent induction of cellular responses by constitutively active phosphatidylinositol-3 kinase. *Science*. 1995; 268(5207):100–2. [PubMed: 7701328]
- Huang C, Borchers CH, Schaller MD, Jacobson K. Phosphorylation of paxillin by p38MAPK is involved in the neurite extension of PC-12 cells. *The Journal of Cell Biology*. 2004; 164(4):593–602. [PubMed: 14970194]
- Ilic D, Furuta Y, Kanazawa S, Takeda N, Sobue K, Nakatsuji N, Nomura S, Fujimoto J, Okada M, Yamamoto T. Reduced cell motility and enhanced focal adhesion contact formation in cells from FAK-deficient mice. *Nature*. 1995; 377(6549):539–44. [PubMed: 7566154]
- Inoue K. Microglial activation by purines and pyrimidines. *Glia*. 2002; 40(2):156–63. [PubMed: 12379903]
- Ishibe S, Joly D, Liu ZX, Cantley LG. Paxillin serves as an ERK-regulated scaffold for coordinating FAK and Rac activation in epithelial morphogenesis. *Mol Cell*. 2004; 16(2):257–67. [PubMed: 15494312]
- Ishibe S, Joly D, Zhu X, Cantley LG. Phosphorylation-dependent paxillin-ERK association mediates hepatocyte growth factor-stimulated epithelial morphogenesis. *Mol Cell*. 2003; 12(5):1275–85. [PubMed: 14636584]
- Klemke RL, Cai S, Giannini AL, Gallagher PJ, de Lanerolle P, Cheres DA. Regulation of cell motility by mitogen-activated protein kinase. *J Cell Biol*. 1997; 137(2):481–92. [PubMed: 9128257]
- Kreutzberg GW. Microglia: a sensor for pathological events in the CNS. *Trends Neurosci*. 1996; 19(8):312–8. [PubMed: 8843599]
- Lee S, Chung CY. Role of VASP phosphorylation for the regulation of microglia chemotaxis via the regulation of focal adhesion formation/maturation. *Mol Cell Neurosci*. 2009; 42(4):382–90. [PubMed: 19733667]

- Lefkowitz RJ, Shenoy SK. Transduction of receptor signals by beta-arrestins. *Science*. 2005; 308(5721):512–7. [PubMed: 15845844]
- Liu ZX, Yu CF, Nickel C, Thomas S, Cantley LG. Hepatocyte growth factor induces ERK-dependent paxillin phosphorylation and regulates paxillin-focal adhesion kinase association. *J Biol Chem*. 2002; 277(12):10452–8. [PubMed: 11784715]
- Mitra SK, Hanson DA, Schlaepfer DD. Focal adhesion kinase: in command and control of cell motility. *Nat Rev Mol Cell Biol*. 2005; 6(1):56–68. [PubMed: 15688067]
- Monami G, Gonzalez EM, Hellman M, Gomella LG, Baffa R, Iozzo RV, Morrione A. Proepithelin promotes migration and invasion of 5637 bladder cancer cells through the activation of ERK1/2 and the formation of a paxillin/FAK/ERK complex. *Cancer Res*. 2006; 66(14):7103–10. [PubMed: 16849556]
- O'Connor KL, Shaw LM, Mercurio AM. Release of cAMP gating by the alpha6beta4 integrin stimulates lamellae formation and the chemotactic migration of invasive carcinoma cells. *J Cell Biol*. 1998; 143(6):1749–60. [PubMed: 9852165]
- Ohsawa K, Irino Y, Nakamura Y, Akazawa C, Inoue K, Kohsaka S. Involvement of P2X₄ and P2Y₁₂ receptors in ATP-induced microglial chemotaxis. *Glia*. 2007; 55(6):604–16. [PubMed: 17299767]
- Ohsawa K, Irino Y, Sanagi T, Nakamura Y, Suzuki E, Inoue K, Kohsaka S. P2Y₁₂ receptor-mediated integrin-beta1 activation regulates microglial process extension induced by ATP. *Glia*. 2010; 58(7):790–801. [PubMed: 20091784]
- Schaller MD. Paxillin: a focal adhesion-associated adaptor protein. *Oncogene*. 2001; 20(44):6459–72. [PubMed: 11607845]
- Sen A, O'Malley K, Wang Z, Raj GV, Defranco DB, Hammes SR. Paxillin regulates androgen- and epidermal growth factor-induced MAPK signaling and cell proliferation in prostate cancer cells. *J Biol Chem*. 2010; 285(37):28787–95. [PubMed: 20628053]
- Shankar H, Garcia A, Prabhakar J, Kim S, Kunapuli SP. P2Y₁₂ receptor-mediated potentiation of thrombin-induced thromboxane A₂ generation in platelets occurs through regulation of Erk1/2 activation. *J Thromb Haemost*. 2006; 4(3):638–47. [PubMed: 16460446]
- Shenoy SK, Drake MT, Nelson CD, Houtz DA, Xiao K, Madabushi S, Reiter E, Premont RT, Lichtarge O, Lefkowitz RJ. beta-arrestin-dependent, G protein-independent ERK1/2 activation by the beta2 adrenergic receptor. *J Biol Chem*. 2006; 281(2):1261–73. [PubMed: 16280323]
- Shenoy SK, Lefkowitz RJ. Seven-transmembrane receptor signaling through beta-arrestin. *Sci STKE*. 2005; (308):cm10. [PubMed: 16267056]
- Shenoy SK, Lefkowitz RJ. beta-arrestin-mediated receptor trafficking and signal transduction. *Trends Pharmacol Sci*. 2011; 32(9):521–33. [PubMed: 21680031]
- Stence N, Waite M, Dailey ME. Dynamics of microglial activation: a confocal time-lapse analysis in hippocampal slices. *Glia*. 2001; 33(3):256–66. [PubMed: 11241743]
- Stork PJ. Does Rap1 deserve a bad Rap? *Trends Biochem Sci*. 2003; 28(5):267–75. [PubMed: 12765839]
- Streit WJ. Microglia as neuroprotective, immunocompetent cells of the CNS. *Glia*. 2002; 40(2):133–9. [PubMed: 12379901]
- Sun Y, Cheng Z, Ma L, Pei G. Beta-arrestin2 is critically involved in CXCR4-mediated chemotaxis, and this is mediated by its enhancement of p38 MAPK activation. *J Biol Chem*. 2002; 277(51):49212–9. [PubMed: 12370187]
- Thomas WE. Brain macrophages: on the role of pericytes and perivascular cells. *Brain Res Brain Res Rev*. 1999; 31(1):42–57. [PubMed: 10611494]
- Webb DJ, Donais K, Whitmore LA, Thomas SM, Turner CE, Parsons JT, Horwitz AF. FAK-Src signalling through paxillin, ERK and MLCK regulates adhesion disassembly. *Nat Cell Biol*. 2004; 6(2):154–61. [PubMed: 14743221]
- Webb DJ, Schroeder MJ, Brame CJ, Whitmore L, Shabanowitz J, Hunt DF, Horwitz AR. Paxillin phosphorylation sites mapped by mass spectrometry. *J Cell Sci*. 2005; 118(Pt 21):4925–9. [PubMed: 16254239]
- Woodrow MA, Woods D, Cherwinski HM, Stokoe D, McMahon M. Ras-induced serine phosphorylation of the focal adhesion protein paxillin is mediated by the Raf-->MEK-->ERK pathway. *Exp Cell Res*. 2003; 287(2):325–38. [PubMed: 12837288]

- Zaidel-Bar R, Ballestrem C, Kam Z, Geiger B. Early molecular events in the assembly of matrix adhesions at the leading edge of migrating cells. *J Cell Sci.* 2003; 116(Pt 22):4605–13. [PubMed: 14576354]
- Zaidel-Bar R, Milo R, Kam Z, Geiger B. A paxillin tyrosine phosphorylation switch regulates the assembly and form of cell-matrix adhesions. *J Cell Sci.* 2007; 120(Pt 1):137–48. [PubMed: 17164291]
- Zhang Z, Baron R, Horne WC. Integrin engagement, the actin cytoskeleton, and c-Src are required for the calcitonin-induced tyrosine phosphorylation of paxillin and HEF1, but not for calcitonin-induced Erk1/2 phosphorylation. *J Biol Chem.* 2000; 275(47):37219–23. [PubMed: 10954702]
- Zimmerman B, Volberg T, Geiger B. Early molecular events in the assembly of the focal adhesion-stress fiber complex during fibroblast spreading. *Cell Motil Cytoskeleton.* 2004; 58(3):143–59. [PubMed: 15146534]

Figure 1. ERK1/2 activity is required for ADP-induced membrane ruffle formation, chemotaxis, and focal adhesion formation in microglia

(A) Lamella dynamics was analyzed by kymographs. For each group, membrane ruffles of at least 15 individual cells were monitored over a 30 min period by capturing images every 12 seconds. Kymographs were assembled using *MetaMorph* software. Representative kymographs are shown and quantifications of ruffle frequency and distance from five independent experiments are shown. * $p < 0.01$ versus ADP; # $p < 0.01$ versus retraction distance of U0126+ADP, Student's t-test. (B) Inhibition of ERK1/2 activity significantly reduced ADP-induced chemotaxis of microglia. Cells were assayed for chemotaxis toward 100 μ M ADP in the continued presence or absence of U0126. Cells that migrated to the bottom of the transwell membrane were stained with crystal violet and quantified using *ImageQuant* software. Quantification from five independent assays is shown in the graphs. * $p < 0.01$ versus ADP. (C) Pretreatment of cells with U0126 caused a significant inhibition of FA formation. BV2 Cells were plated on coverslips coated with 3 μ g/ml fibronectin. After pretreatment with U0126, cells were stimulated with 100 μ M ADP for 10 min. Immunostaining was performed on fixed cells with anti-mouse-paxillin antibody. The size and number of FAs were measured from the images ($n=15$). * $p < 0.01$ versus without ADP; # $p < 0.01$. Error bars indicate $\pm$ SEM. Bar = 10 μ m.

Figure 2. Role of the P2Y₁₂ receptor-dependent phosphorylation of ERK1/2, paxillin at Ser⁸³ and Tyr³¹ for ADP-induced focal adhesion formation

(A) Phosphorylation of ERK1/2 and paxillin at Ser⁸³/Tyr³¹ are mediated by P2Y₁₂ receptor upon ADP stimulation. Cells were pretreated with 50 μ M 2MeSAMP for 10 min or 100 ng/ml Pertussis toxin for 4 hr and then stimulated with 100 μ M ADP for 10 min. Cell lysates were collected and analyzed by western blotting using anti-pERK1/2 and anti-pPaxillin Ser⁸³ and Tyr³¹ antibodies. Quantification of ERK1/2, Paxillin at Ser⁸³ and Tyr³¹ phosphorylation from five independent experiments is shown in the graphs. * $p < 0.05$ versus ADP. (B) Inhibition of ERK1/2 activity blocks paxillin phosphorylation at Ser⁸³, but not at Tyr³¹. Cells were pretreated with 20 μ M U0126 for 20 min. Cells were then stimulated with ADP and cell lysates were analyzed by western blotting using anti-pERK1/2 and anti-pPaxillin Ser⁸³ and Tyr³¹ antibodies. Quantification of ERK1/2, Paxillin at Ser⁸³ and Tyr³¹ phosphorylation from eight independent experiments is shown in the graphs. * $p < 0.01$ versus ADP; # $p < 0.01$ versus BV2 Cont. (C) The kinetics of ERK1/2 phosphorylation and paxillin phosphorylation at Ser⁸³/Tyr³¹. BV2 cells were stimulated for the indicated times with 100 μ M ADP. Phosphorylation of ERK1/2 and paxillin was detected by western blotting with anti-pERK1/2 and anti-pPaxillin Ser⁸³ and Tyr³¹ antibodies. Quantification of five western blots is shown in the graphs.

Figure 3. Kinetics of paxillin formation and disassembly

(A) The number and size of focal adhesions in cells expressing paxillin mutants. Wild type (WT)-, S83A-, or Y31F-paxillin-GFP transiently expressed in Pax-KD cells were imaged upon ADP stimulation in living cells. The region outlined by the box is shown at higher magnification on the bottom panels. The size and number of FAs were measured from the images ($n=10$). $*p < 0.01$ versus WT. Error bars indicate $\pm$ SEM. (B) FA turnover was examined in cells treated with U0126 or Pax-KD cells expressing paxillin mutants. Cells were pretreated with or without 20 μM U0126 for 20 min and then stimulated with 100 μM ADP. Cells were monitored over a 30 min period by capturing images every 30 seconds. To quantify adhesion turnover, the rates of formation and disassembly were determined by measuring the background subtracted changes in fluorescent intensity of individual adhesions as a function of time. Arrows indicate representative adhesion assembly and arrows with dashed line indicate representative adhesion disassembly. (C) The kinetics of paxillin formation and disassembly. I_0 is the initial fluorescent intensity and I is the fluorescent intensity at the indicated times. The rate constants for formation and disassembly were determined from the slope of these graphs. Measurements were obtained for 10–15 individual adhesions from eight to ten cells. Inset shows a western blot of paxillin in control and Pax-KD cells. Bar = 10 μm .

Figure 4. β-Arrestin 2-dependent activation of ERK1/2 is required for ADP-induced paxillin phosphorylation at Ser⁸³

(A) Inhibition of ERK1/2 or Src kinase activity is significantly reduced paxillin phosphorylation at Ser⁸³ or Tyr³¹, respectively. Cells were pretreated with 20 μM LY294002, 20 μM U0126, 30 μM H-89 or 10 μM PP2 for 20 min. Cells were then stimulated with 100 μM ADP for 10 min and cell lysates were analyzed by western blotting using anti-pERK1/2 and anti-pPaxillin Ser⁸³ and Tyr³¹ antibodies. **p* < 0.01 versus ADP. (B) Active Ras levels were determined by using a GST pulldown assay. GST-Raf/RBD-bound agarose beads were mixed with the lysate of BV2 cells that were lysed at an indicated time after stimulation with ADP. Active Ras was detected by immunoblot with the anti-Ras antibody. (C) Overexpression of β-Arrestin 2 in BV2 cells significantly increased basal level of ERK1/2 and paxillin phosphorylation at Ser⁸³. Cells were transfected with FLAG-β-Arr1 or 2 constructs and then cells were lysed and subjected to western blot with anti-FLAG-M2 and anti-β-Arr1/2 and anti-pERK1/2 and anti-pPaxillin/Ser⁸³ antibodies. **p* < 0.05 versus WT. (D) The knockdown of β-Arr2 in cells significantly decreased basal level of ERK1/2 and paxillin phosphorylation at Ser⁸³. β-Arr2-KD cells were lysed and subjected to western blot with anti-β-Arr2 and anti-FLAG-M2 and anti-pERK1/2 and anti-pPaxillin/Ser⁸³ antibodies. **p* < 0.05 versus WT no ADP; #*p* < 0.05 versus WT ADP. Quantification of ERK1/2 and paxillin phosphorylation from seven independent experiments is shown in the graphs. Error bars indicate ± SEM. (E) Coimmunoprecipitation of ERK and β-Arr2 upon ADP stimulation. β-Arr2 were immunoprecipitated with anti-Arr2 antibody, and co-

immunoprecipitated ERK1/2 were detected by Western blotting using anti-ERK1/2 antibody. (F) ERK1/2 and β -Arr2 was translocated to the plasma membrane after ADP stimulation. BV2 cells transfected with Cherry- β -Arr2 were fixed 32 h after transfection and stained with anti-pERK1/2 antibody. Fluorescence intensities of pERK1/2 and β -Arr2 along the membrane, quantified from the image, are shown in the graph. The graph represents three independent experiments in which 10 cells were analyzed for each condition. $*p < 0.01$ versus Cont. Error bars indicate $\pm$ SEM.

Figure 5. Knockdown of β -Arr2 or c-Src is significantly impaired in ADP-induced membrane ruffle formation and microglia chemotaxis

(A) Cells – control BV2 (WT), β -Arr2-KD and c-Src-KD were stimulated with 100 μ M ADP for 10 min and lysates were analyzed by western blotting using anti-pERK1/2 and anti-pPaxillin Ser⁸³ and Tyr³¹ antibodies. $*p < 0.01$ versus WT. Quantification from five independent experiments is shown in the graphs. (B) Membrane ruffle formation in β -Arr2-KD or c-Src-KD cells. Lamella dynamics was analyzed by kymographs. Quantification of ruffle formation and lamellipodia extension from five independent experiments are shown in the graphs. $*p < 0.01$ versus WT; $\#p < 0.01$. (C) Chemotaxis of β -Arr2-KD or c-Src-KD cells. Transwell chamber membranes were coated with 3 μ g/ml fibronectin for 8 hr and cells were plated. Cells were then assayed for migration toward 100 μ M ADP. Quantification from five independent assays is shown in the graphs. $*p < 0.01$ versus WT. Error bars indicate $\pm$ SEM.

Figure 6. β -Arr2 or c-Src knockdown in cells significantly reduced ADP-induced focal adhesion formation

(A) Cells were stimulated with 100 μ M ADP for 10 min and then cells were fixed, permeabilized and stained with anti-paxillin antibody. The region outlined by the box is shown at higher magnification on the bottom panels. The size and number of FAs were measured from the images ($n=15$). $*p < 0.01$ versus WT. Error bars indicate $\pm$ SEM. (B) FA turnover is impaired in β -Arr2-KD and c-Src-KD cells. Paxillin-GFP construct transiently expressed in the control (WT), β -Arr2-KD and c-Src-KD cells was imaged 32 h after transfection in living cells. Cells were stimulated with 100 μ M ADP and then monitored over a 30 min period by capturing images every 30 seconds. To quantify adhesion turnover, the rates of formation and disassembly were determined by measuring the background subtracted changes in fluorescent intensity of individual adhesions as a function of time. I_0 is the initial fluorescent intensity and I is the fluorescent intensity at the indicated times. The rate constants for formation and disassembly were determined from the slope of these graphs. Measurements were obtained for 10–15 individual adhesions from eight to ten cells. Arrows indicate representative adhesion assembly and arrows with dashed line indicate representative adhesion disassembly.

Table 1

Rate constants for formation of paxillin in Pax-KD		
Cell type	Rate Constant for formation (min^{-1})	Fold decrease relative to wild-type paxillin in Pax-KD
Wild-type Paxillin	$(1.9 \pm 0.5) \times 10^{-1}$	-
MEK inhibitor, U012 6	$(1.37 \pm 0.3) \times 10^{-1}$	-
Paxillin ^{S83A}	$(1.4 \pm 0.3) \times 10^{-1}$	-
Paxillin ^{Y31F}	$(2 \pm 0.3) \times 10^{-2}$	9.26

Rate constants for formation of paxillin in BV2		
Cell type	Rate Constant for formation (min^{-1})	Fold decrease relative to wild-type paxillin in BV2
Wild-type BV2	$(1.5 \pm 0.5) \times 10^{-1}$	-
β -Arr2-KD	$(1.18 \pm 0.44) \times 10^{-1}$	-
cSrc-KD	$(3.1 \pm 0.5) \times 10^{-2}$	4.98

Rate constants for disassembly of paxillin in BV2		
Cell type	Rate Constant for disassembly (min^{-1})	Fold decrease relative to wild-type paxillin in BV2
Wild-type BV2	$(1.4 \pm 0.29) \times 10^{-1}$	-
β -Arr2-KD	$(3.43 \pm 0.24) \times 10^{-2}$	3.98
cSrc-KD	$(1.4 \pm 0.35) \times 10^{-1}$	-

Rate constants for disassembly of paxillin in Pax-KD		
Cell type	Rate Constant for disassembly (min^{-1})	Fold decrease relative to wild-type paxillin in Pax-KD
Wild-type Paxillin	$(1.65 \pm 0.24) \times 10^{-1}$	-
MEK inhibitor, U012 6	$(3.4 \pm 0.21) \times 10^{-2}$	4.82
Paxillin ^{S83A}	$(3.1 \pm 0.35) \times 10^{-2}$	4.2
Paxillin ^{Y31F}	$(1.53 \pm 0.3) \times 10^{-1}$	-