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Fibronectin-Mediated Cell Spreading Requires ABBA-Rac1 Signaling

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ABSTRACT

ABBA was reported to be an actin dynamics regulator. However, the molecular mechanism of action of ABBA is still totally obscure. Here, we show that ABBA is ubiquitously expressed in all the examined cultured cells. We found that expression of ABBA in NIH3T3 cells promotes cell spreading. ABBA binds to and markedly promotes cell spreading-induced Rac1 activation. Cell spreading stimulates ABBA activation probably by inducing it tyrosine phosphorylation, which endows ABBA much higher activity to activate Rac1, and attenuates the interaction between ABBA and Rac1. Loss of function suggests that deletion of ABBA in C6-R cells markedly inhibits Rac1 activation and cell spreading; this suggests that and the interaction between ABBA and activated Rac1 is required for ABBA-promoted cell spreading. Taken together, our results indicate that ABBA is activated in response to cell spreading, which markedly promotes cell spreading, and ABBA is required for Rac1 activation and cell spreading. J. Cell. Biochem. 114: 773–781, 2013.

KEY WORDS: ABBA; CELL SPREADING; Rac1 ACTIVATION; MTSS1; MIM

ABBA, MTSS1/MIM, and IRSp53 form an IMD (IRSp53/MTSS1 conserved domain) containing protein family [Machesky and Johnston, 2007; Mattila et al., 2007; Scita et al., 2008]. IRSp53 has been identified as an adaptor protein that links Rac1 with WAVE2 to induce lamellipodia [Miki et al., 2000; Scita et al., 2008] or links Cdc42 with Mena to induce filopodia [Krugmann et al., 2001; Millard et al., 2005]. MTSS1 was initially identified to be a candidate for tumor metastasis suppressor because its expression was missing or down-regulated in metastatic, but present in non-metastatic bladder and prostate cancer cells [Lee et al., 2002; Nixdorf et al., 2004; Utikal et al., 2006; Liu et al., 2010; Wang et al., 2007a; Parr and Jiang, 2009]. Further investigations showed that MTSS1 potentiates Gli-mediated transcription during cell growth and carcinogenesis [Callahan et al., 2004; Gonzalez-Quevedo et al., 2005; Bershteyn et al., 2010]. MTSS1 also acts as a scaffold protein that interacts with Rac1, actin, and protein tyrosine phosphatase delta to modulate actin assembly and cell morphology [Mattila et al., 2003; Woodings et al., 2004; Bompard et al., 2005; Loberg et al., 2005; Lee et al., 2007; Wang et al., 2007; Saarikangas et al., 2011]. The activity of MTSS1 is regulated by tyrosine-phosphorylation [Wang et al., 2007b; Glassmann et al., 2007]. Recently, it was shown that MTSS1 promotes ciliogenesis by antagonizing Src-dependent phosphorylation of Cortactin [Bershteyn et al., 2010]. MTSS1 binds to Daam1 and regulates vertebrate neural tube closure [Liu et al., 2011].

ABBA is also an IMD containing protein, and is a paralog of MTSS1 [Yamagishi et al., 2004]. ABBA contains a WH2 domain, an IMD, a proline-rich domain (PRD) and a serine-rich domain (SRD). High homology between MTSS1 and ABBA suggests that the two proteins could be associated in biological functions [Lee et al., 2007; Machesky and Johnston, 2007]. It was shown that ABBA regulates actin and plasma membrane dynamics in radial glial cells

Xian-Chun Zeng and Xuesong Luo contributed equally to this work.

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[Saarikangas et al., 2008]. However, the molecular mechanism of action of ABBA and its biological significances remain to be characterized.

To explore the cellular functions of ABBA, we prepared an ABBA-specific antibody and examined the endogenous expression of ABBA in normal and tumoral cells. Our results indicated that, unlike MTSS1, which is expressed in a cell-type specific manner, ABBA is expressed in all the examined cells. It suggested that ABBA may play the so called "house keeping" role in a cell. We found that ABBA promotes cell spreading in NIH3T3 cells by promoting cell spreading-induced Rac1 activation. Further experiments showed that cell spreading induces the activation of ABBA, which endows ABBA much higher activity to activate Rac1, and significantly attenuates the interaction between ABBA and Rac1. Loss of function showed that ABBA is required for Rac1 activation and cell spreading. Disruption of the interaction between ABBA and activated Rac1 significantly impaired the spreading of NIH3T3 cells. Our data suggest an indispensable role of ABBA in cell spreading and Rac1 activation, which is probably regulated by tyrosine-phosphorylation.

MATERIALS AND METHODS

ANTIBODY AND REAGENTS

Monoclonal anti-Myc, Rac1 antibody, and 4G10 were purchased from Upstate Biotechnology, Inc. Polyclonal anti-GFP antibody was from Molecular Probes. Anti-ABBA antibody was raised against a peptide: ATPTEETPTPPPAATSD, and was antigen-affinity purified. Rhodamine or fluorescein isothiocyanate-labeled rabbit IgG and mouse IgG, and rhodamine-phalloidin were from Pierce. FBS and FCS were from Hyclone. DMEM medium was from Cambrex. All the other reagents unless otherwise indicated were purchased from Sigma.

PLASMIDS AND CONSTRUCTS

Rac1G12V cDNA was PCR-amplified and cloned into pEF-BOS (Myc-tagged) expression vector. ABBA cDNA was amplified and cloned into pEGFP-N1 vector (BD-Clontech). Retroviral constructs expressing GFP-ABBA, GFP, Myc-Rac1G12V, and Myc-Rac1T17N, respectively, were prepared by cloning the corresponding DNA fragments into *Sac*II and *Not*I sites of retroviral vector MGIN.

CELLS AND CELL CULTURE

NIH3T3 and Hela cells were cultured in DMEM supplemented with 10% FBS. MCF7 and PC3 cells were grown in Ham's F-12/DMEM (1:1) supplemented with 10% FCS. RT4, T24, TccSup, J82, and LNCaP cells were maintained in RPMI supplemented with 10% FCS. NIH3T3 cells stably and moderately expressing GFP-ABBA, GFP, Myc-Rac1G12V, and Myc-Rac1T17N, respectively, were generated by infecting the cells with MGIN retrovirus containing the corresponding DNA fragments, respectively, and screened using G418 at a concentration of 0.6 μg/ml. The cells with GFP fluorescence were sorted by FACS to isolate the cells having moderate GFP fluorescence.

CELL SPREADING ASSAY

Cells were trypsinized, and washed with DMEM medium containing soybean trypsin inhibitor. The cells were collected by centrifugation, and resuspended in DMEM medium containing 10 mM HEPES (pH 7.4), 0.5 mg/ml fatty acid-free bovine serum albumin, and 0.5 mg/ml soybean trypsin inhibitor. The suspended cells were plated onto fibronectin-coated coverslips or cell culture dishes, and cultured at 37°C for 5–180 min.

IMMUNOPRECIPITATION

Cells were lysed with cell lysis buffer (10 mM Tris, pH 7.4, 10 mM NaCl, 5 mM $MgCl_2$, 0.2 mM EGTA, 1 mM Na_3VO_4, 1 mM ammonium molybdate, 0.5% Triton X-100) containing protease inhibitor cocktails (Roche). Cell lysates were precleared by centrifugation at a speed of 15,000 rpm, and then incubated with Myc antibody conjugated agrose beads for 2 h. The beads were washed for three times with lysis buffer. The precipitated proteins were eluted by boiling the samples in 2× SDS dye buffer, and then examined by immunoblotting with an appropriate antibody.

Rac1 ACTIVATION ASSAY

A cDNA fragment coding for PAK-PBD was cloned into pGEX-4T vector to generate a GST fusion protein (GST-PAK-PBD). Production and purification of recombinant GST-PAK-PBD was performed as described previously [Wang et al., 2007b]. The purified GST-PAK-PBD bound to glutathione-Sepharose 4B beads was used to pull down GTP bound Rac1. Precipitated proteins were washed with lysis buffer and then analyzed by Western blot for detection of activated Rac1.

KNOCKDOWN OF ABBA IN C6-R CELLS

C6-R cells were transfected with preannealed ABBA siRNA (targeting ABBA sequence: 5′-AAGGCCATGCGAAAGAGTAT-3′) [Saarikangas et al., 2008] or control siRNA (targeting GFP sequence: 5′-AACAGCCACAACGTCTATATC-3′) using Lipofectamine TM 2000 transfection reagent (Invitrogen). After 24–48 h of culturing, the cells were collected for detection of ABBA expression level.

RESULTS

ABBA IS UBIQUITOUSLY EXPRESSED IN CULTURED CELLS

Because ABBA is highly homologous to MTSS1, it is important to develop a polyclonal antibody that recognizes ABBA, but not MTSS1. Based on the alignment of the amino acid sequences of ABBA and MTSS1, we used a peptide of 17 amino acid residues from ABBA (as shown in light gray color in Fig. 1A) as antigen to raise anti-ABBA antibody in rabbit. The antibody was antigen-affinity purified. We found that ABBA antibody only recognizes GFP-ABBA, but not GFP-MTSS1 (Fig. 1B). ABBA antibody recognized a single band (~80 kDa) in Western blot for J82, 4T1, 4T07, Hela, and NIH3T3 cells, which is exactly consistent with the expected molecular weight of ABBA (Fig. 1C). This data demonstrated good sensitivity and specificity of anti-ABBA antibody, and the constitutive expression of endogenous ABBA in the examined cells.

It was reported that MTSS1 is a candidate for tumor metastasis suppressor, and is only expressed in some non-metastatic tumor cells, and missing or down-regulated in metastatic cells [Wang et al.,

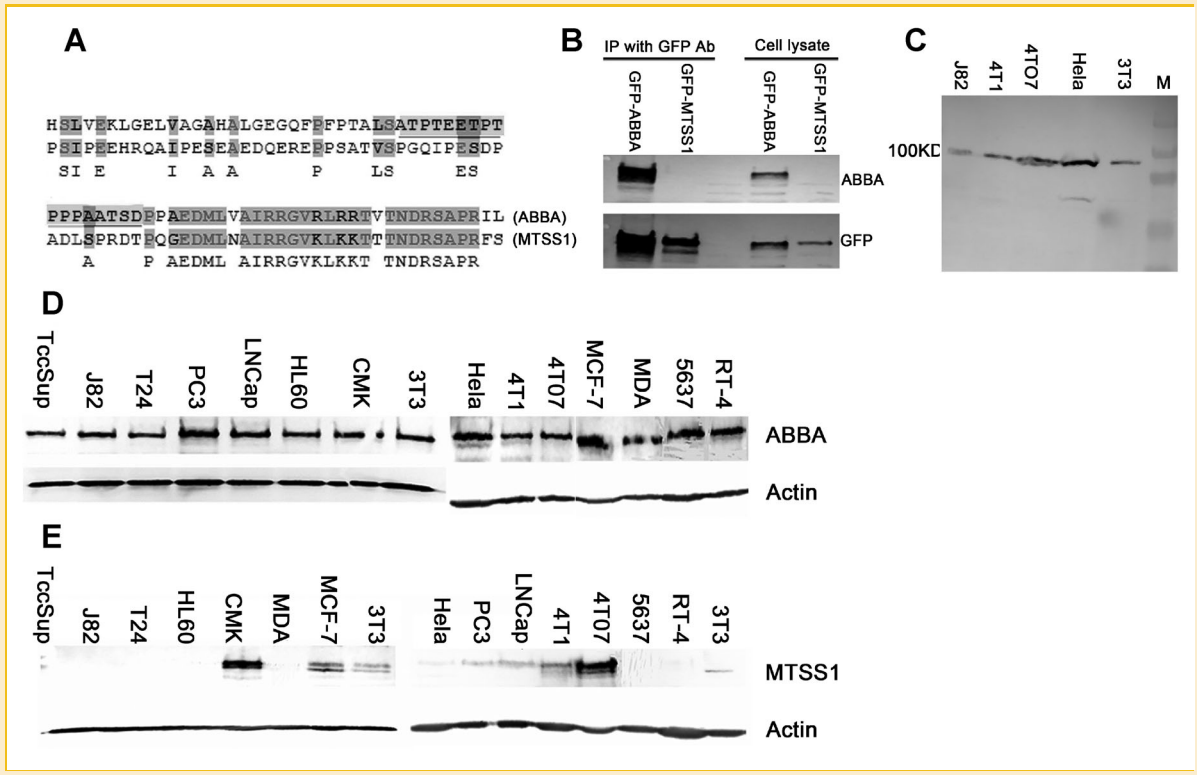

Fig. 1. ABBA is ubiquitously expressed in cultured tumor cells. A–C: The preparation of the ABBA-specific antibody and confirmation of the specificity of the antibody. A: Sequence alignment of the antigen peptide-containing region of ABBA and the corresponding sequence of MTSS1. The light gray region (underlined) stands for the antigen peptide sequence. B: ABBA antibody only recognizes GFP-ABBA, but not GFP-MTSS1. NIH3T3 cells were transfected with GFP-ABBA and GFP-MTSS1 constructs, respectively. The cell lysates were precipitated with GFP antibody. The precipitated proteins were blotted with ABBA antibody (lane 1), and re-blotted with GFP antibody (lane 2). C: ABBA antibody specifically recognized endogenous ABBA and generated only one major band as detected by Western blot. D: Endogenous expression of ABBA in examined cells. Cells were lysed, and the endogenous ABBA was examined by Western blot using ABBA antibody. β-actin was also blotted as a loading control. E: Expression of endogenous MTSS1 in indicated cells. Lysates prepared from the cells was blotted with anti-MTSS1 antibody, and re-blotted with β-actin antibody. MTSS1 antibody was a gift from Dr. Zhan [Wang et al., 2007].

2007; Parr and Jiang, 2009]. ABBA is a close homolog of MTSS1. So it is interesting to examine the expression pattern of endogenous ABBA in different tumor cells. As shown in Figure 1D, ABBA expression is readily detected by Western blot in all the examined cells, including human bladder carcinoma cells (TCCSUP, T24, J82, 5637, and RT-4), human prostate adenocarcinoma cells (PC3 and LNCaP), human promyelocytic leukemia cells (HL60), human megakaryoblastic cell (CMK), mouse fibroblasts NIH-3T3 cell, human cervical cancer cell (Hela), mouse breast cancer cells (4T1 and 4T07) and human breast adenocarcinoma cells (MCF-7, and MDA-MB-231). These cells include normal, non-metastatic and highly metastatic tumor cells from human and mouse. This result indicated that ABBA is ubiquitously expressed in tumor cells, and its expression is not affected by metastatic status of the cells. In contrast, MTSS1 is expressed in a cell type-specific manner (Fig. 1E). This suggests that, although ABBA and MTSS1 are highly homologous, their expressions are differentially regulated.

ABBA PROMOTES CELL SPREADING

Given that ABBA is localized at the leading edge and membrane ruffle of NIH3T3 cells (data not shown), it is likely that ABBA could be involved in cell spreading. To investigate this possibility, we made use of NIH3T3 cells transiently transfected with GFP-ABBA. Cells were trypsinized, washed, plated on fibronectin-coated coverslips and cultured at 37°C. Five minutes later, cells were fixed and stained with GFP antibody and rhodamine-phalloidin. As shown in Figure 2A, GFP-ABBA expressing cells demonstrated robust cell extension, showing a flat, well-spread morphology with smooth membrane edge and without any filopodia seen at the periphery of the cells. In contrast, the neighboring non-transfected cells showed an irregular shape with extensive filopodia at the cell periphery. The cell area of GFP-ABBA expressing cells is much larger than that of the non-transfected cells. These data suggest that over-expression of GFP-ABBA in NIH3T3 cells induces fast spreading at the early stage of cell spreading.

Over-expression of GFP-ABBA may induce abnormal change of physiological conditions in NIH3T3 cells. Therefore, we developed a NIH3T3 cell line stably and moderately expressing GFP-ABBA. We also generated a NIH3T3 cell line stably expressing GFP as a parallel control. Cell spreading assay was performed with the cells. Twenty and sixty minutes after plating, cells were fixed for immunofluorescent staining. Confocal images showed that, 20 min after plating, GFP-ABBA expressing cells exhibited a well-spread disk-like morphology with smooth curvatures in the cell periphery indicating fast and robust spreading of the cells, whereas the control cells demonstrated an atypical morphology with some membrane

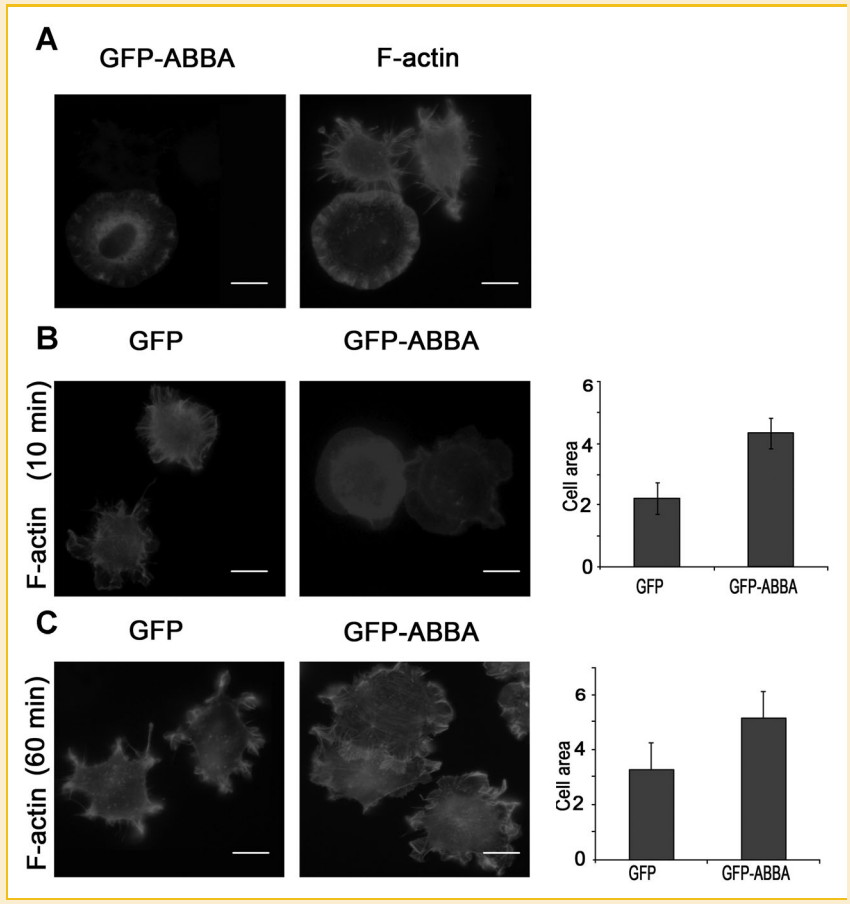

Fig. 2. ABBA promotes cell spreading. A: Over-expression of GFP-ABBA induces fast and robust cell extension at the early stage of cell spreading. NIH3T3 cells were transfected with GFP-ABBA. Cells were trypsinized, washed, and plated on fibronectin-coated coverslips. Ten minutes after plating, the cells were fixed, stained with GFP antibody and FITC-conjugated anti-rabbit IgG, and rhodamine-phalloidin. Fluorescent images were collected by Confocal microscope. B,C: Moderate and stable expression of GFP-ABBA in NIH3T3 cells markedly promotes cell spreading. Cells stably expressing GFP-ABBA or GFP were trypsinized and allowed for spreading for 10 and 60 min, respectively. Cells were then stained with rhodamine-phalloidin for actin. Images were analyzed with ImageJ software. The error bars represent standard error of three independent experiments, with $n > 60$.

protrusions or filopodia seen in the periphery of the cells; 60 min after plating, GFP-ABBA expressing cells demonstrated much more membrane ruffles in the periphery of the cells than the control cells, and some of control cells, but not GFP-ABBA expressing cells, displayed filopodia structures (Fig. 2B,C). We quantitatively measured the two-dimensional cell surface area. We found that, 20 min after plating, the mean area of the cells expressing GFP-ABBA was approximately 90% bigger than that of the control cells (Fig. 2B); and 60 min after plating, the area of GFP-ABBA expressing cells was approximately 60% bigger than that of the control cells (Fig. 2C). This suggests that the expression of GFP-ABBA markedly promotes cell spreading, and the cell area difference between GFP-ABBA expressing cells and the control cells becomes less and less evident as cell spreading progresses. The increase in the area of NIH3T3 cells expressing GFP-ABBA can be explained by a speed-up for cell spreading as we found that the cells expressing GFP-ABBA reached its maximal area approximate 120 min after plating, whereas the cells expressing GFP plateaued around 180 min after plating, and the difference in their maximal areas is undetectable (data not shown).

These results clearly indicated that the expression of ABBA in NIH3T3 cells markedly promotes fibronectin-induced cell spreading. We also examined the effect of MTSS1 on cell spreading. We found that MTSS1 is unable to promote cell spreading (data not shown), although it is highly homologous to ABBA.

ABBA IS ACTIVATED IN RESPONSE TO CELL SPREADING AND MARKEDLY PROMOTES CELL SPREADING-INDUCED Rac1 ACTIVATION

It is well known that cell spreading is regulated by small GTPase Rac1 and both MTSS1 and ABBA bind to and activates Rac1 [Saarikangas et al., 2008; Zheng et al., 2010]. To further analyze the potential of ABBA to promote cell spreading-induced Rac1 activation, we performed Rac1 activation assay and made semi-quantitative analysis for the amount of activated Rac1 in different kinds of cells. The Western blot bands were imaged and quantified using VersaDoc system and Quantity One software (Bio-Rad, Hercules, CA). Cells stably and moderately expressing GFP-ABBA (or GFP alone) were used for cell spreading assay for 0 and 60 min, respectively. Cells were collected for detection of the activated Rac1.

We found that, 0 min after plating, Rac1 activity in the cells expressing GFP-ABBA is approximately 50% higher than that in the GFP-expressing cells (Fig. 3A,B); it suggests that GFP-ABBA significantly promotes Rac1 activation in non-spreading cells. This observation is consistent with our previous finding [Zheng et al., 2010].The Rac1 activity in GFP expressing cells that underwent spreading for 60 min is approximately 80% higher than that in non-spreading GFP-expressing cells (Fig. 3A,B). Thus, it can be inferred that if ABBA had kept the same Rac1-activation activity during cell spreading as that in non-spreading cells, the Rac1 activity in GFP-ABBA expressing cells that underwent spreading for 60 min would be approximately 130% (50 + 80%) higher than that in the quiescent GFP-expressing cells. However, it is fascinating to find that the actual Rac1 activity in GFP-ABBA expressing cells that underwent spreading for 60 min is approximately more than two times higher than that in non-spreading GFP-expressing cells (Fig. 3A,B).This data clearly demonstrates that the expression of GFP-ABBA markedly promotes cell spreading-induced Rac1 activation, and the cell spreading endows ABBA much higher Rac1-activation activity. This strongly suggests that ABBA is activated during cell spreading. It is most likely that ABBA molecules were modified and changed into a new configuration that favored Rac1 activation in response to cell spreading.

CELL SPREADING INDUCES PHOSPHORYLATION OF ABBA AND ATTENUATES THE INTERACTION BETWEEN ABBA AND Rac1

To characterize the nature of the configuration that represents activated ABBA, we examined whether or not cell spreading induces tyrosine-phosohorylation of ABBA. Cells stably expressing GFP-ABBA were used for cell spreading assay for 0 and 60 min, respectively. Cells were collected and lysed. GFP-ABBA was immuno-precipitated from the cell lysates, and blotted with 4G10. The membrane was also blotted with GFP antibody to see if the amount of GFP-ABBA in the samples are similar or not. The result showed that before the cells start to spread, GFP-ABBA is non-phosphorylated; and 60 min after plating, GFP-ABBA becomes tyrosine-phosphorylated (Fig. 4A).

We further examined whether or not the phosphorylation of ABBA affects the interaction between ABBA and Rac1 during cell spreading. Cell spreading assay was performed with the NIH3T3 cells stably expressing Myc-Rac1G12V, which were transfected with GFP-ABBA. Zero and sixty min after plating, cells were lysed and immuno-precipitated with Myc antibody agrose beads. The precipitated proteins were blotted with GFP and Myc antibody, respectively (lanes 1 and 2 in Fig. 4B). Phosphorylation of GFP-ABBA was examined by re-blotting with 4G10 (data not shown). The expression levels of GFP-ABBA in the cell lysates were examined by blotting with GFP antibody (lane 3). We found that tyrosine phosphorylation of GFP-ABBA significantly attenuated the interaction between GFP-ABBA and activated Rac1 (Fig. 4B,C).

This finding was confirmed by pervanadate treatment for the cells expressing GFP-ABBA and Myc-Rac1G12V. The treated cells were immuno-precipitated with anti-Myc antibody agrose beads. Pervanadate is a powerful phosphatase inhibitor that leads to the accumulation of phosphorylated proteins and the activation of kinases that are normally retained in inactive forms by dephosphorylation. As we expected, after pervanadate treatment of the cells, GFP-ABBA was strongly phosphorylated, which markedly attenuated the interaction between GFP-ABBA and Myc-Rac1G12V (Zeng et al., unpublished data).

Taken together, our data suggest that cell spreading induces tyrosine-phosphorylation of ABBA, which may endow ABBA much higher activity to activate Rac1, and attenuates the interaction between ABBA and Rac1.

KNOCKDOWN OF ABBA EXPRESSION INHIBITS Rac1 ACTIVATION AND CELL SPREADING

To further examine whether or not ABBA is indispensible to Rac1 activation and cell spreading, we knocked down ABBA expression with siRNA. Unfortunately, we failed to delete the endogenous expression of ABBA using a set of different siRNAs in NIH3T3 cells.

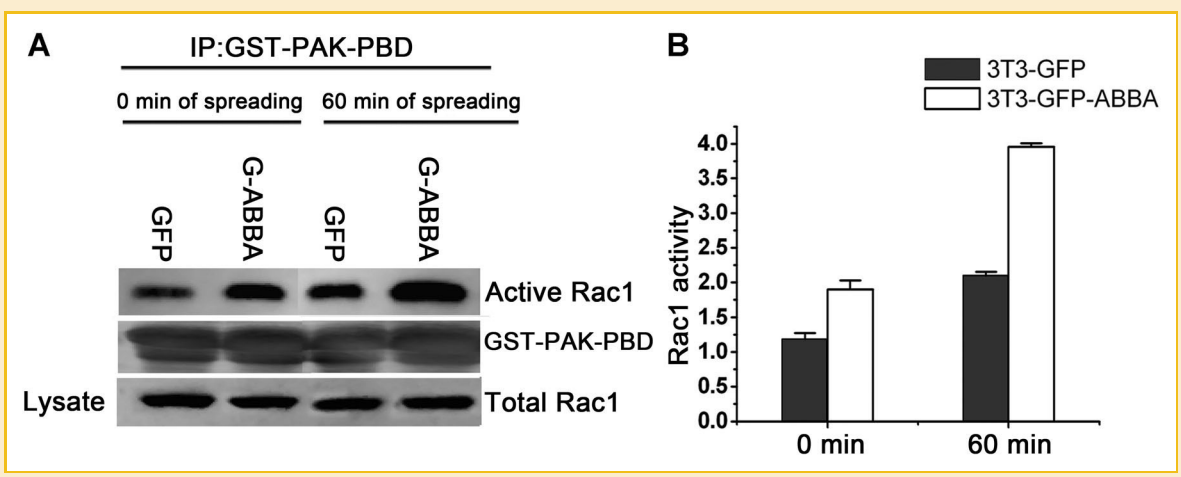

Fig. 3. ABBA is activated in response to cell spreading and markedly promotes cell spreading-induced Rac1 activation. A: ABBA markedly promotes cell spreading-induced Rac1 activation. Cell spreading was performed with NIH3T3 cells stably expressing GFP-ABBA or GFP, respectively. 0 and 60 min after plating, the cells were collected for Rac1 activation assay. B: The Western blot bands were imaged and quantified using VersaDoc system and Quantity One software (Bio-Rad, Hercules, CA).

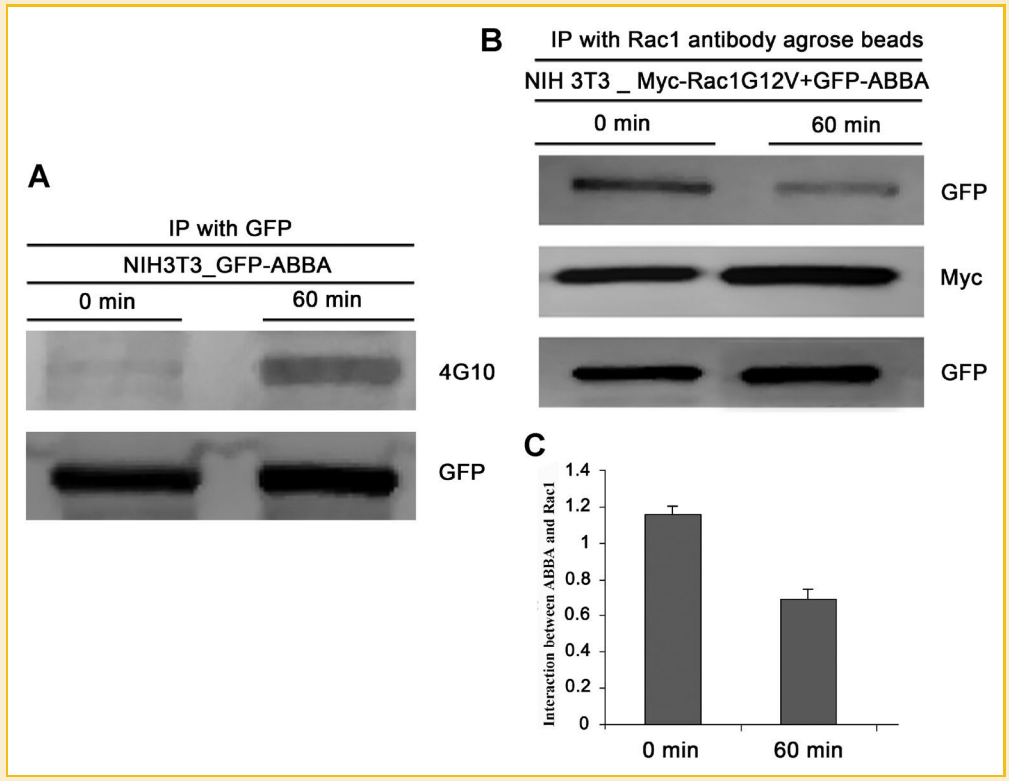

Fig. 4. Cell spreading induces phosphorylation of ABBA and attenuates the interaction between ABBA and Rac1. A: Cell spreading induces tyrosine phosphorylation of GFP-ABBA. NIH3T3 cells stably expressing GFP-ABBA were used for cell spreading assay. Zero and sixty minutes after plating, the cells were collected and lysed. Cell lysates were precipitated with GFP antibody. The precipitated proteins were then blotted with 4G10, and re-blotted with GFP antibody. B: Cell spreading attenuates the interaction between ABBA and Rac1. NIH3T3 cell stably expressing Myc-Rac1G12V was transfected with GFP-ABBA construct. Cell spreading assay was performed with the cells. Zero and sixty minutes after plating, the cells were lysed. Cell lysates were precipitated with Rac1 antibody agarose beads. The precipitated proteins were blotted with GFP antibody (lane 1) and re-blotted with Myc antibody(lane 2). The cell lysates were blotted with GFP antibody to show the expression levels of GFP-ABBA in the samples (lane 3). C: The Western blot bands were imaged and quantified using VersaDoc system and Quantity One software (Bio-Rad, Hercules, CA).

We next tried to knock down the endogenous expression of human ABBA from C6-R cells as described previously [Saarikangas et al., 2008]. As shown in Figure 5A, after 24 h of siRNA transfection, the expression level of endogenous ABBA markedly decreased. Cell spreading and Rac1 activation assay were then performed with the ABBA-deleted and control cells (Fig. 5B). The result showed that 0 min after cell plating, knockdown of ABBA expression markedly inhibited Rac1 activation; 60 min after plating, loss of ABBA abolished cell spreading-induced Rac1 activation (Fig. 5B). Co-expression of ABBA siRNA, and GFP-ABBA mutant (containing some nonsense mutations in siRNA targeting region, and thereby resistant to ABBA siRNA) [Saarikangas et al., 2008] significantly rescued Rac1 activation (Fig. 5B). Our results suggest that ABBA is required for Rac1 activation in quiescent cells, and for cell spreading-induced Rac1 activation.

As shown in Figure 5C and 5D, knockdown of ABBA in C6-R cells markedly inhibited cell spreading; this strongly suggests that ABBA is also required for fibrolectin-induced cell spreading.

INTERACTION BETWEEN ABBA AND ACTIVATED Rac1 IS REQUIRED FOR ABBA-PROMOTED CELL SPREADING

It was reported that a basic patch in IMD is critical for the interaction between ABBA and activated Rac1. If all the 13 lysine residues within the basic patch were mutated into glutamine residues, ABBA would fail to bind to Rac1 [Zheng et al., 2010]. To explore whether or not the interaction between ABBA and activated Rac1 is required for ABBA-promoted cell spreading, we prepared GFP-ABBA$^{K/D}$ mutant. Co-immunoprecipitation was performed to examine the interaction between GFP-ABBA$^{K/D}$ and activated Rac1. NIH3T3 cells stably expressing Myc-Rac1G12V were transfected with GFP-ABBA$^{K/D}$ and GFP-ABBA, respectively. Lysates prepared from these cells were immunoprecipitated with Rac1 antibody agrose beads. Western blot analysis for the precipitated proteins indicated that the interaction between GFP-ABBA$^{K/D}$ and Myc-Rac1G12V markedly decreased (Fig. 6A).

We next investigated the effect of the expression of GFP-ABBA$^{K/D}$ on cell spreading. NIH3T3 cells were transfected with GFP-ABBA$^{K/D}$, GFP-ABBA, and GFP constructs, respectively. Cells were plated on fibronectin-coated coverslips and allowed to spread for 60 min in the incubator. Cells were fixed and stained using GFP antibodies and rhodamine-phalloidin for F-actin. We found that GFP-ABBA$^{K/D}$ expressing cells displayed much poorer spread than GFP-ABBA expressing cells (Fig. 6B). We also found that there were no significant differences between the mean area of the cells expressing GFP-ABBA$^{K/D}$ and that of the cells expressing GFP (Fig. 6B,C). These

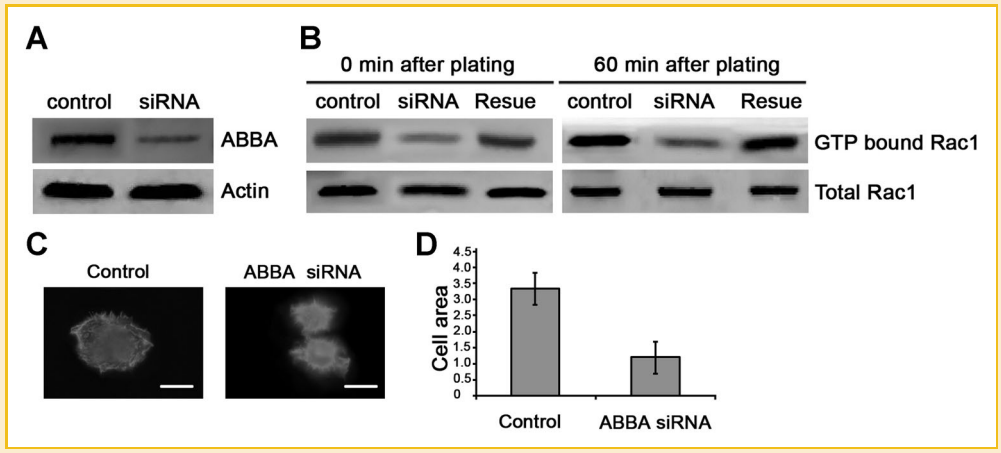

Fig. 5. Deletion of ABBA impaired Rac1 activation. A: Knockdown of endogenous ABBA1 by siRNA. C6-R cells were transfected with ABBA siRNA, and control siRNA, respectively. After 24 h of incubation, cells were lysed, and the endogenous ABBA was examined by Western blot using anti-ABBA1 antibody; β-actin was also blotted as loading control. B: Knockdown of endogenous ABBA inhibits the activation of Rac1. C6-R cells were treated by ABBA siRNA and control siRNA, respectively. C6-R cells were also transfected with both ABBA siRNA and GFP-ABBA mutant that is resistant to ABBA siRNA [Saarikangas et al., 2008] as a control (rescue experiment). After 24 h of culturing, cells were plated for spreading for 0 and 60 min, respectively. The cells were then collected for preparation of total cell lysates. GTP-bound Rac1 was immuno-precipitated with GST-PAK-PBD bound to glutathione-Sepharose 4B beads. The precipitated GTP-bound (lane 1) and total Rac1 (lane 2) were examined by Western blot with anti-Rac1 antibody. The bands were imaged and quantified using VersaDoc system and Quantity One software (Bio-Rad, Hercules, CA). C: Images were collected by Confocal microscope. D: Comparison of the area of the cells treated with ABBA siRNA and control siRNA, respectively.

data suggest that the interaction between ABBA and Rac1 is required for cell spreading.

DISCUSSION

The BAR (Bin-Amphiphysin-Rvs) is a highly conserved protein domain that has been shown to participate in cellular membrane remodeling [Zhao et al., 2011]. There are three distinct families of BAR domain-containing proteins: classical BAR, F-BAR, and I-BAR. Each BAR domain dimer can induce distinct degrees of membrane curvature. I-BAR domain, which is also known as IMD (IRSp53/MTSS1 homology domain), was shown to induce plasma membrane protrusions such as filopodia and lammellipodia [Saarikangas et al., 2009; Robens et al., 2010]. In mammalian cells, I-BAR domain-containing proteins can be classified into two evolutionarily distinct

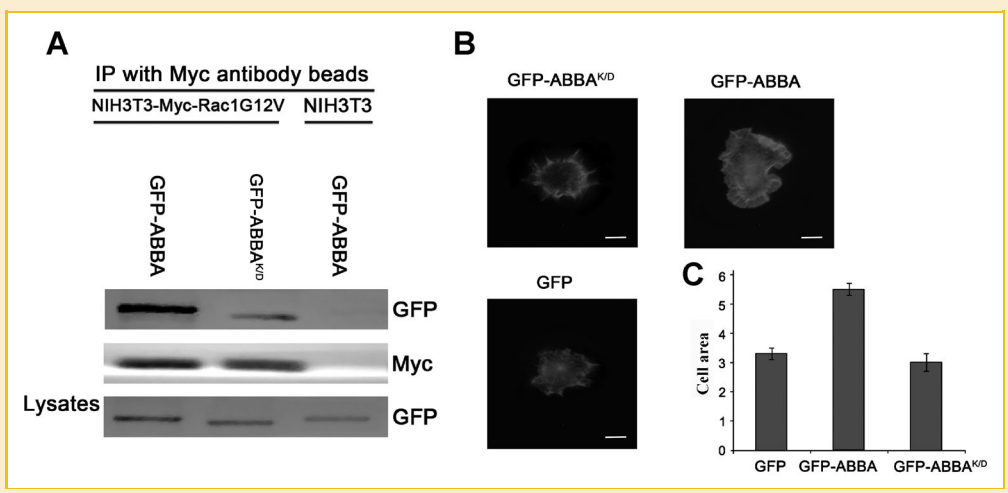

Fig. 6. Interaction between ABBA and activated Rac1 is required for ABBA-promoted cell spreading. A: Mutant GFP-ABBA$^{K/D}$ almost failed to interact with activated Rac1. NIH3T3 cells stably expressing Myc-Rac1G12V were transfected with GFP-ABBA and GFP-ABBA$^{K/D}$, respectively. NIH3T3 cells were transfected with GFP-ABBA as a negative control. Cell lysates prepared from the cells were immuno-precipitated with Myc monoclonal agrose beads. The precipitated proteins were blotted with GFP antibody (lane 1), and re-blotted with Myc antibody (lane 2). The cell lysates were also blotted with GFP antibody showing the expression levels of GFP-ABBA, GFP-ABBA$^{K/D}$ or GFP in the cells, respectively (lane 3). B: NIH3T3 cells stably expressing GFP-ABBA, GFP-ABBA$^{K/D}$ or GFP were used for cell spreading assay. After being cultured for 60 min, the cells were stained with GFP antibody and rhodamine-phalloidin. Images were collected by Confocal microscope. C: Comparison of the area of the cells expressing GFP, GFP-ABBA and GFP-ABBA$^{K/D}$, respectively.

subfamilies: one including MTSS1 and ABBA, and the other one consisting of IRSp53, IRTKS, and FLJ22582 [Millard et al., 2007]. It was shown that I-BAR domain binds to Rac1. We thus speculated that these proteins could potentiate Rac1-mediated cell spreading. However, our data demonstrated that only ABBA, but neither MTSS1 nor IRSp53 is able to significantly promote cell spreading.

ABBA is a close homolog of MTSS1. We showed that their expression patterns in cultured tumoral cells are quite different. ABBA is constitutively expressed and abundant in all the examined cells; in contrast, MTSS1 is only expressed in a cell type-specific manner (Fig. 1D). It strongly suggests that ABBA and MTSS1 would display different biochemical properties. Our data demonstrated that MTSS1 has much higher activity to activate Rac1 than ABBA in non-spreading NIH3T3 cells (Zeng et al., unpublished results). However, we found that ABBA markedly promotes cell spreading, whereas MTSS1 doesn't display any cell spreading-promoting activity (data not shown). This suggests that ABBA and MTSS1 could be involved in different signaling cascades.

It is striking to find that cell spreading induces the activation of ABBA probably by tyrosine phosphorylation, which endows ABBA much higher Rac1-activation activity than that of the ABBA molecules in non-spreading cells. Activated ABBA demonstrates relatively weaker binding affinity to activated Rac1. It is interesting to further figure out the detailed mechanism by which the ABBA molecule is activated in response to cell spreading.

Moreover, we found that deletion of ABBA impaired Rac1 activation in C6-R cells. We also found that GFP-ABBA$^{K/D}$ mutant almost failed to interact with activated Rac1, and thus failed to promote cell spreading in NIH3T3 cells (Fig. 6B). These observations suggest an indispensable role of ABBA in Rac1-mediated cell spreading in NIH3T3 cells.

Rac1 is ubiquitously expressed and regulates cell adhesion, migration, transcription, proliferation, and differentiation [Benninger et al., 2007; Rose et al., 2007]. Deregulated expression or activation patterns of Rac1 can result in aberrant cell signaling and numerous pathological conditions [Fritz and Kaina, 2006; Chae et al., 2008; Bosco et al., 2009]. Our data support a unique role of ABBA as a signaling transducer, linking Rac1 signaling to cell spreading.

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