A mammalian Rho-specific guanine-nucleotide exchange factor (p164-RhoGEF) without a pleckstrin homology domain

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Rho GTPases, which are activated by specific guanine-nucleotide exchange factors (GEFs), play pivotal roles in several cellular functions. We identified a recently cloned human cDNA, namely KIAA0337, encoding a protein containing 1510 amino acids (p164). It contains a RhoGEF-specific Dbl homology (DH) domain but lacks their typical pleckstrin homology domain. The expression of the mRNA encoding p164 was found to be at least 4-fold higher in the heart than in other tissues. Recombinant p164 interacted with and induced GDP/GTP exchange at RhoA but not at Rac1 or Cdc42. p164-ΔC and p164-ΔN are p164 mutants that are truncated at the C- and N-termini respectively but contain the DH domain. In contrast with the full-length p164, expression of p164-ΔC and p164-ΔN strongly induced actin stress fibre formation and activated serum response factor-mediated and Rho-dependent gene transcription. Interestingly, p164-ΔN2, a mutant containing the C-terminus but having a defective DH domain, bound to p164-ΔC and suppressed the p164-ΔC-induced gene transcription. Overexpression of the full-length p164 inhibited M_3 muscarinic receptor-induced gene transcription, whereas co-expression with $G\beta_1\gamma_2$ dimers induced transcriptional activity. It is concluded that p164-RhoGEF is a Rho-specific GEF with novel structural and regulatory properties and predominant expression in the heart. Apparently, its N- and C-termini interact with each other, thereby inhibiting its GEF activity.

Key words: Dbl family, guanine-nucleotide exchange factor, Rho protein, signal transduction.

INTRODUCTION

GTPases of the Rho family, which consists of at least 15 distinct proteins, control a large variety of cellular processes. First identified as regulators of specific changes of the actin cytoskeleton, RhoA, Rac1 and Cdc42 are now considered to be pivotal regulators of several signalling networks, including activation of serum response factor (SRF)-dependent gene transcription [1–4].

Like other small GTPases, Rho GTPases cycle between an inactive GDP-bound state and an active GTP-bound state. This cycling of Rho GTPases is controlled by three distinct classes of regulatory proteins, namely (1) guanine-nucleotide dissociation inhibitors, which stabilize the inactive form; (2) guanine-nucleotide exchange factors (GEFs), which catalyse the GDP/GTP exchange; and (3) GTPase-activating proteins, which stimulate the low, intrinsic GTPase activity of Rho GTPases [1–3]. The GEFs for Rho GTPases form a protein family with more than 30 members [5–7]. A common feature of GEFs for Rho GTPases is the Dbl homology (DH) domain responsible for exchange activity, followed by a pleckstrin homology (PH) domain supposed to be involved in subcellular localization of the GEFs [5–8]. The release of GDP from the GTPases is apparently initiated by an intermediate complex of the GEF with the guanine nucleotide-free state of the GTPase [9]. Several GEFs for Rho GTPases are preferentially expressed in specific cells and tissues, suggesting cell- and tissue-specific effects of these GEFs [5–7]. In addition, some GEFs are specific for individual Rho GTPases, e.g. Lfc for Rho, Tiam1 for Rac, and Fgd1 for Cdc42; on the other hand, some GEFs were found to act on several Rho GTPases, e.g. Dbl and Ost, which act on both Rho and Cdc42 [9–13]. In the present study, we report the characterization of p164-RhoGEF, a novel RhoGEF. It contains a DH domain but lacks the PH domain and is under a basal autoinhibitory constraint.

MATERIALS AND METHODS

Expression plasmid construction

The cDNA of KIAA0337 (GenBank® accession no. AB002335), kindly provided by Dr T. Nagase (Kazusa DNA Research Institute, Chiba, Japan), was subcloned into the *Eco*RI site of pCMV (cytomegalovirus)-Tag3C (Stratagene, Heidelberg, Germany) to obtain pCMV-Tag3–p164-FL (p164-FL, full-length p164). For pCMV-Tag3–p164-ΔC, an *Eco*RI–*Pvu*II fragment was subcloned into the *Eco*RI and *Eco*RV sites of pCMV-Tag3C. pCMV-Tag3–p164-ΔN resulted from the subcloning of an *Xho*I fragment of pCMV-Tag3–p164-FL into pCMV-Tag3B. To obtain pCMV-Tag3C–p164-ΔN2, a *Bam*HI–*Xho*I fragment of pCMV-Tag3C–p164-ΔN was subcloned. The ΔDH mutant pCMV3-Tag3C–p164-ΔDH was obtained by digesting pCMV-Tag3–p164-FL with *Eco*RI, *Bam*HI and *Bgl*II. The *Eco*RI–*Bgl*II fragment (453–2130 bp) and the *Bam*HI–*Eco*RI fragment (2430–5357 bp) were ligated to pCMV-Tag3C.

Procurement of tissue

Myocardium was obtained from patients with serious heart failure who had undergone orthotopic heart transplantation. Non-failing hearts were obtained from prospective organ donors whose hearts could not be transplanted due to technical or

Abbreviations used: ANF, atrial natriuretic factor; DH, Dbl homology; GEF, guanine-nucleotide exchange factor; GST, glutathione S-transferase; GTP[S], guanosine 5′-*O*-[γ-thio]triphosphate; h, human; m, mouse; p164-FL, full-length p164; PH, pleckstrin homology; RPA, RNase protection assay; SRE, serum response element; SRF, serum response factor; TRITC, tetramethylrhodamine β-isothiocyanate.

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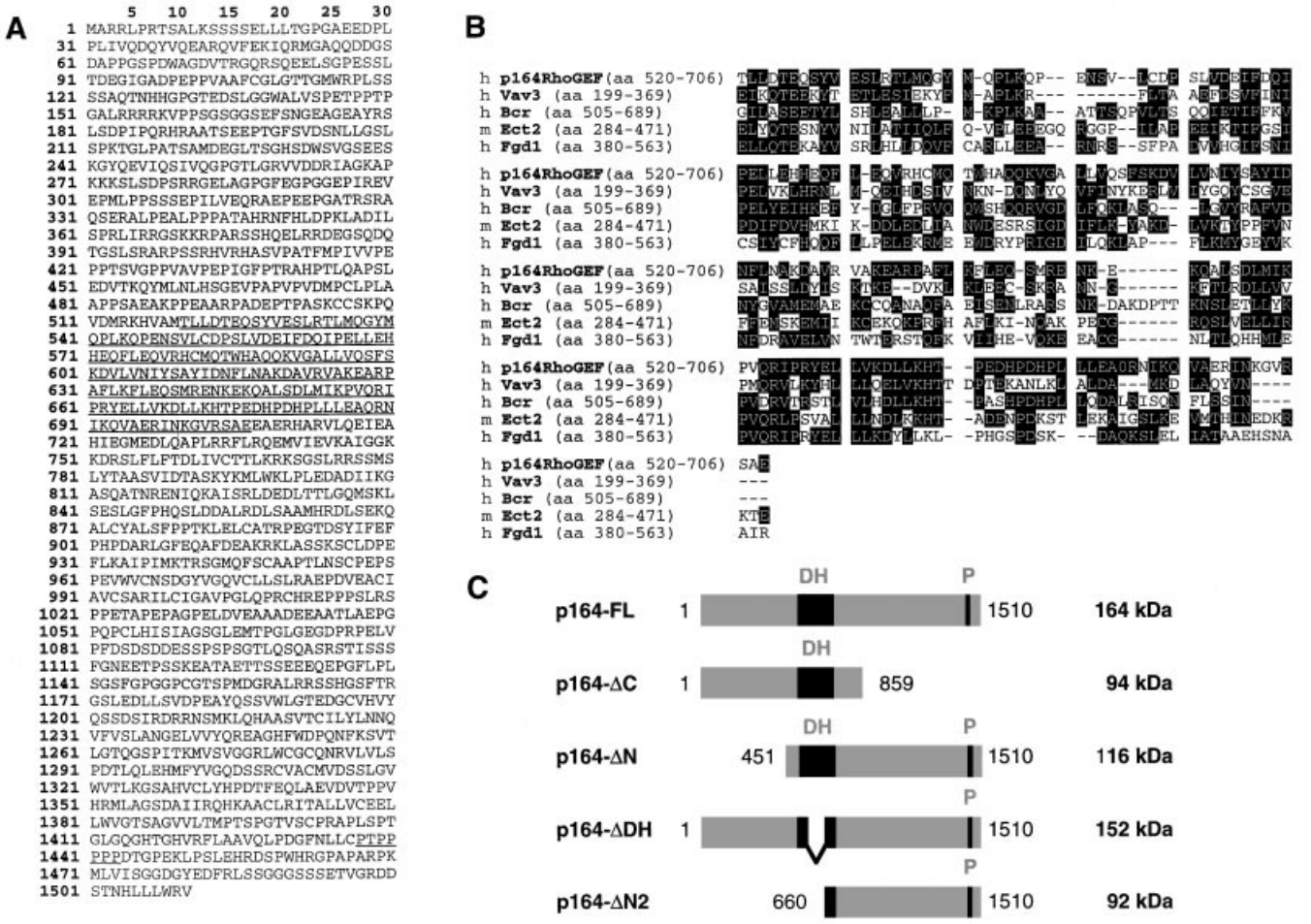

Figure 1 Deduced amino acid sequence, alignment of its DH domain with other RhoGEFs and schematic representation of p164-RhoGEF and the used truncated variants

(**A**) The predicted amino acid sequence of the coding sequence in the cDNA clone KIAA0337 is shown. Amino acid numbers are given on the left-hand side. The DH domain (amino acids 520–706) and the proline-rich region (amino acids 1437–1444) are indicated by underlining. (**B**) The DH domains of p164-RhoGEF, hVav3, hBcr, mEct2 and hFgd1 were aligned by Blast search. White-on-black residues represent sequence homologies, i.e. conservative amino acid changes within the following functionally similar groups: hydrophobic residues (I, V, L and M), aromatic residues (F, W and Y), acidic residues (E and D), basic residues (K and R) and uncharged polar residues (N and Q). (**C**) p164-FL–RhoGEF and its truncated variants used in the present study are shown with their respective amino acid numbers and the calculated molecular masses.

clinical reasons. Hepatic tissue was obtained from a patient undergoing liver transplantation. Human cortex was obtained from the Pathology Department of the Universitätsklinikum Hamburg-Eppendorf (Hamburg, Germany). Myometrial samples were taken at hysterectomies for benign gynaecological disorders. Tissues were immediately frozen and stored at −80 °C. Written informed consent was taken from all patients before surgery or from relatives before section. Permission for these studies was obtained from the Research Ethics Committee of Hamburg.

Preparation of RNA, Northern-blot analysis and RNase protection assay (RPA)

A human multiple-tissue Northern blot (ClonTech Laboratories, Heidelberg, Germany) was hybridized with an [α-^{32}P]dCTP-labelled cDNA probe encompassing nucleotides 988–1294 of the KIAA0337 sequence. Hybridization and washing were performed according to the manufacturer's instructions. Total RNA extraction and Northern-blot analysis of 18 S RNA expression was performed as described previously [14]. A 247 bp fragment (*Bam*HI–*Kpn*I) of the KIAA0337 cDNA was subcloned into pBluescript SK (Stratagene), and [^{32}P]UTP-labelled antisense RNA was transcribed with T7 RNA polymerase (Roche, Mannheim, Germany). RPAs were performed with the RPA II® kit (Ambion Inc., Austin, TX, U.S.A.), using 10 μg of total RNA [15,16]. Data were quantified by two-dimensional densitometry after phosphoimaging using AIDA software (FLA-3000; Fuji).

Cell culture and transfection

Culture of HEK-293 cells and human bladder carcinoma (J82) cells and transfection of the cells were performed as described previously [17,18].

Purification of proteins

Glutathione S-transferase (GST) fusion proteins of RhoA, Rac1 and Cdc42 were expressed in *Escherichia coli* and purified [18]. (Vectors were kindly provided by Dr A. Hall, University College

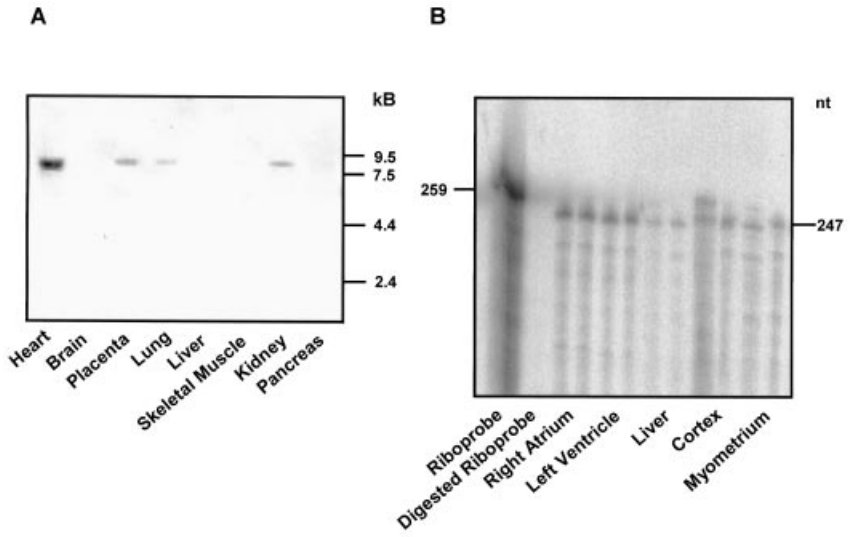

Figure 2 Expression of p164-RhoGEF mRNA

(**A**) Northern-blot analysis was performed with a radiolabelled cDNA probe of p164-RhoGEF for hybridization to polyadenylated [poly(A)$^+$] RNA of the indicated human tissues. The positions of molecular-mass markers (kb) are shown on the right-hand side. (**B**) Total RNA (10 μg) from right atrium or left ventricle of human myocardium, liver, cortex and myometrium was hybridized with a p164-RhoGEF riboprobe. The molecular masses of the riboprobe and the protected fragment are indicated. Assay duplicates of the RPA are shown.

London, London, U.K.) For expression of GST–p164-ΔC in *Spodoptera frugiperda* (Sf9) cells, the *Eco*RI–*Pvu*II fragment of the KIAA0337 cDNA described above was subcloned into the shuttle vector pAcGHLT-B (Pharmingen, San Diego, CA, U.S.A.). Production of baculoviruses and expression and purification of recombinant GST–p164-ΔC by affinity chromatography on glutathione–Sepharose (Amersham Pharmacia Biotech) were performed as described previously [17,18].

Protein binding and GDP/GTP exchange assays

Binding of p164-FL to Rho GTPases was performed as described previously [17,18]. Proteins were detected by Western blotting with an anti-Myc antibody (9E10; Calbiochem) and the enhanced chemiluminescence system (Amersham). To study the interaction of GST–p164-ΔC with p164-ΔN2, 150 μg of purified GST–p164-ΔC was bound to gluthatione–Sepharose beads. The beads were initially incubated with the cell lysate from uninfected HEK-293 cells (700 μg of protein) in order to saturate unspecific binding sites, then the beads were incubated for 48 h at 4 °C with lysate (4 mg of protein) of HEK-293 cells expressing p164-ΔN2. The beads were extensively washed, and bound proteins were subjected to SDS/PAGE and Western blotting.

The GDP/GTP exchange assays with recombinant proteins were performed as described previously [17,18].

Fluorescence microscopy

Fluorescence microscopy of Myc-tagged p164 proteins expressed in J82 cells with anti-Myc antibody and FITC-labelled goat anti-mouse IgG antibody, and of actin filaments with tetramethylrhodamine β-isothiocyanate (TRITC)-conjugated phalloidin was performed 48 h after transfection as described previously [17,18].

SRF activation assay

Rho-dependent activation of SRF was measured in HEK-293 cell extracts with the Dual Luciferase Reporter Assay System (Promega, Mannheim, Germany). HEK-293 cells seeded on 12-well plates were co-transfected with different expression plasmids, together with pSRE.L-luciferase reporter plasmid (a gift from Dr J. Mao and Dr D. Wu, University of Rochester, Rochester, New York, U.S.A.) and pRL-TK control reporter vector [17]. Transfection was stopped, and luciferase activities were determined with a Lumat counter (Berthold, Pforzheim, Germany) as described previously [17]. The activity of the experimental reporter was normalized against the activity of the control vector.

RESULTS

Identification and sequence analysis of p164-RhoGEF

In search of new GEFs for Rho GTPases, we examined protein databases with the DH domains of known RhoGEFs. We identified a human cDNA clone KIAA0337 (GenBank® accession no. AB002335), which encodes a DH domain having a moderate degree of homology to those of known Dbl family members [50% to human (h)Vav-3, 48% to hBcr, 45% to mouse (m)Ect2 and 37% to hFdg1; see Figure 1]. KIAA0337 contains an open reading frame between bp 455 and 4987. The deduced protein consists of 1510 amino acids with a calculated molecular mass of 164 kDa; thus the protein is termed p164-RhoGEF. The DH domain is located between amino acids 520 and 706. In contrast with all other known mammalian RhoGEFs, p164-RhoGEF is apparently lacking a PH domain. We could not detect the PH domain consensus motif within the deduced amino acid sequence using several bioinformatic tools (BlastP, Scan PROSITE or InterPro). When several known PH domains were aligned with p164-RhoGEF using ExPASy SIM, the highest similarity of 77.7% was detected in a small 9 amino acid overlap (amino acids 750–759) to the PH domain found in hVav-3. A further alignment of the hVav-3 PH domain with p164-RhoGEF by GeneStream align [19], in the region where the putative PH domain of the DH/PH tandem motif should be located (amino

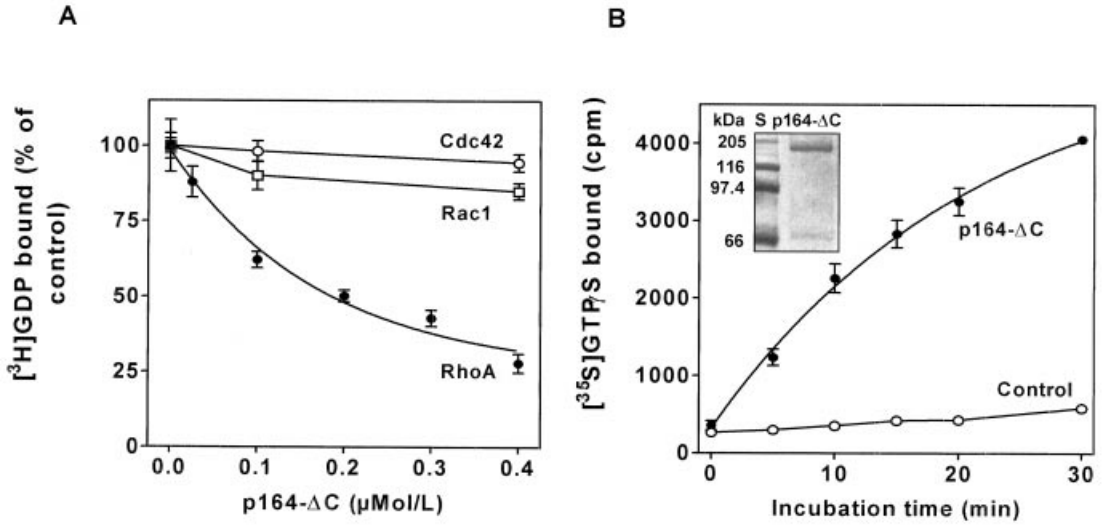

Figure 3 Rho-specific GDP/GTP exchange activity of p164-RhoGEF

(**A**) [^{3}H]GDP release from GST–RhoA, GST–Rac1 or GST–Cdc42 (0.6 μM each) induced by GST–p164-ΔC at the indicated concentrations after 40 min at room temperature. Bound [^{3}H]GDP is expressed as the percentage of control, i.e. binding in the absence of p164-ΔC. (**B**) Time course of [^{35}S]GTP[S] binding to GST–RhoA (0.6 μM) measured in the absence (Control) and presence of GST–p164-ΔC (0.3 μM). Data shown are means $\pm$ S.E.M. ($n = 4$). Inset, Coomassie Blue-stained SDS/PAGE of purified GST–p164-ΔC, with molecular-mass markers (S) indicated on the left-hand side.

acids 730–840), again detected the 9 amino acid overlap, but only a global alignment score of 5 was calculated. For comparison, the global alignment score for the hVav-3 DH domain with p164-RhoGEF reached 220.

Using identical sequence information, De Toledo et al. [20] reported an open reading frame between 534 and 6288 bp, which should be translated into a protein containing 1609 amino acids, and a DH domain between amino acids 616 and 799, with a maximum of 30 % similarity to other DH domains. Our alignment to the DH consensus motif produced a much higher degree of similarity (up to 50 %). Furthermore, as there is no ATG for translation start at positions 534–537 in the cDNA sequence, the information on the resulting protein reported by De Toledo et al. [20] is apparently not correct. We detected one additional motif, i.e. a proline-rich region located between amino acids 1437 and 1444. By searching several human genome databases, the gene encoding p164-RhoGEF can be mapped to the long arm of chromosome 11 at position 11q12–14.

Preferential expression of p164-RhoGEF in the heart

The expression of p164-RhoGEF in human tissues was examined by Northern-blot analysis. We detected a transcript of approx. 9 kb (Figure 2A), the expression being the highest in the heart and significantly lower in placenta, lungs, kidneys and the pancreas. For quantification, the mRNA content was studied in RNA obtained from human heart (right atrium, left ventricle), liver, cortex and myometrium by RPA. A specific protected fragment of 247 nucleotides (Figure 2B) was observed in all tissues. When normalized to the non-differing expression of 18 S RNA, the expression of p164-RhoGEF mRNA was found to be at least 4-fold higher in the heart than in other tissues (results not shown). Similar expression levels of p164-RhoGEF mRNA were measured in RNA prepared from non-failing donor hearts ($n = 4$) and failing hearts ($n = 5$) with end-stage cardiomyopathy (results not shown).

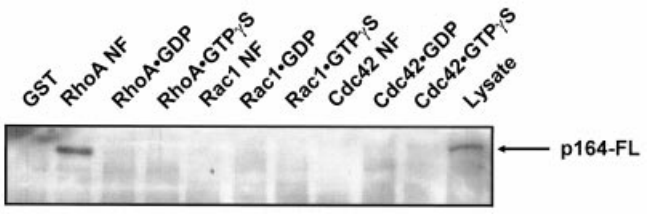

Figure 4 Rho-specific binding of p164-RhoGEF

Binding of GST–RhoA, GST–Rac1 and GST–Cdc42, in the nucleotide-free state (NF), GDP-bound state or GTP[S]-bound state respectively, with Myc-tagged p164-FL expressed in HEK-293 cells. Bound p164-FL was resolved by SDS/PAGE and identified with an anti-Myc antibody.

Rho-specific GEF activity of p164-RhoGEF

We aimed at studying the potential GEF activity of p164-RhoGEF and its specificity towards distinct Rho family members. Therefore purified recombinant GST-fused p164-ΔC was added to purified GST–RhoA, GST–Rac1 or GST–Cdc42, and binding of [^{3}H]GDP and ^{35}S-labelled guanosine 5′-*O*-[γ-thio]-triphosphate (GTP[S]) to these GTPases was measured. As shown in Figure 3(A), p164-ΔC induced a substantial release of [^{3}H]GDP from RhoA but not from Rac1 and Cdc42 (0.6 μM each). Half-maximal and maximal release of [^{3}H]GDP (72 ± 3 %; $P < 0.0001$ versus control; $n = 4$) from RhoA was observed at 0.12 and $\geqslant 0.4$ μM of p164-ΔC respectively. p164-ΔC (0.3 μM) significantly accelerated the dissociation of [^{3}H]GDP from RhoA ($t_{1/2} = 10$ min), in contrast with a slow release in its absence ($t_{1/2} = 65$ min) (results not shown). In agreement with the [^{3}H]GDP binding data, p164-ΔC (0.3 μM) significantly promoted binding of [^{35}S]GTP[S] to RhoA (0.6 μM; Figure 3B).

Protein binding assays with GST fusion proteins of RhoA, Rac1 and Cdc42, each in the nucleotide-free, GDP- or GTP[S]-bound

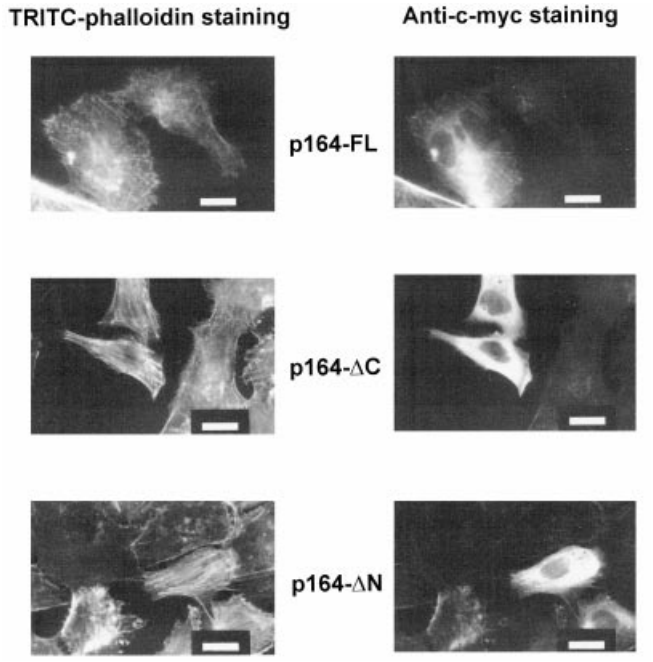

Figure 5 Effects of different variants of p164-RhoGEF on the actin cytoskeleton

TRITC-phalloidin staining revealing the actin cytoskeleton (left panel) and anti-Myc antibody immunofluorescence (right panel) of J82 cells transfected with Myc-tagged p164-FL, p164-ΔC or p164-ΔN, as indicated. Scale bar, 20 μm.

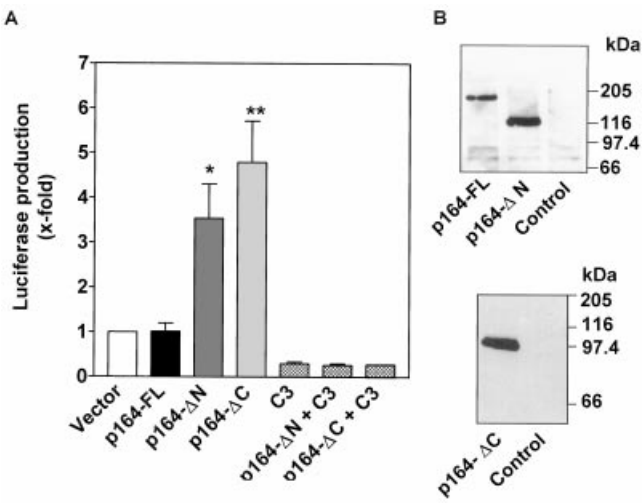

Figure 6 Stimulation of SRF-mediated gene transcription by different variants of p164-RhoGEF

HEK-293 cells were co-transfected both with pSRE.L-luciferase reporter plasmid (0.5 μg) expressing firefly luciferase and with pRL-TK control reporter vector (0.1 μg) expressing *Renilla* luciferase, in the absence and presence of expression plasmids for p164-FL, p164-ΔN or p164-ΔC (1 μg each), as indicated, without and with plasmid DNA for C3 transferase (1 μg). (**A**) Firefly luciferase activities normalized against the level of expressed *Renilla* luciferase. Data are means $\pm$ S.E.M. ($n = 4$). Statistical analysis was performed by one-way ANOVA followed by Tukey's multiple-comparison test (**$P < 0.01$; *$P < 0.05$ versus vector control). (**B**) Expression of p164 proteins verified by immunoblots with an anti-Myc antibody.

state and immobilized on glutathione–Sepharose beads, and Myc-tagged p164-FL expressed in HEK-293 cells corroborated the activity measurements. p164-FL specifically bound to RhoA but not to Rac1 or Cdc42 and it bound only in its nucleotide-free form. No interaction with the GDP- or GTP[S]-bound state of RhoA was detected (Figure 4).

Induction of stress fibres and transcriptional activity by p164-RhoGEF

We first studied potential GEF activity of p164-RhoGEF for Rho GTPases in intact cells by examining the cytoskeletal changes induced by overexpression of p164-RhoGEF in J82 cells, which endogenously express p164-RhoGEF (results not shown). Overexpression of Myc-tagged p164-FL, detected by an anti-Myc antibody, induced no, or only minimal, formation of actin stress fibres (Figure 5). In contrast, expression of the DH domain containing truncated mutants, namely p164-ΔC and p164-ΔN (see Figure 1C), strongly induced actin stress fibres detected by TRITC–phalloidin staining. Formation of filopodia, microvilli, lamellipodia or membrane ruffles was not seen in cells transfected with either construct.

As activated RhoA effectively stimulates SRF, resulting in activation of the c-*fos* serum response element (SRE) [4], we decided to investigate the ability of p164-RhoGEF to induce gene expression via SRF and SRE. Hence, we used a firefly-luciferase expression vector controlled by SRE.L, a derivative of the c-*fos* SRE that contains an intact high-affinity binding site for SRF but cannot bind ternary complex factor [4,21]. As reported previously [17], co-expression of constitutively active V14RhoA in HEK-293 cells induced an approx. 20-fold increase in luciferase reporter production. Consistent with the data obtained on stress fibre formation in J82 cells, overexpression of p164-FL in HEK-293 cells, which endogenously express p164-RhoGEF (results not shown), did not induce SRE.L-dependent gene transcription. In contrast, the truncated mutants p164-ΔN and p164-ΔC increased luciferase production by 3.5 ± 0.7- and 4.8 ± 0.9-fold respectively (Figure 6). This luciferase production was fully abrogated by co-expression of *Clostridium botulinum* C3 transferase, which inactivates RhoA-C [22].

Interaction of the N- and C-termini of p164-RhoGEF

The fact that the basal activity of p164-FL was negligible compared with its truncated mutants suggested that there was an autoinhibition of its GEF activity. To test this possibility, we constructed a second N-terminal truncated form p164-ΔN2, lacking most of the DH domain (see Figure 1), and we then examined its interaction with p164-ΔC, a C-terminally truncated mutant. As shown in Figure 7(A), GST–p164-ΔC, immobilized on glutathione–Sepharose beads, bound Myc-tagged p164-ΔN2 expressed in HEK-293 cells, whereas no interaction with GST was observed. This interaction with ΔC obviously occurs in intact cells as shown in Figure 7(B). Consistent with a defective DH domain, expression of p164-ΔN2 by itself did not induce any transcriptional activity. However, luciferase production induced by p164-ΔC was strongly inhibited (by approx. 50%) by co-expression of p164-ΔN2.

Influence of p164-RhoGEF on receptor- and G-protein-induced gene transcription

Activation of M_3 muscarinic acetylcholine receptors stably expressed in HEK-293 cells by the agonist carbachol (1 mM), strongly stimulated the Rho-controlled luciferase expression

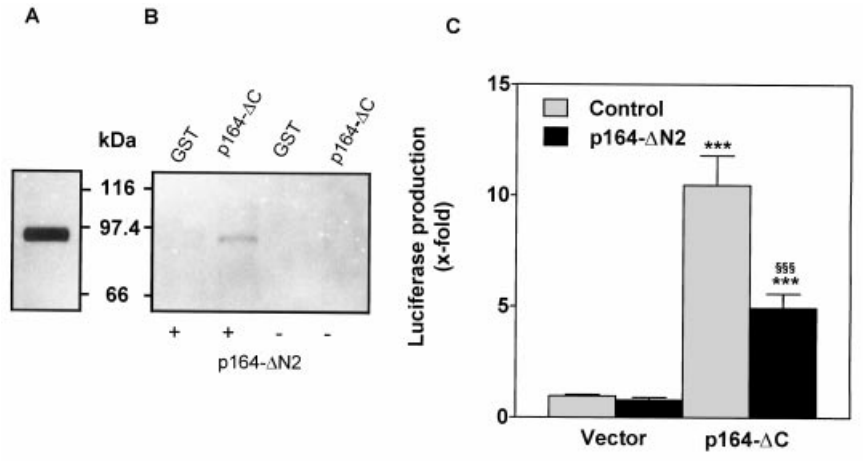

Figure 7 Inhibitory interaction of the N- and C-termini of p164-RhoGEF

(**A**) Expression of p164-ΔN2 in HEK-293 cells verified by immunoblotting with an anti-Myc antibody. (**B**) Binding of purified GST–p164-ΔC to Myc-tagged p164-ΔN2 expressed in HEK-293 cells. Bound p164-ΔN2 was resolved by SDS/PAGE and identified with an anti-Myc antibody. (**C**) HEK-293 cells were co-transfected with pSRE.L (0.5 μg), pRL-TK (0.1 μg), in the absence (Vector) and presence of the expression plasmid for p164-ΔC (0.5 μg/well), without (Control) and with plasmid DNA for p164-ΔN2 (0.5 μg). Results are means ± S.E.M. ($n = 4$). Statistical analysis was performed by two-way ANOVA (***$P < 0.001$ versus vector control; $^{§§§}P < 0.001$ versus p164-ΔC alone).

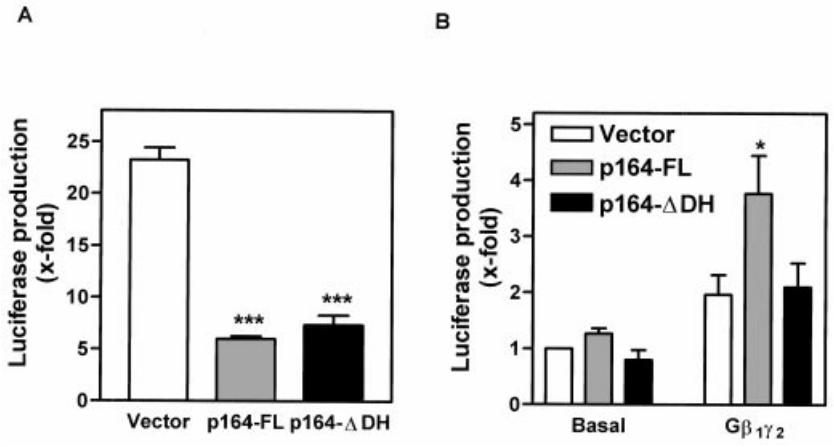

Figure 8 Influence of p164-RhoGEF on receptor- and G-protein-induced gene transcription

(**A**) HEK-293 cells stably expressing the M_3 muscarinic receptor were co-transfected with pSRE.L (0.5 μg), pRL-TK (0.1 μg) and with or without the indicated constructs of p164 (0.5 μg each). Cells were stimulated by 1 mM carbachol for 12 h. (**B**) HEK-293 cells were co-transfected with pSRE.L (0.5 μg), pRL-TK (0.1 μg), pCMV3-Tag3C–p164-ΔDH or pCMV3-Tag3C–p164-FL (0.5 μg each) and without (Basal) or with pCis-Gβ_1 plus pCis-Gγ_2 (0.2 μg each), as indicated. Results are means ± S.E.M. (**A**, $n = 4$; **B**, $n = 10$). Statistical analysis was performed by ANOVA followed by Tukey's multiple-comparison test (***$P < 0.001$ versus vector control; *$P < 0.05$ versus all other conditions).

(Figure 8). Expression of p164-ΔDH, partially lacking the DH domain (see Figure 1), significantly decreased (by approx. 70 %) the carbachol-induced luciferase production. Interestingly, overexpression of p164-FL inhibited agonist-induced transcriptional activity to a similar extent. In contrast, co-expression of G$\beta_1\gamma_2$ with p164-FL but not with p164-ΔDH significantly increased (approx. 2-fold) transcriptional activity, although G$\beta_1\gamma_2$ by itself was rather ineffective.

DISCUSSION

Sequence alignment of p164-RhoGEF with other Dbl family members revealed a rather moderate degree of similarity to other RhoGEFs within its catalytic DH domain and even more diversity outside the DH domain. It is noteworthy that this protein represents, to our knowledge, the first mammalian Dbl family member without a PH domain and, thus, not exhibiting the classical DH/PH tandem module [5–7]. Nevertheless, there are members of the Dbl family in *Drosophila melanogaster*, Pbl [23] and GEF64C [24], as well as in *S. cerevisiae*, Bud3p [25], Rom1p, Rom2p [26] and Fus2p [27], that also apparently lack the PH domain.

Both *in vitro* and intact cell data indicate that p164 is a Rho-specific GEF. Studies with purified recombinant proteins demonstrated that p164-RhoGEF strongly increases the GDP/GTP exchange of RhoA but not Cdc42 and Rac1. De Toledo et al. [20] recently reported that the DH domain of p164-RhoGEF stimulates GDP release from RhoA but not from RhoG. Consistent

with its GEF activity towards RhoA, p164-RhoGEF exhibited specific binding to nucleotide-free RhoA but not to GDP- or GTP[S]-bound RhoA. The specificity of p164-RhoGEF for RhoA was confirmed by the induction of two distinct RhoA-mediated cellular effects. First, similar to activated RhoA, expression of p164-RhoGEF in J82 cells promoted actin stress fibre formation, without an induction of Rac- or Cdc42-specific cytoskeletal changes. Secondly, overexpression of p164-RhoGEF in HEK-293 cells efficiently induced transcriptional activation by SRF, an effect completely abrogated by *C. botulinum* C3 transferase, which inactivates Rho but not Rac and Cdc42 [22].

Besides their specificity for distinct Rho GTPases, a typical feature of several Dbl family members is their preferential expression in specific cells and tissues [5–7]. At least three Dbl family members, Graf [28], p63RhoGEF [29] and obscurin [30,31], have been reported to be highly expressed in the heart. Nevertheless, Graf and p163RhoGEF were expressed at high levels in the brain, whereas obscurin was detected at high levels in the heart and skeletal muscles. We found a predominant expression of p164-RhoGEF mRNA in the heart. Together with its specificity for RhoA, this is of particular interest. RhoA has been implicated as mediator of hypertrophic signalling in cardiac myocytes. In the model of cultured, neonatal rat cardiac myocytes, G_q-coupled receptor agonists activate RhoA and thereby induce hypertrophic responses, such as increase in cell size, organization of myofilaments and re-expression of embryonic genes, including atrial natriuretic factor (ANF) and myosin light chain 2v [32–34]. Like the SRE-regulated luciferase expression studied herein, the RhoA-induced ANF expression in cardiac myocytes is mediated by SRE-like elements in the ANF promoter [35]. Moreover, RhoA regulates the delayed rectifier potassium channel Kv1.2 in cardiac myocytes [36], and this interaction has been suggested to cause sinus and atrioventricular nodal dysfunction in mice overexpressing RhoA in the heart [37]. Finally, a previous study [38] has provided evidence that RhoA mediates the stimulation of cardiac phospholipase D by adenosine A_3 receptors, which are involved in adenosine-induced cardioprotection. Thus p164-RhoGEF, a Dbl family member with a predominant expression in the heart, may act as an important mediator in one or several of these pathways.

Interestingly, p164-FL-RhoGEF exhibited no, or only modest, GEF activity in intact cells, whereas the truncated mutants p164-ΔC and p164-ΔN markedly activated Rho-dependent effectors. Several of the Dbl family members appear to exist in an inactive, basal state controlled by autoinhibitory domains within the molecule [7]. For example, the N-terminus of Vav has been reported to form an α-helix that binds to the binding pocket for Rho GTPases in the DH domain, and upon phosphorylation of Tyr-174 residue by Src-like kinases, the N-terminal peptide is restructured and released from the DH domain, resulting in Vav activation [39]. Another mechanism for autoinhibition has recently been reported for Dbl. Within this GEF, the N-terminal domain, probably forming a coiled-coiled-like structure, is binding to the PH domain, thereby allosterically hindering the access of Rho GTPases to the catalytic DH domain [40]. The data reported herein suggest that p164-RhoGEF, which has no PH domain, is also under an autoinhibitory constraint. In contrast with Vav, the N-terminus of p164-RhoGEF is apparently not interacting with its DH domain, since p164-ΔC, containing the complete N-terminus and the DH domain, was similarly active as p164-ΔN, in which more than 90% of the N-terminus has been deleted. Our data indicate that the N- and C-termini of p164-RhoGEF are binding to each other and thereby prevent GEF activity, although, at least *in vitro*, p164-RhoGEF is able to bind RhoA (see Figure 4).

Recent studies on the mechanism of activation of Rho GTPases by RhoGEFs have provided some mechanisms by which such GEFs can be activated. As mentioned above, Vav can be activated by phosphorylation at a specific tyrosine residue [39]. Other Dbl family members, like p115-RhoGEF and its analogues, seem to directly interact with activated $G\alpha_{13}$ proteins through an N-terminal RGS homology domain, resulting in increased GEF activity, although a hitherto unknown tyrosine kinase may also be involved in this process [20,41,42]. p114-RhoGEF, a RhoGEF not related to p115-RhoGEF, can amplify Rho-dependent gene transcription by G-protein-coupled receptors, but the mechanism by which this GEF is activated is unclear [17]. Moreover, $G\beta\gamma$ dimers were recently found to bind to the N-terminus of Dbl, but without changing its GEF activity [43]. Expression of p164-RhoGEF did not enhance Rho-dependent gene transcription induced by the M_3 muscarinic receptor, which couples to G_q- and G_{12}-type G-proteins in HEK-293 cells [44]. In contrast, luciferase production was inhibited when the DH domain-defective mutant p164-ΔDH was expressed or when p164-FL was expressed. Luciferase production induced by expression of recombinant $G\alpha_{12}$, $G\alpha_{13}$ or $G\alpha_q$ was also strongly impaired by co-expression of p164-ΔDH (results not shown). In view of the fact that p164-FL is able to bind RhoA *in vitro*, these data suggest that overexpressed p164-FL and p164-ΔDH, which only partially lacks the DH domain, may sequester RhoA and, thereby, blunt transcriptional activation by the M_3 muscarinic receptor and activated G-proteins. A significant, although modest, increase in luciferase production was observed by co-expression of $G\beta_1\gamma_2$ dimers with p164-FL but not with p164-ΔDH, suggesting that p164-RhoGEF may contribute to the activation of RhoA by G-protein-coupled receptors. Investigations into additional mechanisms leading to activation of Rho GTPases by p164-RhoGEF are in progress.

In summary, a novel Rho-specific GEF has been characterized, which is the first mammalian RhoGEF lacking a PH domain. It is predominantly expressed in the heart and exhibits a basal, catalytically silent state, which is apparently due to an autoinhibitory interaction of its N- and C-termini.

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