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Role of Dbl's big sister in the anti-mitogenic pathway from α1B-adrenergic receptor to c-Jun N-terminal kinase

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Abstract

We previously reported that the α1B-adrenergic receptor leads to activation of Rho family small GTPases, and in turn, c-Jun N-terminal kinase (JNK), which results in the inhibition of cell proliferation. Here, we show the involvement of the Rho family guanine nucleotide exchange factor (GEF) Dbl's Big Sister (Dbs) in the signaling pathway. Transfection of a Dbl-homology (DH) and pleckstrin-homology (PH) domain-deficient form of Dbs into cells blocked the α1B-adrenergic receptor-induced activation of JNK. Conversely, transfection of an isolated DH domain of Dbs induced JNK activation. Stimulation of the α1B-adrenergic receptor enhanced an intrinsic Cdc42-GEF activity of Dbs in a manner dependent on Src family tyrosine kinases. Additionally, DH and PH domain deficient Dbs blocked the receptor-induced inhibition of cell proliferation, while DH domain of Dbs inhibited cell proliferation via the JNK-dependent pathway. Taken together, Dbs may play an important role in the anti-mitogenic JNK pathway downstream of the α1B-adrenergic receptor.

Keywords: Dbs; α1B-adrenergic receptor; Src; Cdc42; JNK

G protein-coupled receptors (GPCRs) are the largest known family of human cell-surface receptors, encompassing ~1000 distinct receptors. GPCRs are activated by a variety of natural ligands, such as hormones, neurotransmitters, and chemokines [1,2]. Growing evidence suggests that GPCRs stimulate the activity of c-Jun N-terminal kinase (JNK), a subfamily of mitogen-activated protein kinases (MAPKs) [3,4]. JNK activation by GPCRs/G proteins often involves certain tyrosine kinases and Rho family small GTPases RhoA, Rac1, and/or Cdc42 [3–8]. We previously reported that activation of the prototypic Gq-coupled α1B-adrenergic receptor, which stimulates a phospholipase C/protein kinase C cascade, induces to activation of a JNK kinase MKK4, and in turn JNK, resulting in inhibition of cell proliferation [7,8]. The anti-mitogenic pathway from the α1B-adrenergic receptor to JNK requires activation of Src family tyrosine kinases and Rho family small GTPases; the Src family tyrosine kinases acting upstream of the Rho family small GTPases [7]. However, it remains unclear how Src family tyrosine kinases activate Rho family small GTPases in the signal transduction pathway downstream of GPCRs, including the α1B-adrenergic receptor.

Rho family small GTPases form a subfamily of low molecular weight guanine nucleotide-binding proteins distinct from the Ras family [9,10]. Rho family small GTPases exist as an active GTP-bound or an inactive GDP-bound state upon stimulation of extracellular signals. The activities of Rho family small GTPases are controlled positively by their guanine nucleotide exchange factors (GEFs), which stimulate dissociation of GDP from Rho family · GDP to form Rho family · GTP, and negatively by their GTPase activating proteins (GAPs), which accelerate their GTPase activities. RhoA, Rac1, and Cdc42 of Rho family small

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GTPases participate in the regulation of dynamic assembly of cytoskeletal components. These small GTPases stimulate upstream kinases of JNK, as Ras upregulates the activities of Raf kinases. Therefore, Rho family small GTPases can convey signals controlling gene expression, cell cycle, and cytokinesis.

Rho family GEFs, which are known as Dbl family proteins, contain a Dbl homology (DH) domain, responsible for the exchange activity and a pleckstrin homology (PH) domain possibly involved in the regulation of the exchange activity [9,10]. Despite the identification of numerous members of Rho family GEFs, the mechanism by which GPCRs activate the GEFs is not fully understood, except in the case of Rho subfamily-specific GEFs containing the regulator of G protein signaling (RGS) domain and Rac subfamily-specific GEFs [11–16]. However, these Rho family GEFs are activated directly by G proteins through tyrosine kinase-independent mechanism [11–16].

During a study of the anti-mitogenic pathway through JNK in cells expressing the α1B-adrenergic receptor [7,8], we found that the receptor-induced JNK activation required a Rho family GEF Dbl's Big Sister (Dbs), which is ubiquitously expressed in human tissues. Stimulation of the α1B-adrenergic receptor enhanced a Cdc42-GEF activity of Dbs in a manner dependent on Src family tyrosine kinases. We also found that Dbs was involved in the anti-mitogenic response downstream of the α1B-adrenergic receptor. These findings provide insights into our understanding of GPCR-mediated Rho family GEF activation and the cellular function.

Materials and methods

Antibodies. A Mouse monoclonal antibody M2 against FLAG-peptide was purchased from Sigma–Aldrich (St. Louise, MO). A mouse monoclonal antibody B-14 against *Schistosoma japonicum* glutathione-*S*-transferase (GST) was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). A mouse monoclonal antibody 1E4 against *Aequorea victoria* green fluorescence protein (GFP) was purchased from Medical and Biological Laboratories (Nagoya, Japan). A mouse monoclonal anti-phosphorylated tyrosine antibody 4G10 was obtained from Upstate Biotechnology (Lake Placid, NY). Rabbit polyclonal antibodies C-19, SRC2, and C-17 against Gαq/11, Src family tyrosine kinases, and JNK1, respectively, were purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-mouse and anti-rabbit IgG antibodies conjugated with horseradish peroxidase were obtained from Amersham Biosciences (Buckinghamshire, UK).

Inhibitors. PP1 and PP2, inhibitors of Src family tyrosine kinases, were purchased from Biomol (Plymouth Meeting, PA) and Calbiochem–Novabiochem (San Diego, CA), respectively.

Plasmids. The cDNA of KIAA0362, a human ortholog of mouse Dbs [17], was provided by Drs. F. Miki and T. Nagase (Kazusa DNA Research Institute, Chiba, Japan). The region encoding the Dbs protein was subcloned into the *Bgl*II/*Bam*HI restriction sites of the mammalian FLAG-tag expression vector pCMV-FLAG [5]. The pCMV-FLAG-Dbs (ΔDHPH) plasmid lacking amino acids 725–918 of Dbs was constructed by digestion with the endogenous *Bgl*II restriction sites of Dbs cDNA. A portion (amino acids 639–845) of Dbs was amplified from Dbs cDNA as a template and ligated into the *Bam*HI restriction site of the mammalian GST-tag expression vector pCMV-GST [5], generating pCMV-GST-Dbs-DH. The cDNAs of RhoA, Rac1, and Cdc42 were amplified from total RNA of human kidney 293T cells, using the method of reverse transcriptase-polymerase chain reaction, and were inserted into pCMV and the *Escherichia coli* GST-tag expression vector pET42a. The pEGFP-N3-α1B-adrenergic receptor plasmid was constructed as described previously [7,8]. pUSE-CA-Src (a constitutively activated mutant of c-Src) was purchased from Upstate Biotechnology (Lake Placid, NY). pCMV-GST-MKK4, pCMV-FLAG-MKK4K95R, pCMV-FLAG-Cdc42G12V, and pCMV-FLAG-Cdc42T17N were constructed as described previously [5]. The *E. coli* expression plasmid encoding a kinase-deficient form of Mpk2, a MAPK of *Xenopus laevis*, was provided by Dr. E. Nishida (Kyoto University, Kyoto, Japan). The *E. coli* expression plasmid encoding N-terminus (amino acids 1–221) of c-Jun was provided by Dr. M. Karin (University of California, San Diego, CA). All DNA sequences were confirmed using a MegaBASE 1000 DNA sequencer (Amersham Biosciences, Buckinghamshire, UK), according to manufacturer's protocol.

Recombinant proteins. Recombinant GST-tagged Rho family small GTPases were purified using *E. coli* BL21 (DE3) pLysS (Novagen, Madison, WI). *E. coli* cells treated with 0.4 mM isopropyl-1-thio-β-D-galactopyranoside for 3 h were harvested by centrifugation and a cell-free extract was made by sonication in extraction buffer A (50 mM Tris–HCl (pH 7.5), 5 mM $MgCl_2$, 1 mM dithiothreitol, 1 mM phenylmethyl sulfonyl fluoride, 1 μg/ml leupeptin, and 1 mM EDTA) containing 0.5% NP-40, 0.1 μM GDP, and 70 U/ml DNase I (Takara, Kyoto, Japan) on ice. All purification steps were performed at 4 °C. The cell extracts were clarified by centrifugation (150,000*g*, 30 min). The supernatants were applied to glutathione–Sepharose 4B (Amersham Biosciences, Buckinghamshire, UK) and the resin was washed with elution buffer A (100 mM Tris–HCl (pH 8.0), 2 mM $MgCl_2$, 1 mM dithiothreitol, 1 mM phenylmethyl sulfonyl fluoride, and 1 μg/ml leupeptin) containing 0.1 μM GDP. The GST-tagged proteins were eluted with elution buffer A containing 0.1 μM GDP and 20 mM glutathione, and dialyzed against dialysis buffer A (10 mM HEPES–NaOH (pH 7.5), 2 mM $MgCl_2$, 150 mM NaCl, 1 mM dithiothreitol, 1 mM phenylmethyl sulfonyl fluoride, and 1 μg/ml leupeptin) containing 0.1 μM GDP. Samples were stored at −80 °C until use. Recombinant hexahistidine-tagged Mpk2 and GST-tagged c-Jun were purified as described previously [9–11]. Recombinant c-Src, which was expressed in Sf9 insect cells and purified, was purchased from Upstate Biotechnology (Lake Placid, NY).

Cell culture, transfection, and counting. Human kidney epithelial-like 293T cells and 293T cells stably expressing GFP-tagged α1B-adrenergic receptor (293T-α1B) were maintained as described previously [8]. The GFP-tagged α1B-adrenergic receptor binds to agonists and antagonists, and stimulates phosphatidylinositol/Ca^{2+} signaling in a similar fashion to the wild type receptor [18,19]. Plasmid DNAs were transfected into 293T cells using Calphos Mammalian Transfection Kit (BD Bioscience Clontech, Palo Alto, CA), according to manufacturer's protocol. The final amount of the transfected DNA for a 10-cm dish was adjusted to 25 μg by addition of empty vector, pCMV. 293T cells were co-transfected with 1 μg pCMV-FLAG-Dbs, 10 μg pCMV-FLAG-Dbs (ΔDHPH), 10 μg pCMV-GST-Dbs-DH, 3 μg pCMV-GST-MKK4, 10 μg pCMV-FLAG-MKK4K95R, 0.3 μg pEGFP-N3-α1B-adrenergic receptor, or 3 μg pUSE-CA-Src. The medium was replaced 24 h after transfection and 293T cells were starved in serum-free medium for 24 h before the addition of 20 μM phenylephrine for 15–20 min. For cell proliferation assay, 293T-α1B and parental 293T cells were seeded at 0.5×10^6 cells per well into 6-well plates. Cells were incubated overnight and then transfected using Lipofectamine Plus (Invitrogen, Carlsbad, CA), according to manufacturer's protocol. The final amount of the transfected DNA was adjusted to 2 μg by addition of empty vector, pCMV. Cells were co-transfected with 1 μg pCMV-FLAG-Dbs (ΔDHPH), pCMV-GST-Dbs-DH, pCMV-FLAG-MKK4K95R, pCMV-FLAG-Cdc42T17N, or pCMV-FL

AG-Cdc42G12V. Cells were treated with or without 20 μM phenylephrine 24 h post-transfection and cultured for a further 24 h. 293T-α1B and 293T cells were stained with 0.3% trypan blue and viable cells were manually counted using a hemacytometer, as described previously [20]. Trypan blue-incorporating cells numbered fewer than 1% in each experiment.

Immunoprecipitation and immunoblotting. Transfected 293T cells were lysed in 900 μl lysis buffer (20 mM HEPES–NaOH (pH 7.5), 3 mM $MgCl_2$, 100 mM NaCl, 1 mM dithiothreitol, 1 mM phenylmethyl sulfonyl fluoride, 1 μg/ml leupeptin, 1 mM EGTA, 1 mM Na_3VO_4, 10 mM NaF, 20 mM β-glycerophosphate, and 0.5% NP-40) for a 10-cm dish, and the lysates were centrifuged, as described previously [5–8]. Aliquots (800 μg) of the supernatants were mixed with protein G–Sepharose CL-4B (Amersham Biosciences, Buckinghamshire, UK) pre-absorbed with 0.5 μg anti-JNK1 antibody or 1 μg anti-FLAG antibody, or with glutathione–Sepharose 4B. The immune- or affinity-complexes were precipitated by centrifugation and washed twice with lysis buffer and twice with kinase buffer A (20 mM HEPES–NaOH (pH 7.5), 10 mM $MgCl_2$, 1 mM dithiothreitol, 0.1 mM phenylmethyl sulfonyl fluoride, 0.1 μg/ml leupeptin, 0.1 mM EGTA, 10 μM Na_3VO_4, and 2 mM β-glycerophosphate), kinase buffer B (20 mM HEPES–NaOH (pH 7.5), 10 mM $MgCl_2$, 3 mM $MnCl_2$, 1 mM dithiothreitol, 0.1 mM phenylmethyl sulfonyl fluoride, 0.1 μg/ml leupeptin, 0.1 mM EGTA, 10 μMNa_3VO_4, and 2 mM β-glycerophosphate), or exchange buffer (20 mM HEPES–NaOH (pH 7.5), 5 mM $MgCl_2$, 150 mM NaCl, 1 mM dithiothreitol, 0.1 mM phenylmethane sulfonylfluoride, 0.1 μg/ml leupeptin, and 1 mM EDTA). To compare the amounts of immuno- or affinity-precipitate or expressed protein in the cell lysates in each transfection, the precipitates or aliquots of the cell lysates were boiled in Laemmli sample buffer and then separated on 8% or 15% SDS–polyacrylamide gels. The electrophoretically separated proteins were transferred to a PVDF membrane. The membranes were blocked with Block Ace (Dainihon-Seiyaku, Osaka, Japan) or phosphate-buffered saline containing 0.05% Tween 20 and 5 mg/ml bovine serum albumin and immunoblotted with various antibodies. The bound antibodies were detected using the ECL or ECL Plus system (Amersham Biosciences, Buckinghamshire, UK) with anti-rabbit or anti-mouse IgG antibodies, conjugated with horseradish peroxidase, according to manufacturer's protocol. Images of protein bands were captured using an Epson GT-7000U scanner and Adobe Photoshop 5.0 plug-in software. The band intensities of kinases and exchangers were semi-quantified using NIH Image 1.61. The representatives of at least three separate experiments are shown in figures.

Kinase assays. After the addition of 20 μM phenylephrine for 20 min, transfected 293T cells were lysed in 900 μl lysis buffer for a 10-cm dish and the lysates were centrifuged, as described above. Aliquots (800 μg) of the supernatants were used for JNK or MKK4 assay. The activities of the immobilized JNK and MKK4 proteins were measured as the radioactivity incorporated into c-Jun and Mpk2, respectively, using a BAS2500 imaging analyzer (Fujifilm, Tokyo, Japan), as described previously [5–8]. Activities were normalized to the amounts of kinases in the immuno- or affinity-precipitates.

Exchange assays. After the addition of 20 μM phenylephrine for 15 min, transfected 293T cells were lysed in 900 μl lysis buffer for a 10-cm dish and the lysates were centrifuged, as described above. Aliquots (800 μg) of the supernatants were used for exchange assays. For binding assays, as described previously [20,21], the affinity precipitated DH domain of Dbs and the immunoprecipitated Dbs protein were incubated in 30 μl exchange buffer containing 16 ng/μl Rho family small GTPase, 50 ng/μl bovine serum albumin, and 3 μM [α-^{32}P]GTP (0.1 μCi/μl) at 30 °C for 0, 20, or 40 min (Fig. 3) and 20 min (Fig. 4). The reactions were stopped by adding 1 ml ice-cold wash buffer (20 mM HEPES–NaOH (pH 7.5) and 10 mM $MgCl_2$) and filtered through BA85 nitrocellulose membranes (Schleicher & Schuell, Dassel, Germany). The membranes were immediately washed three times with ice-cold wash buffer and air-dried. The radioactivity remaining on each membrane was measured by an LSC3500 liquid scintillation counter (Aloka Japan, Tokyo, Japan). For release assays, the Cdc42 · [β-^{32}P]GDP complex was obtained by incubation with exchange buffer containing 125 ng/μl Cdc42, 250 ng/μl bovine serum albumin, and 3 μM [β-^{32}P]GDP (0.75 μCi/μl) at 30 °C for 90 min, as described previously [22]. The reactions were stopped by adding 5 mM $MgCl_2$ and the mixtures were immediately cooled on ice. The immunoprecipitated Dbs protein was incubated in 30 μl exchange buffer containing 16 ng/μl Cdc42 · [β-^{32}P]GDP, 33 ng/μl bovine serum albumin, and 1 μM cold GDP at 30 °C for 0, 10, or 20 min. The reactions were stopped by adding ice-cold wash buffer and filtered through nitrocellulose membranes. The membranes were immediately washed with wash buffer and air-dried. The radioactivity remaining on each membrane was determined as before. Activities were normalized to the amounts of exchangers in the immuno- or affinity-precipitates.

In vitro tyrosine phosphorylation. Transfected 293T cells were lysed in 900 μl of lysis buffer for a 10-cm dish, and the lysates were centrifuged, as described above. Aliquots (800 μg) of the supernatants were used for an in vitro tyrosine phosphorylation reaction using recombinant c-Src. The immobilized Dbs protein was incubated with recombinant c-Src (9 U) in 30 μl kinase buffer B containing 20 μM ATP at 30 °C for 15 min and chilled on ice. The tyrosine phosphorylated Dbs protein was washed twice with lysis buffer and twice with exchange buffer, and used in an exchange reaction for Cdc42.

Statistical analysis. Statistical analysis was performed using Microsoft Excel 98 and SAS StatView 5.0. Values shown represents means ± SE from at least three separate experiments.

Results and discussion

Involvement of Dbs in the signaling pathway from the α1B-adrenergic receptor to JNK

To investigate the involvement of Dbs in the anti-mitogenic pathway from the Gq-coupled α1B-adrenergic receptor to JNK, 293T cells were co-transfected with the plasmids encoding the α1B-adrenergic receptor with Dbs (ΔDHPH), a mutated form which lacks the DH and PH domains. It is known that in other Rho family GEFs, the truncated form lacking the DH and PH domains and the full-length form carrying a mutation within the DH domain show a dominant-inhibitory effect [12,23,24]. Using an anti-JNK antibody, JNK was immunoprecipitated from the cell lysate and the in vitro kinase activity of JNK was assayed as the ^{32}P radioactivity incorporated into recombinant c-Jun. Addition of phenylephrine, a specific agonist of α1-adrenergic receptors, stimulated JNK activity (Fig. 1). Pretreatment of cells with prazosin, an antagonist of the α1-adrenergic receptors, abolished JNK activation by phenylephrine (data not shown). JNK activation induced by phenylephrine was inhibited by co-transfection of Dbs (ΔDHPH) (Fig. 1A), suggesting that Dbs is necessary for JNK activation induced by the α1B-adrenergic receptor.

Next, we examined the effect of Dbs (ΔDHPH) on MKK4 activation by the α1B-adrenergic receptor. Activation of the α1B-adrenergic receptor stimulates only a JNK activator MKK4, but not another JNK activator MKK7 [7]. Cells were co-transfected with the plasmid encoding GST-MKK4 and the expressed GST-MKK4

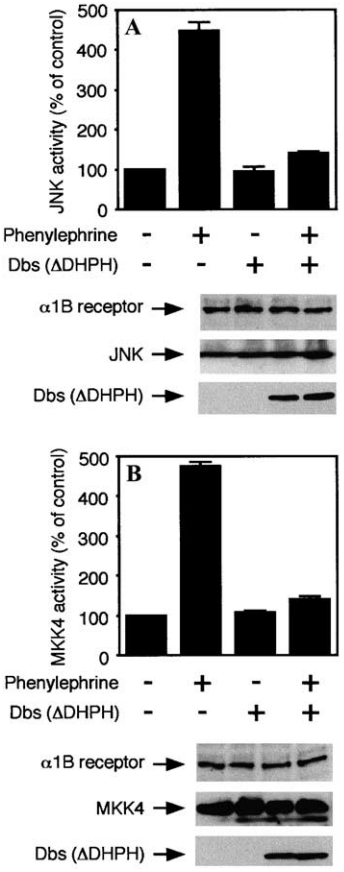

Fig. 1. Involvement of Dbs in the pathway from the α1B-adrenergic receptor to JNK. (A) Cells were transfected with the plasmids encoding the α1B-adrenergic receptor and Dbs (ΔDHPH), and treated with phenylephrine. JNK activity was assayed as described in Materials and methods. JNK was immunoprecipitated with an anti-JNK antibody from the cell lysates and immunoblotted with an anti-JNK antibody. (B) Cells were transfected with the plasmids encoding the α1B-adrenergic receptor, Dbs (ΔDHPH), and MKK4, and treated with phenylephrine. MKK4 activity was assayed as described in Materials and methods. GST-MKK4 was precipitated with glutathione–Sepharose 4B from the cell lysates and immunoblotted with an anti-GST antibody. Expression of α1B-adrenergic receptor and Dbs (ΔDHPH) is shown.

was affinity precipitated from the cell lysate, using glutathione-resin. The in vitro kinase activity of GST-MKK4 was assessed as the ^{32}P radioactivity incorporated into recombinant Mpk2. As shown in Fig. 1B, phenylephrine-induced MKK4 activation was blocked by co-transfection of Dbs (ΔDHPH). These results suggest that Dbs is necessary for activation of the JNK cascade induced by the α1B-adrenergic receptor.

We studied whether an isolated catalytic DH domain of Dbs (Dbs-DH) can lead to JNK activation. It has been demonstrated that the isolated DH domain of Rho family GEF can function as an activator of Rho family small GTPases [25,26]. As shown in Fig. 2A, transfection of the plasmid encoding Dbs-DH resulted in an increase of JNK activity, suggesting that Dbs has the ability to activate JNK.

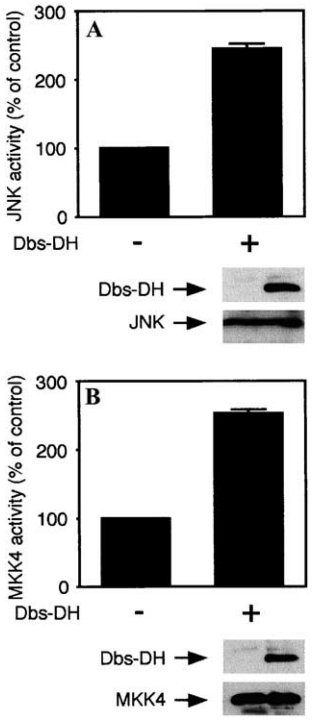

Fig. 2. DH domain of Dbs can stimulate JNK cascade. (A) Cells were transfected with the plasmid encoding Dbs-DH. JNK activity was assayed as described in Materials and methods. JNK was immunoprecipitated with an anti-JNK antibody from the cell lysates and immunoblotted with an anti-JNK antibody. (B) Cells were transfected with the plasmids encoding Dbs-DH and MKK4. MKK4 activity was assayed as described in Materials and methods. GST-MKK4 was precipitated with glutathione–Sepharose 4B from the cell lysates and immunoblotted with an anti-GST antibody. Expression of Dbs-DH is shown.

Likewise, as shown in Fig. 2B, Dbs-DH was able to increase MKK4 activity, indicating that Dbs can activate the JNK cascade. Considering the results from Fig. 1, activation of the JNK cascade induced by the α1B-adrenergic receptor may involve Dbs.

Enhancement of the Cdc42-GEF activity of Dbs by the α1B-adrenergic receptor

To examine which Rho family small GTPase is activated by the catalytic DH domain of Dbs, the ability to incorporate [^{32}P]GTP into recombinant Rho family small GTPases RhoA, Rac1, and Cdc42 was determined. As shown in Figs. 3A–C, the isolated DH domain activated RhoA and Cdc42, and to a lesser extent, Rac1. These results suggest that Dbs has the ability of RhoA- and Cdc42-GEF, and to a lesser extent, of Rac1-GEF, consistent with the result of [17].

To clarify whether GEF activity of Dbs is enhanced by stimulation of the α1B-adrenergic receptor, we tried to measure GEF activity of the immunoprecipitated Dbs. Following stimulation of the α1B-adrenergic receptor, GEF activity of Dbs for Cdc42 was enhanced,

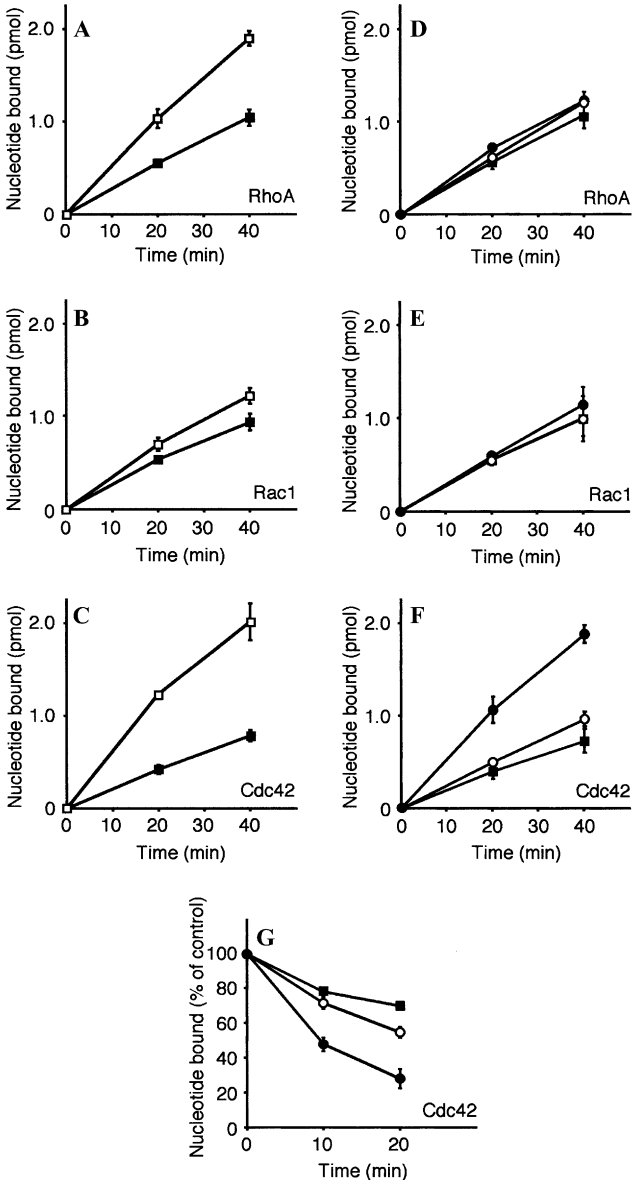

Fig. 3. Activation of the Cdc42-GEF activity of Dbs upon stimulation of the α1B-adrenergic receptor. (A–C) Cells were transfected with mock plasmid (close square) or the plasmid encoding Dbs-DH (open square). Binding of guanine nucleotide to RhoA (A), Rac1 (B), and Cdc42 (C) was assayed as described in Materials and methods. (D–G) Cells were transfected with mock plasmid (close square) or the plasmids encoding α1B-adrenergic receptor and Dbs (open circle and close circle), and treated without (open circle) or with phenylephrine (close circle). Binding of guanine nucleotide to RhoA (D), Rac1 (E), and Cdc42 (F) of Dbs was assayed as described in Materials and methods. Release of guanine nucleotide from Cdc42 (G) was assayed as described in Materials and methods.

but no effect was observed for RhoA or Rac1 (Figs. 3D–F). Phenylephrine-induced activation of the Cdc42-GEF activity of Dbs was blocked by pretreatment with prazosin (data not shown). We analyzed GEF activity to induce the release of [^{32}P]GDP from Cdc42. Dbs enhanced the release of [^{32}P]GDP from Cdc42 upon stimulation of the α1B-adrenergic receptor (Fig. 3G). These results suggest that activation of the α1B-adrenergic receptor enhances GEF activity only for Cdc42 of Dbs.

Tyrosine kinase-dependent activation of the Cdc42-GEF activity of Dbs by the α1B-adrenergic receptor

It is known that tyrosine kinases act as the upstream regulator of Rho family small GTPases in certain signaling pathways [6,9,10]. Additionally, we previously showed that Src family tyrosine kinases are required for activation of Rho family small GTPases in the pathway downstream of the α1B-adrenergic receptor [7]. Thus, we investigated whether the α1B-adrenergic receptor-induced activation of the Cdc42-GEF activity of Dbs requires Src family tyrosine kinases. As shown in Fig. 4A, phenylephrine-induced activation of the Cdc42-GEF activity of Dbs was inhibited by pretreatment of PP1, an inhibitor of Src family tyrosine kinases. Similarly, pretreatment of PP2, another inhibitor of Src family tyrosine kinases, blocked phenylephrine-induced activation of the Cdc42-GEF activity (data not shown). It is likely that Src family tyrosine kinases are necessary for the receptor-induced activation of the Cdc42-GEF activity of Dbs in cells.

Therefore, we examined whether Src family tyrosine kinases can increase the Cdc42-GEF activity of Dbs in cells. We co-transfected the plasmid encoding CA-Src, a constitutively activated mutant of c-Src, with Dbs, into cells. As shown in Fig. 4B, CA-Src was able to increase the Cdc42-GEF activity of Dbs. Taken together with Fig. 4A, Src family tyrosine kinases may mediate the α1B-adrenergic receptor-induced activation of the Cdc42-GEF activity of Dbs in cells.

We investigated whether activation of the α1B-adrenergic receptor induces tyrosine phosphorylation of Dbs in cells. As shown in Fig. 4C, the α1B-adrenergic receptor induced tyrosine phosphorylation of Dbs. Additionally, the receptor-induced tyrosine phosphorylation of Dbs was inhibited by pretreatment of PP1, suggesting that Src family tyrosine kinases are necessary for the receptor-induced tyrosine phosphorylation of Dbs in cells. Dbs was tyrosine phosphorylated even in the unstimulated condition, when co-transfected with the α1B-adrenergic receptor (Fig. 4C). However, it was unlikely that Src family tyrosine kinases contributed to the tyrosine phosphorylation of Dbs, because pretreatment of PP1 failed to suppress it (Fig. 4C).

We examined whether Src family tyrosine kinases can induce tyrosine phosphorylation of Dbs in cells. As shown in Fig. 4D, CA-Src was able to induce tyrosine phosphorylation of Dbs in cells. Additionally, CA-Src-induced tyrosine phosphorylation of Dbs was inhibited by pretreatment of PP1. Taken together with Fig. 4C, Src family tyrosine kinases may mediate the α1B-

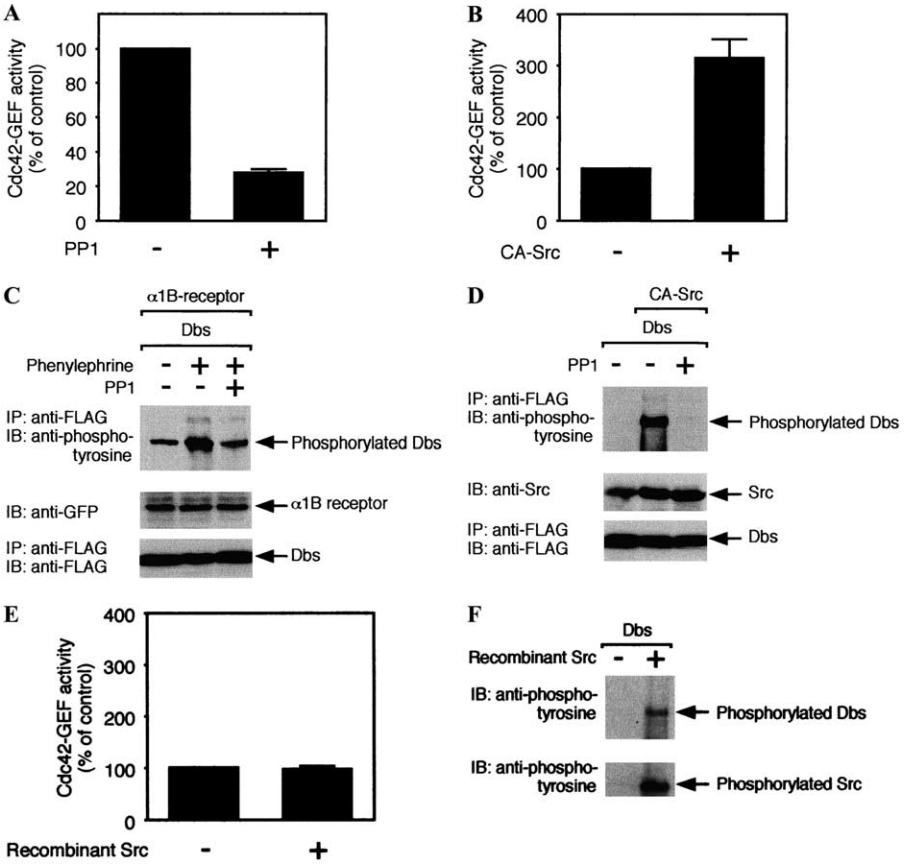

Fig. 4. Src family tyrosine kinase-dependent activation of the Cdc42-GEF activity of Dbs upon stimulation of the α1B-adrenergic receptor. (A) Cells were transfected with the plasmids encoding α1B-adrenergic receptor and Dbs, and pretreated with 10 μM PP1 for 1 h before addition of phenylephrine. Binding of guanine nucleotide to Cdc42 was assayed as described in Materials and methods. (B) Cells were transfected with the plasmids encoding CA-Src and Dbs. Binding of guanine nucleotide to Cdc42 was assayed as described in Materials and methods. (C) Cells were transfected with the plasmids encoding α1B-adrenergic receptor and Dbs, and pretreated with 10 μM PP1 for 1 h before the addition of phenylephrine. FLAG-Dbs was immunoprecipitated with an anti-FLAG antibody from the cell lysates and immunoblotted with an anti-phosphorylated tyrosine (upper) or an anti-FLAG antibody (lower). Expression of α1B-adrenergic receptor is shown (middle). (D) Cells were transfected with the plasmids encoding CA-Src and Dbs, and treated with 10 μM PP1 for 18 h. FLAG-Dbs was immunoprecipitated with an anti-FLAG antibody from the cell lysates and immunoblotted with an anti-phosphorylated tyrosine (upper) or an anti-FLAG antibody (lower). Expression of CA-Src is shown (middle). (E) The immobilized Dbs was incubated with recombinant c-Src and binding of guanine nucleotide to Cdc42 was assayed as described in Materials and methods. (F) The immobilized Dbs was incubated with recombinant c-Src and its tyrosine phosphorylation was analyzed using immunoblotting with anti-phosphorylated tyrosine antibody (upper). Tyrosine phosphorylated c-Src is shown (lower).

adrenergic receptor-induced tyrosine phosphorylation of Dbs in cells.

The well-characterized mechanism whereby Rho family small GTPases are activated involves direct tyrosine phosphorylation of Rho family GEF such as Vav family [15]. Thus, we tested the possibility that the immunoprecipitated Dbs might be phosphorylated and activated by recombinant c-Src in vitro. Under the experimental condition of this study, Dbs was not co-immunoprecipitated with detectable tyrosine kinase activity, as judged from the in vitro kinase assay using [^{32}P]ATP and the immunoblotting using anti-phosphorylated tyrosine antibody (data not shown). As a result, the in vitro tyrosine phosphorylated Dbs (Fig. 4F) failed to elevate the Cdc42-GEF activity of Dbs (Fig. 4E). It is therefore unlikely that c-Src directly activates the Cdc42-GEF activity of Dbs. However, we could not rule out the possibility that tyrosine kinases other than c-Src directly phosphorylate Dbs and activate its Cdc42-GEF activity in vitro.

We previously reported that JNK activation by the α1B-adrenergic receptor requires RhoA and Rac1 as well as Cdc42 in a manner dependent on Src family tyrosine kinases in cells [7]. GEFs for RhoA and Rac1 in this signaling pathway have remained to be identified. For example, it is possible that Vav family GEFs Vav2 and Vav3 may participate in the α1B-adrenergic receptor-induced RhoA activation, because these GEFs show tyrosine phosphorylation-dependent activation of the RhoA-GEF activity and have a relatively broad expression profile [15].

Involvement of Dbs in the anti-mitogenic pathway induced by the α1B-adrenergic receptor

We previously reported that the α1B-adrenergic receptor leads to inhibition of cell proliferation through the JNK pathway [8]. In this study, the α1B-adrenergic receptor-induced activation of the JNK pathway involves Dbs. Therefore, to clarify whether Dbs is involved in the receptor-induced anti-mitogenic response, we examined the effect of Dbs (ΔDHPH) on cells expressing the α1B-adrenergic receptor. In the absence of phenylephrine, incubation of cells for 24 h gave ~4.0×10^6 cells/well (Fig. 5A), while treatment of cells with phenylephrine led to a marked reduction in cell numbers, ~35% relative to control values (Fig. 5A and [8]). In contrast, transfection of Dbs (ΔDHPH) blocked phenylephrine-induced inhibition of cell proliferation (Fig. 5A). Additionally, the inhibition of cell proliferation induced by CA-Src, but not by constitutively activated form of Cdc42 (Cdc42G12V), was suppressed by Dbs (ΔDHPH) (Figs. 5B and C). These results suggest that the α1B-adrenergic receptor may inhibit cell proliferation through Dbs.

To investigate whether Dbs can inhibit cell proliferation, we transfected the plasmid encoding Dbs-DH into cells. Dbs-DH inhibited cell proliferation (Fig. 6A), as did the α1B-adrenergic receptor (Fig. 5A). In contrast, MKK4K95R, which is a kinase-deficient mutant and shows a dominant-inhibitory effect [9–12], blocked Dbs-DH-induced inhibition of cell proliferation (Fig. 6A), suggesting that Dbs inhibits cell proliferation through the JNK pathway. Additionally, Dbs-DH-induced inhibition of cell proliferation was suppressed by dominant-inhibitory mutant of Cdc42 (Cdc42T17N), but not by pretreatment of PP1 (Figs. 6B and C). It therefore seems likely that Dbs can regulate the anti-mitogenic pathway through JNK.

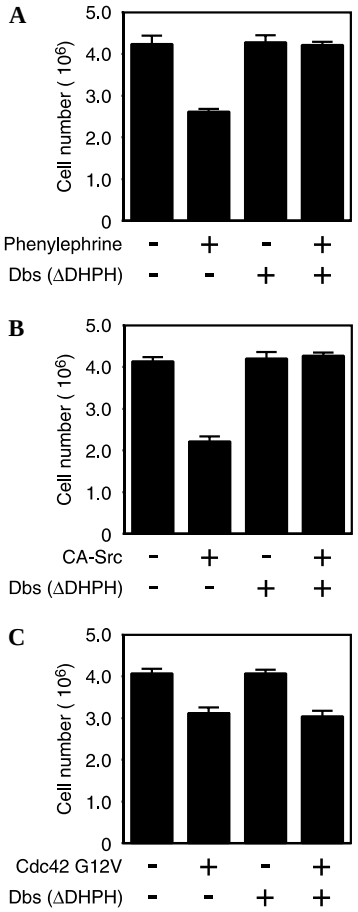

Fig. 5. Involvement of Dbs in the anti-mitogenic pathway induced by the α1B-adrenergic receptor. (A) Cells stably expressing α1B-adrenergic receptor were transfected with the plasmid encoding Dbs (ΔDHPH) and cultured in the presence of phenylephrine for 24 h. (B) Cells were transfected with the plasmids encoding CA-Src and Dbs (ΔDHPH), and cultured further. (C) Cells were transfected with the plasmids encoding Cdc42G12V and Dbs (ΔDHPH), and cultured further. Cells were then counted as described in Materials and methods.

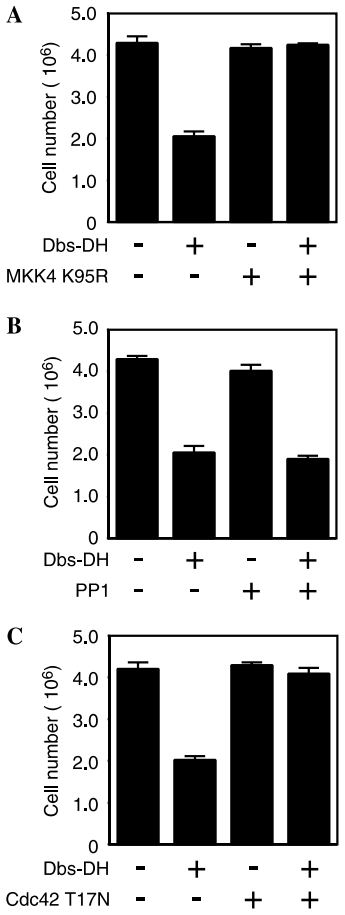

Fig. 6. Dbs can inhibit cell proliferation through JNK pathway. (A) Cells were transfected with the plasmids encoding Dbs-DH and MKK4K95R, and further cultured. (B) Cells were transfected with the plasmid encoding Dbs-DH and incubated with PP1. (C) Cells were transfected with the plasmids encoding Dbs-DH and Cdc42 T17N and further cultured. Cells were then counted as described in Materials and methods.

Chen et al. [27] reported that the α1-adrenergic receptors, other than the α1B type, attenuate cell proliferation in rat arterial smooth muscle cells. Keffel et al. [28] also reported that the α1A- but not the α1B-adrenergic receptor mediates anti-mitogenicity in CHO cells expressing the α1-adrenergic receptors. The α1B-adrenergic receptor-induced inhibition of cell proliferation may differ depending on the cell type. However, it remains unclear whether α1A- and α1D-adrenergic receptors cause inhibition of cell proliferation through a Dbs/Cdc42/JNK pathway in rat arterial smooth muscle and CHO cells.

In this study, we demonstrate Src family tyrosine kinase-dependent activation of the Cdc42-GEF activity of Dbs in the anti-mitogenic pathway from the α1B-adrenergic receptor to JNK. Further study to clarify how the Src family tyrosine kinase activates Dbs might promote our understanding of the general mechanism by which GPCRs activate Rho family small GTPases and in turn JNK, and thereby helping to elucidate the cellular function of the pathway.

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