Human p63RhoGEF, a novel RhoA-specific guanine nucleotide exchange factor, is localized in cardiac sarcomere

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Summary

The Rho small GTPases are crucial proteins involved in regulation of signal transduction cascades from extracellular stimuli to cell nucleus and cytoskeleton. It has been reported that these GTPases are directly associated with cardiovascular disorders. In this context, we have searched for novel modulators of Rho GTPases, and here we describe p63RhoGEF a new Dbl-like guanine nucleotide exchange factor (GEF). P63RhoGEF encodes a 63 kDa protein containing a Dbl homology domain in tandem with a pleckstrin homology domain and is most closely related to the second Rho GEF domain of Trio. Northern blot and in situ analysis have shown that p63RhoGEF is mainly expressed in heart and brain. In vitro guanine nucleotide exchange assays have shown that p63RhoGEF specifically acts on RhoA. Accordingly, p63RhoGEF expression induces RhoA-dependent stress fiber formation in fibroblasts and in H9C2 cardiac myoblasts. Moreover, we show that p63RhoGEF activation of RhoA in intact cells is dependent on the presence of the PH domain. Using a specific anti-p63RhoGEF antibody, we have detected the p63RhoGEF protein by immunocytochemistry in human heart and brain tissue sections. Confocal microscopy shows that p63RhoGEF is located in the sarcomeric I-band mainly constituted of cardiac sarcomeric actin. Together, these results show that p63RhoGEF is a RhoA-specific GEF that may play a key role in actin cytoskeleton reorganization in different tissues, especially in heart cellular morphology.

Key words: GEF, RhoA, Cardiac sarcomere

Introduction

The Rho family of small GTPases act as molecular switches to control a wide range of cellular processes in eukaryotic cells, such as normal growth, transformation, gene regulation and actin cytoskeletal organization (Hall, 1998). Each member of the Rho family has a different influence on the cytoskeletal structure and on cellular morphology. In fibroblasts, RhoA induces the formation of stress fibers associated with focal adhesions, Rac1 produces lamellipodia or membrane ruffling, and Cdc42 evokes filopodia (Nobes and Hall, 1995; Ridley et al., 1992; Ridley and Hall, 1992). In other cell types, these Rho-like GTPases trigger tissue-specific responses by their action on the actin cytoskeleton. The Rho GTPases are molecular switches that cycle between an inactive (GDP-bound) and an active (GTP-bound) conformation. They are regulated by guanine nucleotide exchange factors (GEFs) that activate them by accelerating the GDP/GTP exchange, while the GTPase-activating proteins (GAPs) stimulate GTP hydrolysis (Stam and Collard, 1999). To date, more than 30 mammalian GEFs of the Dbl family have been identified. These proteins are characterized by a Dbl homology (DH) domain of about 180 residues based on sequence homology with the central portion of the Dbl oncogene, the first identified Rho GEF active on Cdc42 and RhoA (Hart et al., 1994). In tandem with the DH domain is invariably found a pleckstrin homology (PH) domain, generally recognized as a membrane targeting module by its capacity to bind to phosphoinositides (Lemmon and Ferguson, 2000). More recently, PH domains have been shown to directly link PH-containing proteins to the actin cytoskeleton, by binding to actin (Yao et al., 1999) or to an actin-binding protein such as filamin in the case of Trio (Bellanger et al., 2000). PH domains have also been proposed to modulate the DH-mediated catalytic activity, depending on the nature of the phosphoinositides they bind (Han et al., 1998).

The invariable association of a DH with a PH domain within the Rho GEF family suggests that the DH-PH tandem represents the functional unit responsible for activation of the Rho GTPases. In addition to this tandem module, Rho GEFs often present other structural or functional domains, predicted to play regulatory roles in the localization or the control of the GEF activity (Stam and Collard, 1999) and in the involvment of Rho GEFs in specific signal transduction networks.

Several 3D structures of GEF-DH domains have been resolved (Liu et al., 1998) and indicate that the DH domain is entirely composed of α helices. PH domains show amino acid sequences with very low similarity although they share a common 3D scaffold (Lemmon and Ferguson, 2000). Outside the DH/PH tandem, the Rho-GEFs do not display structural similarity (Whitehead et al., 1997).

Most of the Rho GEFs isolated so far display activity on Rho GTPases, at least in vitro. Some Rho GEFs appear to exhibit in vivo selectivity for a specific GTPase (e.g. Lbc for Rho, Tiam1 for Rac, and FGD1 for Cdc42), whereas others seem to act on several GTPases (e.g. Vav, Dbl and Trio) (Stam and Collard, 1999). Interestingly, the Rho GEF Trio possesses two GEF domains: the N-terminal (Trio1) shows a RhoG/Rac1 specificity inducing ruffles formation whereas the second domain (Trio2) is an exchange factor for RhoA and induces the formation of stress fibers (Debant et al., 1996).

In the cardiovascular field, Rho GTPases play a key role in several signaling pathways activated by G-protein-coupled receptors such as lysophosphatidic acid (LPA) (Blomquist et al., 2000), endothelin-1 (Gohla et al., 2000; Shome et al., 2000), angiotensin II (Aoki et al., 1998) and phenylephrin (Sah et al., 1996). It has been demonstrated that Rho is required for α1-adrenergic receptor-mediated hypertrophy in cardiomyocytes with an increase of gene expression for ANF, MLC-2, β-MHC, skeletal α-actin (Sah et al., 1996). In addition, the Rho family of small G proteins plays a critical role in mechanical stress-induced hypertrophic responses of cardiac myocytes (Aikawa et al., 1999).

In the present study, we report the identification of a new member of the Rho GEF subfamily, called p63RhoGEF, from a proprietary database of human sequences. P63RhoGEF encodes a 63 kDa protein (580 amino acids) containing the conserved structural feature of a DH domain in tandem with a PH domain. P63RhoGEF is most closely related to the Rho GEF Trio2 with an identity score of 70%. Northern blot and in situ hybridization analysis have shown that p63RhoGEF is mainly expressed in heart and brain. P63RhoGEF functions as a GEF for RhoA in vitro and its expression induces an increase in stress fiber formation in REF-52 cells and H9C2 embryonic cardiac cells. Moreover, we show that the PH domain is necessary for p63RhoGEF-mediated RhoA activation in intact cells. Using a specific anti-p63RhoGEF antibody, we have detected the p63RhoGEF protein by immunocytochemistry experiments performed in human heart and brain tissue sections. Interestingly, p63RhoGEF protein was detected by confocal microscopy in the sarcomere of the cardiac fibers, more precisely located in the I-band mainly constituted of cardiac sarcomeric actin.

Materials and Methods

Database search and protein sequence analysis

The complete p63RhoGEF sequence has been scanned against the collection of mammalian databases and the following sequences with significant relatedness were identified as human ESTs (AI677902, AI338780, AI684570, AI141311, AI928887, AW264299, AI366527, AW136644, AA463333, AI089627, AI298483, U47343, AI372875, AW129570, AA463846, BE677351, W26816, AW305132, AI374899, AW134722, AA611831, AA031465, AI652923, AW204313, AI654427, BE773133, AW205086, D81166, AI217285, D80712, AI382756, AA906309); mouse ESTs (AW412210, BE913522, BE656230, AI596069, BE310842, AA268669, AA612007, AA048218, AW146362, AL362651, AA171122, AI747537, AA119224, W98059, BE371560, AA119606, AI877153, AU014694, AW226613, AI663076, AI574348, AA170980, AI115767, AA198251); mouse cDNA (BAB26951); bovine ESTs (AV611879, BF043452, BE667062, AV608536, AV605397, AV611878, AV608537, AV605398); and porcine ESTs (BE030495, BE236200).

Bioinformatic or computational searches of both public and private EST and genomic databases (collaboration with Human Genome Sciences, Rockville, MD) were used to identify various sequences that encode structural characteristics of putative GEFs.

SMART method (Schultz et al., 2000) was used for identification and cartoon representation of the tandem DH-PH domain. Low compositional complexity was determined by the SEG program available with SMART. Multiple alignments have been obtained with Clustal W 1.7 method (Thompson et al., 1994) and other more sophisticated methods such as hydrophobic cluster analysis (Callebaut et al., 1997). The evolutionary trace method (Lichtarge et al., 1996) was used to generate a sequence identity dendrogram and is available from Binding Site Analysis module implemented in InsightII 2000 program (Molecular Simulations Inc., San Diego, CA).

Plasmid constructions

The cDNA sequence (2118 bp *Srf*I-*Xho*I fragment) containing p63RhoGEF ORF (580 amino acids) was cloned into pBluescript KS(+) plasmid (Stratagene) *Eco*RV-*Xho*I digested to give BSKS-p63RhoGEF plasmid.

To perform mammalian cell transfection, the 1878 bp *Ehe*I-*Eco*RV fragment encoding p63RhoGEF was subcloned into pCDNA3-HisB expression vector (Invitrogen) linearized by *Eco*RV to generate pCDNA3-p63RhoGEF plasmid. This plasmid contains Xpress and His tags in frame with p63RhoGEF at the N-terminal. To perform guanine nucleotide exchange assay, the 663 bp *Bam*HI-*Eco*RI fragment encoding p63RhoGEF-DH domain (amino acids 149-374) was subcloned into pGEX-6P3 GST-fusion vector (Pharmacia) *Bam*HI-*Eco*RI digested to give GST-p63RhoGEF DH plasmid.

The mutant p63RhoGEF L301E was constructed using the Quick change site-directed mutagenesis kit from Stratagene according to the manufacturer's instructions. This mutation was performed both on mammalian expression vector pCDNA3-p63RhoGEF and on GST-p63RhoGEF pGEX plasmid and the constructs were verified by sequencing.

RNA hybridization

Human MTN blot (Clontech) was pre-hybridized for 30 minutes at 65°C in 5 ml of ExpressHyb buffer (Clontech), then hybridized for 1 hour at 65°C in the same buffer containing denatured [α-^{32}P] dCTP-labeled probe (1-2×10^6 cpm/ml). The filter was then washed twice for 15 minutes at 65°C in 50 ml of 2× SSC, 0.1% SDS, and washed once for 15 minutes at 52°C in 50 ml of 0.2× SSC, 0.1% SDS. It was sequentially hybridized with p63RhoGEF and β-actin cDNA probes.

C3 exoenzyme production and purification

C3 exoenzyme was expressed in the pET prokaryotic expression vector (generous gift of P. Bocquet, INSERM U452, Nice, France) and purification of the recombinant protein was performed by anion exchange chromatography as previously described (Dillon and Feig, 1995).

Cell culture, transfections and immunofluorescence microscopy

REF-52 cells and H9C2 were maintained as described elsewhere

(Gauthier-Rouviere et al., 1998; Kimes and Brandt, 1976). Both cell lines were transfected with the lipofectamine plus reagent according to Life's technology instructions. After fixation in 3.7% formaldehyde and permeabilization in 0.1% Triton in PBS for 3 minutes, cells were stained with the appropriate primary antibodies followed by FITC-coupled anti-mouse immunoglobulins and with rhodamine-conjugated phalloidin for F-actin staining. Cells were observed under a DMR Leica microscope using a 40× planapochromat lens. All transfections were repeated at least three times, and an average of 100 cells were examined each time.

Guanine nucleotide exchange assays

Recombinant GST-fusion proteins for p63RhoGEF DH domain (amino acids 149-374), Dbl and the Rho GTPases were produced using standard procedures. GDP release and GTP binding assays were performed as described elsewhere (Debant et al., 1996; Vignal et al., 2000). Experiments were repeated at least three times, and each point was done in duplicate.

In situ hybridization

Probe synthesis

RNA probe synthesis was carried out by means of a RNA transcription Kit (Stratagene, La Jolla, CA). Plasmid (pBluescript, Stratagene) containing p63RhoGEF-DH domain (amino acids 149-374) was linearized to give rise to the antisense and sense probes. 1 μg of the linearized DNA template was incubated for 2 hours at 37°C in a solution containing transcription buffer 1×, dithiothreitol (30 mM), rATP (0.4 mM), rGTP (0.4 mM), rCTP (0.4 mM), [α-^{35}S]UTP (5 μCi/μl), RNAse inhibitor (1.6 U/μl) and RNA polymerase (0.4 U/μl) T7 (sense) and T3 (antisense). The DNA template was then digested with RQ-1 DNAse (10 U) for 15 minutes at 37°C. After incubation, 10 μg yeast tRNA were added to the sample. Probe isolation was achieved on a sephadex G50 column. After precipitation, the probe was dissolved in hybridization mix (50% formamide, 0.3 M NaCl, 20 mM Tris-HCl, pH 8.5, 5 mM EDTA, 10% dextran sulfate, 1× Denhardt's solution, 0.5 μg/μl yeast tRNA and 10 mM DTT) at a final concentration of 2.10^4 cpm/μl and stored at –70°C until hybridization.

Slide treatment

Wax sections (brain and heart) were obtained from Novagen (Hybrid Ready Tissues). Radioactive in situ hybridization was performed on paraffin sections as previously described (Mazurais et al., 1999). After treatment with xylene (3× 5 minutes) to remove paraffin and rehydratation through an ethanol series, sections were rinsed in 0.85% NaCl and PBS (0.1 M, pH 7.4), postfixed in 4% paraformaldehyde and then treated with proteinase K (20 μg/ml) diluted in TE buffer (50 mM Tris-HCl, 5 mM EDTA, pH 8). After a rinse in PBS, the sections were refixed in 4% paraformaldehyde to stop proteinase K activity and then rinsed in PBS before acetylation with acetic anhydride (0.25% in triethanolamine 0.1 M, pH 8). Finally, sections were rinsed in distilled water before dehydration through ethanol series. After air drying, tissue sections were hybridized under coverslips with radioactive probe diluted in hybridization buffer (2.10^4 cpm/μl) overnight at 55°C in a humid chamber. After hybridization, coverslips were removed in 5× standard saline citrate (SSC 1×: trisodium citrate 15 mM, NaCl 150 mM, pH 7), 10 mM DTT at 55°C for 15 minutes and the slides were washed (30 minutes) with 2× SSC, 50% formamide, 10 mM DTT at 65°C. After a rinse (10 minutes) in NTE buffer (10 mM Tris-HCl, 0.5 mM NaCl, 5 mM EDTA, pH 8), sections were treated with RNAse A (20 μg/ml in NTE) for 30 minutes at 37°C. Slides were than washed in NTE for 15 minutes at room temperature and incubated for 30 minutes in 2× SSC, 50% formamide, 10 mM DTT at 65°C. Before autoradiography, the tissues were rinsed in 2× SSC and 0.1× SSC at room temperature and dehydrated in an ethanol series containing 0.3 M ammonium acetate. Autoradiography was performed by dipping the slides in Ilford K5 nuclear track emulsion and exposing the slides in the dark for 28 days at 4°C. After development, the sections were counterstained with toluidine blue and mounted in Pertex (Microm, France).

Peptide synthesis and polyclonal antibody production

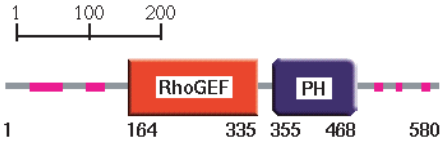
The deduced amino acid sequence of p63RhoGEF protein was analyzed for highly antigenic regions using the Jameson-Wolf antigenic index and checked for the absence of sequence homology with other proteins. Peptide 699, comprising amino acids 428 to 442 (CRFALTSRGPEGGIQ) of p63RhoGEF, was synthesized (Peptide Synthesizer Model 431A, Applied Biosystems), purified and conjugated to keyhole limpet hemocyanin using *m*-maleimidobenzoyl-N-hydroxysuccinimide as the coupling agent. Two 14-week-old New Zealand rabbits were injected with peptide-carrier conjugate (150 μg/injection) in complete Freund's adjuvant on day 0 and every two weeks with peptide-carrier conjugate (50 μg/injection) in incomplete Freund's adjuvant. Animals were bled 7 days after boosts (J39, J69 and J95) and their sera were tested at various dilutions on the unconjugated peptide coated ELISA plates. The immunoglobulins fraction from the anti-p63RhoGEF immune serums (Ab699) were obtained by affinity chromatography on protein A-sepharose and used for immunocytochemistry. The antibody Ab699 was validated by western immunoblot using a GST-p63RhoGEF fusion protein; Immunocomplex was abolished in presence of the 699 peptide (from 0.5 μg/ml to 50 μg/ml) in a concentration-dependent manner (data not shown).

Immunohistochemistry

Wax human sections were obtained from Novagen (Hybrid-Ready Tissues). After treatment with xylene and rehydratation through an ethanol series and PBS, the specimens were heated in target retrieval solution, pH 9.9 (Dako), 40 minutes at 95°C and then left to cool for 20 minutes on the bench. Competition was performed by overnight incubation of the primary antibody (1:750 and 1:20 dilutions for p63RhoGEF and β-myosin heavy chain (β-MHC) detection, respectively) with or without related 699 peptide (three times in excess compared with antibody concentration) used to generate anti-p63RhoGEF rabbit polyclonal antibody Ab699. After two washes of 2 minutes in TBS solution (Dako), sections were incubated for two hours at room temperature with primary antibody. Secondary antibody and the tyramide signal amplification peroxydase immunohistochemistry detection kit (Dako) were used according to the manufacter's instructions with diaminobenzidine as a substrate. Stained immunocytochemical sections were analyzed on a Nikon (Eclipse E800, Sony camera DXC-950P) microscope.

Immunofluorescence studies were performed on confocal microscope (Olympus Fluoview IX70) using primary antibodies: either Ab699 (1:750 dilution), monoclonal antibody anti-β-MHC at 1:20 dilution (Chemicon International) and monoclonal antibody anti-vinculin at 1:250 dilution (Sigma-Aldrich). For detection of p63RhoGEF, biotin-SP-conjugated AffiniPure F(ab′)$_2$ fragment Donkey anti-Rabbit IgG (H+L) (Jackson ImmunoResearch Laboratories) at 1:200 dilution followed by Cy2-conjugated streptavidin (1:360 dilution) were used. For detection of β-MHC, Rhodamine (TRITC)-conjugated AffiniPure F (ab′)$_2$ fragment Donkey anti-Mouse IgG (H+L) (Jackson ImmunoResearch Laboratories) was used at 1:50 dilution.

A



B

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h_tiam1     1043 K--VICELLETE-RTYVKDLNCLMERYLKPLQK-----ETFLTQDELDVLFGNLTEMVEFQVEFLKTLEDGVRLVPDLEKLEKVDQFKKVLFSLGGSFLYYA--DRFKLYSAFCAIHTKV---PKVLVKAKT
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h_vav1      199 RCCCLREIQQTE-EKYTDTLGSIQQHFLKPLQR--------FLKPQDIEIIFINIEDLLRVHTHFL-----------KEMKEALGTPG-APNLYQVFIKYK--ERFLVYGRYCSQVESA---SKHLDRVAA
h_sos1      200 TYYDLVKAFMAEIRQYIRELNLIIKVFREPFVS-----NSKLFSANDVENIFSRIVDIHELSVKLLG---------HIEDTVEMTDEGSP-HPLVGSCFEDLAEELAFDPYESYARDILRPGFHDRFLSQLSK
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h_trio2          ------TFFEDLKQR--LGHRLQLTDLLIKPVQRIMKYQLLLKDFLKYSKKASLDTSELERAVEVMCIVPRRCNDMM--NVGRLQG--FDGKIVAQ-GK----------LLLQDTFLVTD-----------Q
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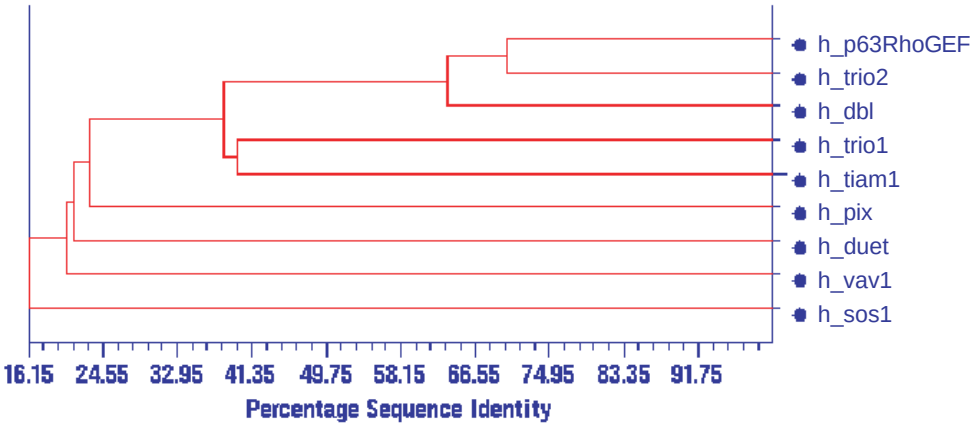
C



Results

Identification of p63RhoGEF, a novel guanine nucleotide exchange factor for Rho proteins

We set out to identify new GEF candidates for the Rho family of small GTPases. To that end, using in-house bioinformatics software we searched proprietary databases (SmithKline-Beecham Pharmaceuticals) for a consensus amino acid sequence motif derived from the DH domain of known GEFs for Rho-related GTPases (Boguski and McCormick, 1993). This search revealed the existence of a number of characterized or uncharacterized proteins exhibiting DH-like domains that are putative GEFs for Rho-like small GTPases. One of them, corresponding to Project Id ESTs: HFTCU45, HHFTC16 and HCE3W04 (HGS, Human Genome Science), was of particular interest and was further characterized. The cDNA containing an open reading frame encoding a protein of 580 amino acids called p63RhoGEF (GenBank accession number AXO02224) was obtained by merging overlapping fully sequenced ESTs.

Protein sequence analysis performed on p63RhoGEF led to the identification of the characteristic GEF tandem domain composed of the DH and PH domains (Fig. 1A). P63RhoGEF was then compared with well characterized GEFs (Fig. 1B). Moreover, the DH domain was classified with SMART (Schultz et al., 2000) as Rho GEF domain and thus, was putatively considered as an upstream regulator of the Rho GTPases family. The evolutionary trace method, which allows partitioning of groups of sequences (Lichtarge et al., 1996) highlighted the high identity score of 70% between p63RhoGEF and Trio2 (Fig. 1C). This result emphasized the putative linkage between p63RhoGEF and Rho GTPases, as Trio2 has been reported to be a RhoA-specific GEF (Debant et al., 1996).

P63RhoGEF is expressed in human brain and heart

We next examined the pattern of expression of p63RhoGEF mRNA. Hybridization of a 1.9 Kb p63RhoGEF probe to a northern blot membrane from multiple human tissues revealed the presence of a single mRNA product of about 2.6 Kb mainly in heart and brain tissues (Fig. 2). P63RhoGEF mRNA was also detected to a small extent in human small intestine but not in colon, thymus, spleen or peripheral blood leukocytes (data not shown).

P63RhoGEF displays in vitro exchange activity towards RhoA

To identify the Rho GTPase targets of p63RhoGEF, we performed in vitro guanine nucleotide release assays using bacterially expressed GST-p63RhoGEF DH domain (amino acids 149-374), GST-Dbl as a positive control and GST-Rho-GTPases. The guanine nucleotide exchange activity of p63RhoGEF was measured on various recombinant [^{3}H]GDP-loaded GTPases in the presence of GTP. As described elsewhere, Dbl preferentially stimulated guanine nucleotide release on RhoA, Cdc42, and RhoG, and to a lesser extent on Rac (Hart et al., 1994). As shown in Fig. 3A, p63RhoGEF specifically displayed exchange activity towards RhoA. p63RhoGEF did not promote nucleotide exchange on the other tested GTPases, but rather stabilized GDP binding on Rac and Cdc42. This observation suggests that p63Rho-GEF may bind ineffectively to these two GTPases in vitro, therefore preventing GDP release.

p63RhoGEF stimulated complete GDP dissociation from RhoA within 20 minutes (Fig. 3C) and consistently stimulated [^{35}S]GTPγS binding on the GDP-loaded GTPase (Fig. 3D). We also produced a p63RhoGEF protein containing a point mutation on leucine 301, residue located in one α-helix of the DH domain facing the GTPase partner (Liu et al., 1998) and highly conserved among the known Rho GEFs. This mutation is predicted to strongly affect exchange activity leading to an inactive protein (Alberts and Treisman, 1998). Indeed, the

Fig. 1. P63RhoGEF protein. (A) The human protein p63RhoGEF (GenBank accession number AXO02224) and identification by SMART method of the characteristic tandem domain, DH (for Dbl homology domain annotated by SMART as Rho GEF) and PH (pleckstrin homology domain). Segments in purple represent zones of low compositional complexity determined by the SEG program implemented in SMART (Schultz et al., 2000). (B) Multiple alignment of human p63RhoGEF DH-PH domains with other well-characterized human GEFs. Stars, double and single points indicate conserved residues, conservative substitutions and similar residues, respectively. Numbering indicates positions of start and end residues for each fragment of sequences used in the alignment. The DH domain is indicated by a solid line, while the PH domain is indicated by a dotted line. The two GEF domains of the protein Trio are displayed as h_trio1 and h_trio2 (AAC34245) (Debant et al., 1996). h_dbl (CAA21955; C. Bird, direct submission). h_tiam1 (NP_003244) (Habets et al., 1995). h_pix (NP_003890) (Nagase et al., 1995). h_duet (AB011422) (Kawai et al., 1999). h_vav (P15498) (Katzav et al., 1989). h_sos (Q07889) (Chardin et al., 1993). (C) Sequence identity dendrogram of DH-PH tandem domains obtained with the evolutionary trace method (Lichtarge et al., 1996).

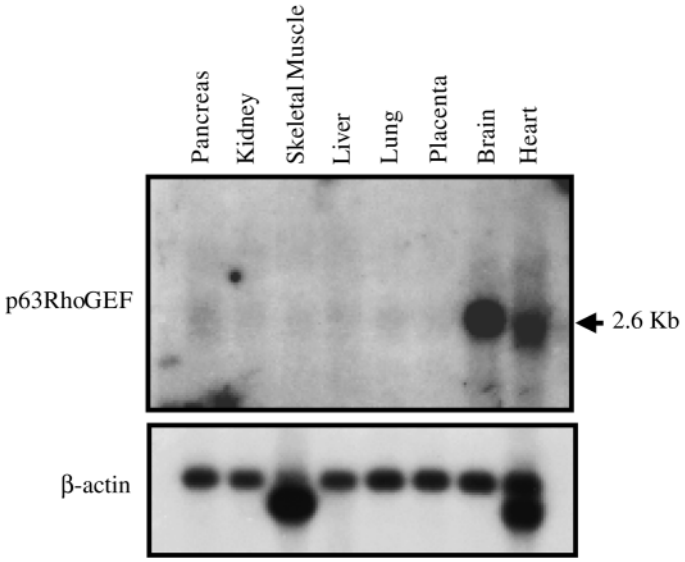


Fig. 2. P63RhoGEF mRNA distribution in human tissues. Human multiple tissue northern blot (Clontech) containing approximately 2 μg of poly(A)+ RNA per lane from eight different human tissues was sequentially hybridized with ^{32}P-labeled full-length p63RhoGEF and β-actin (control for the amount of RNA loaded) cDNA probes. Membranes were exposed for 16 hours (p63RhoGEF) and for 6 hours (β-actin). The arrow indicates the size of the p63RhoGEF transcript.

mutation L301E in p63RhoGEF completely abolished its exchange activity on RhoA (Fig. 3B).

P63RhoGEF induces stress fiber formation via RhoA activation in fibroblasts

In fibroblasts, Cdc42 and Rac induce filopodia and lamellipodia formation, respectively, while RhoA promotes stress fiber formation (Hall, 1998). We then measured the effect of p63RhoGEF expression on the actin cytoskeleton reorganization of REF-52 cells. REF-52 cells present basal stress fiber formation as illustrated by phalloidin staining of non-transfected cells. The expression of p63RhoGEF strongly induced cell retraction and an enhancement of stress fiber formation in 70% of the transfected cells, as did an activated form of RhoA (RhoAV14) (Fig. 4A,B). This effect was shown to be dependent on the p63RhoGEF GEF activity, since the L301E p63RhoGEF mutant-expressing cells do not exhibit enhanced stress fiber formation. Indeed, only 20% of the transfected cells by the p63RhoGEF mutant presented the p63RhoGEF wild-type phenotype. Similarly, treatment of the cells with the Rho-specific inhibitor exoenzyme C3 dramatically inhibits the induction of stress fiber formation by the wild-type p63RhoGEF. In addition, deletion of the PH domain abrogates the capacity of p63RhoGEF to induce an increase in stress fiber formation, suggesting that this domain is absolutely required for p63RhoGEF function in vivo. All together, these data indicate that RhoA is likely to be the target of p63RhoGEF in vivo.

P63RhoGEF induces stress fiber formation in cardiac myoblasts

In order to further investigate the putative role of p63RhoGEF in cardiac tissue, we investigated the effect of p63RhoGEF expression on the actin cytoskeleton reorganization of the rat cardiomyocytes-derived cell line H9C2. As it was observed in REF-52 fibroblasts, the expression of p63RhoGEF strongly induced an increase of stress fiber formation in the transfected cardiac myoblasts, as did an activated form of RhoA (RhoAV14) (Fig. 5). These data suggest that p63RhoGEF may be able to promote RhoA activation in intact cardiac myoblasts.

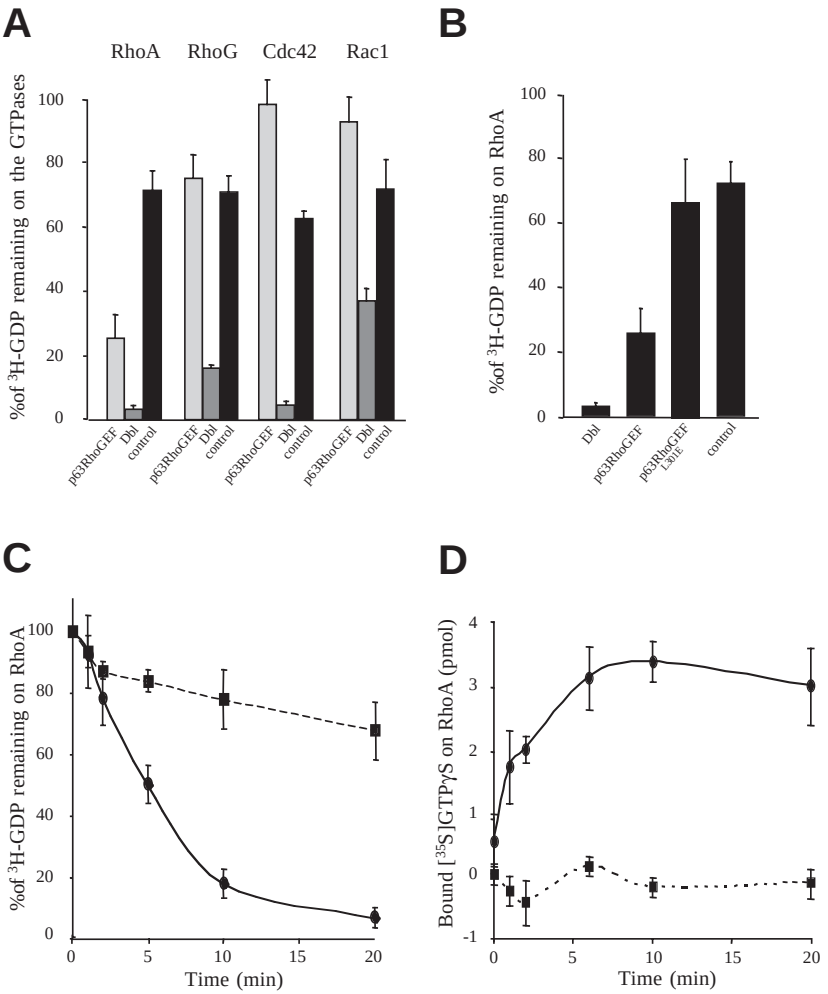


Fig. 3. P63RhoGEF specifically displays in vitro exchange activity towards RhoA. (A) [^{3}H]GDP nucleotide release assays on different recombinant Rho GTPases (0.4 μM) in absence (control) or in presence of GST-p63RhoGEF DH domain (2.8 μM), or GST-Dbl (1.2 μM). The exchange activity is expressed as the [^{3}H]GDP remaining on the GTPases after 15 minutes of reaction. The experiment presented here is representative of at least three independent assays. Means and standard deviations are shown. (B) [^{3}H]GDP nucleotide release assays on RhoA (0.4 μM) in absence (control) or in presence of GST-Dbl (1.2 μM), GST-p63RhoGEF DH (2.8 μM), or GST-p63RhoGEF L301E (2.8 μM). The exchange activity is represented as in A. (C) Time course study of guanine nucleotide exchange activity on the RhoA GTPase (0.4 μM) in absence (■) or in presence (●) of GST-p63RhoGEF DH (4 μM). The exchange activity is expressed as in A. (D) Kinetic of association of [^{35}S]GTPγS to GDP-loaded RhoA (1 μM) in absence (■) or in presence (●) of p63RhoGEF DH (10 μM). Means and standard deviations are shown.

Detection of p63RhoGEF transcript in human heart and brain by in situ hybridization

Sections of human cerebellar cortex and heart were subjected to an in situ hybridization using a p63RhoGEF antisense probe. The expression pattern was remarkably consistent from one experiment to the other. In all cases, with respect to the northern blot analysis, the p63RhoGEF mRNA was shown to be expressed in brain and in heart. No specific signal could be detected in the liver, lung and kidney (data not shown).

In the brain, the expression was detected in cell bodies of astrocytes and oligodendrocytes localized in the cerebellar cortex (Fig. 6Aa,b,e). Absence of labelling on adjacent sections hybridized with the sense probe consistently demonstrated the specificity of the p63RhoGEF probe (Fig. 6Ac,d,f).

In the heart, the results obtained by light microscopy showed that the p63RhoGEF mRNA had a widespread distribution within the left ventricle but that the labeling was restricted to the cardiomyocytes (Fig. 6Ba,b,e) as no specific signal could be detected in vessels and fibroblasts (data not shown). The

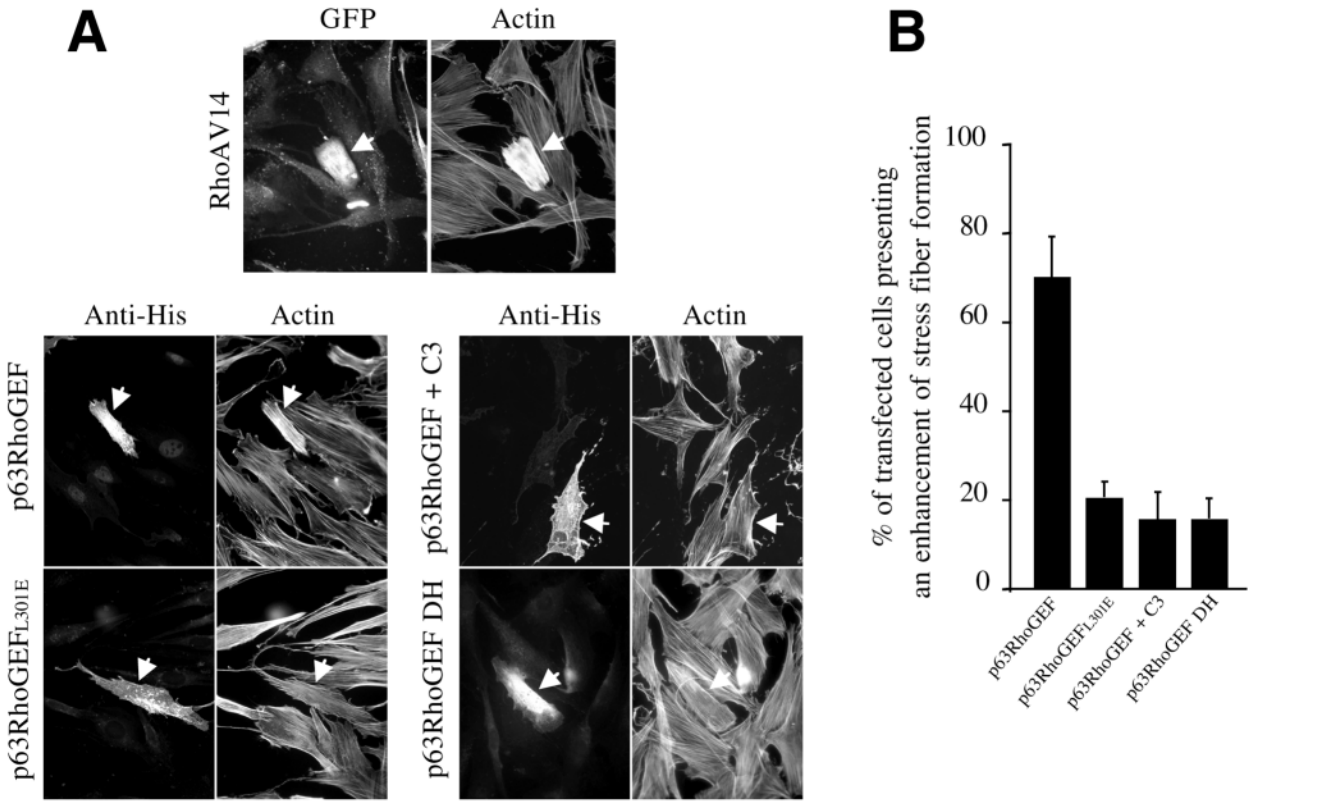


Fig. 4. P63RhoGEF induces RhoA-mediated stress fiber formation. (A) REF-52 cells were transfected with the different constructs as indicated and, 15 hours later, cells were fixed and permeabilized as described in Materials and Methods. The expression of p63RhoGEF was detected by an anti-His monoclonal antibody followed by FITC-coupled anti-mouse immunoglobulins, whereas F-actin was detected by rhodamine-conjugated phalloidin. Expression of GFP-RhoAV14 was visualized directly. When indicated, purified C3 exoenzyme was added in cell medium (0.5 μg/ml) 15 hours before fixation. This experiment is representative of at least three independent experiments, and 100 cells were examined each time. Transfected cells are indicated by white arrows. (B) Quantification of enhancement of stress fiber formation in REF-52 cells transfected as indicated.

specificity of the signal was confirmed by the absence of signal on the control slides (Fig. 6Bc,d,f).

P63RhoGEF protein is detected in human brain tissue sections

Analysis of human cerebellar cortex using Ab699 polyclonal antiserum revealed that p63RhoGEF protein is present at relatively high levels in the different cell populations (Fig. 7A). The staining was strong and uniformly distributed through the sections. Homogenous punctuated staining was observed in the external hypocellular layer containing astrocytes and proximal dendrites, in the intermediate Purkinje cell layer (Fig. 7Ab, arrow) and in the deep hypercellular granular cell layer. By contrast, no appreciable staining in the different cell populations was detected in the section using rabbit control nonspecific IgG.

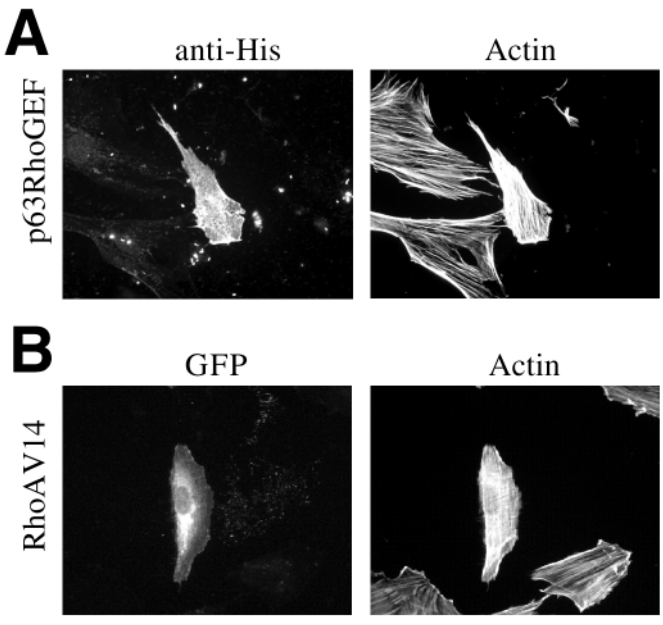


Fig. 5. P63RhoGEF induces RhoA-mediated stress fiber formation in cardiac myoblasts. After 48 hours of transfection, H9C2 cells expressing His-tagged p63RhoGEF (A) or GFP-RhoAV14 (B) were directly fixed and permeabilized as described in Materials and Methods. The expression of p63RhoGEF was detected by the anti-His antibody followed by FITC-coupled anti-mouse immunoglobulins, whereas F-actin was detected by rhodamine-conjugated phalloidin. This experiment is representative of at least three independent experiments.

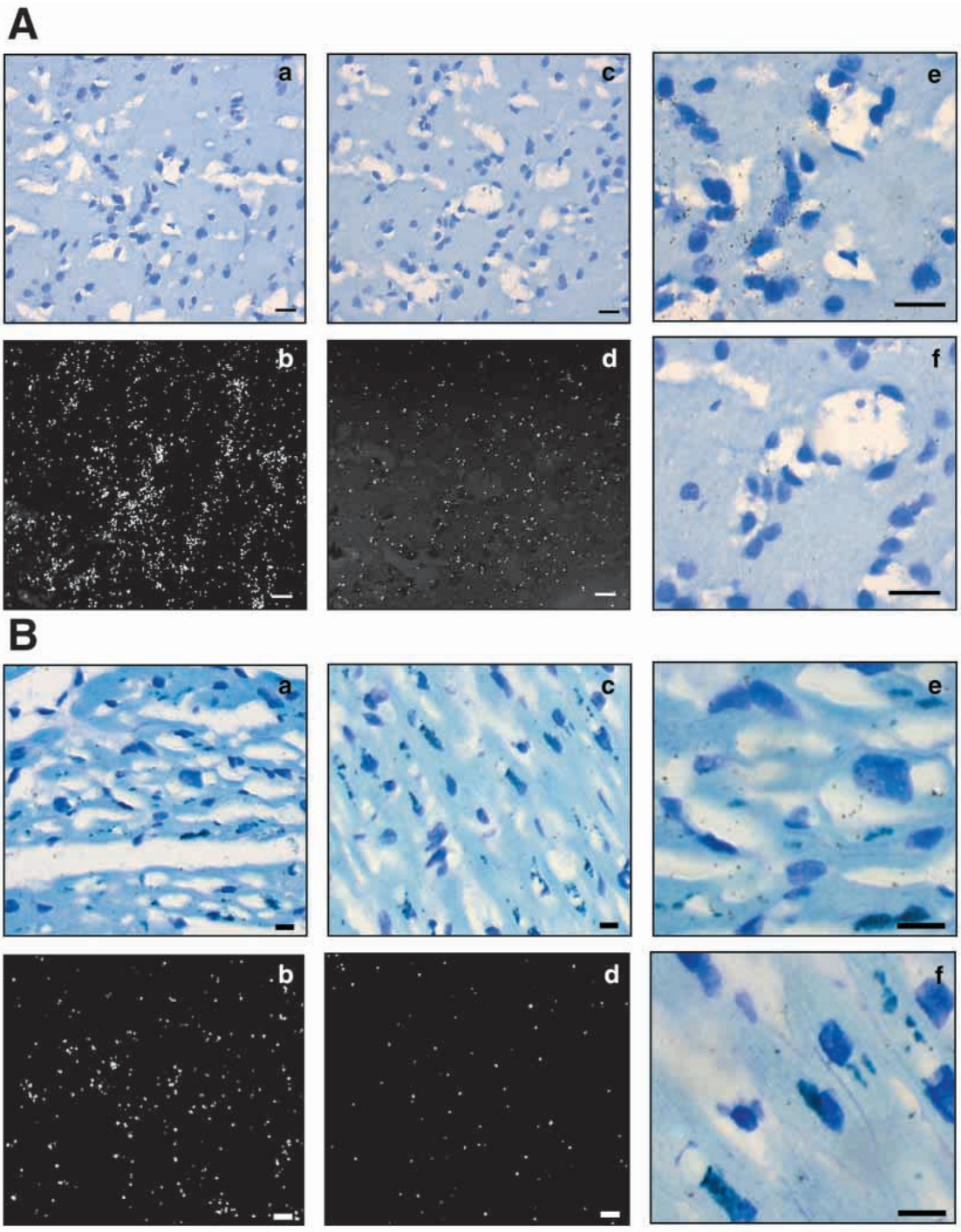


Fig. 6. Detection of p63RhoGEF mRNA by in situ hybridization in human heart and brain tissue sections. (A) Light microscopy of p63RhoGEF in situ hybridization in human brain. Brightfield (a,e) and darkfield (b) illuminations show the specific hybridization signal observed in body cells localized in the cerebral cortex. Control serial sections were incubated with the sense probe (c,d,f). Bars, 20 μm. (B) Light microscopy of p63RhoGEF in situ hybridization in human heart left ventricle. Brightfield (a,e) and darkfield (b) illuminations show the weak but specific hybridization signal observed in cardiomyocytes. Control sections were incubated with the sense probe (c,d,f). Bar, 10 μm.

P63RhoGEF protein is detected in human heart tissue sections

Analysis of human heart left ventricular section using Ab699 polyclonal antiserum revealed that p63RhoGEF protein was present at high levels in the vast majority of cardiomyocytes, when observing the fibers in transversal and longitudinal orientations (Fig. 7Ba,b), whereas a control rabbit IgG showed no appreciable staining (Fig. 7Bc). Most of the signal was

abolished by pre-incubation with the immunogenic 699 peptide that was used to generate the anti-p63RhoGEF rabbit polyclonal antibody (Fig. 7Bd).

Moreover, at the transversal incidence, a homogenous dotted staining was detected (Fig. 7Ba). Interestingly, the p63RhoGEF staining depicted in longitudinal fibers was represented by a brown repetitive striated staining, consistent with a sarcomeric organization (Fig. 7Bb,e). β-MHC monoclonal antibody used as a control of the endogenous sarcomeric striated pattern exhibited a similar arrangement (Fig. 7Bf). Different layers of blood vessels showed faint or no detectable staining with Ab699 (Fig. 7Ba, asterisk).

The striated pattern of immunolabelling exhibited by p63RhoGEF led us to analyze its sarcomeric distribution by means of double immunostaining. Confocal microscopic analysis was performed using rabbit p63RhoGEF polyclonal antibody and either mouse anti-β-MHC or mouse anti-vinculin in human left ventricle (Fig. 7C). Myosin, the principal component of the thick filaments, and vinculin localized in the extrasarcomeric cytoskeleton are good markers of the cardiac sarcomeric structure (Chen and Chien, 1999).

Fluorescence immunohistochemistry demonstrated that p63RhoGEF exhibit striated labelling organized in doublets (Fig. 7Ca). Moreover, with respect to myosin distribution, β-MHC immunolabelling was detected in doublets in transverse A-bands on both sides of the M-line (Fig. 7Cb, labeled as a and m, respectively). To determine potential colocalization between myosin and p63RhoGEF, dual immunostaining of p63RhoGEF and β-MHC in the double channel scan was performed. This experiment shows the alternative localization of the two proteins in the sarcomere indicating potential localization of p63RhoGEF in the I-band (Fig. 7Cc).

To confirm this hypothesis, double immunolabelling using anti-vinculin antibody was performed. With respect to extrasarcomeric cytoskeleton organization, vinculin was found to be localized in Z-disks and at the level of the intercalated disk (Fig. 7Ce). Double immunostaining analysis revealed that vinculin signal is found between p63RhoGEF immunolabelling (Fig. 7Cf). These results could indicate that p63RhoGEF is localized in the I-band of human cardiac sarcomeres.

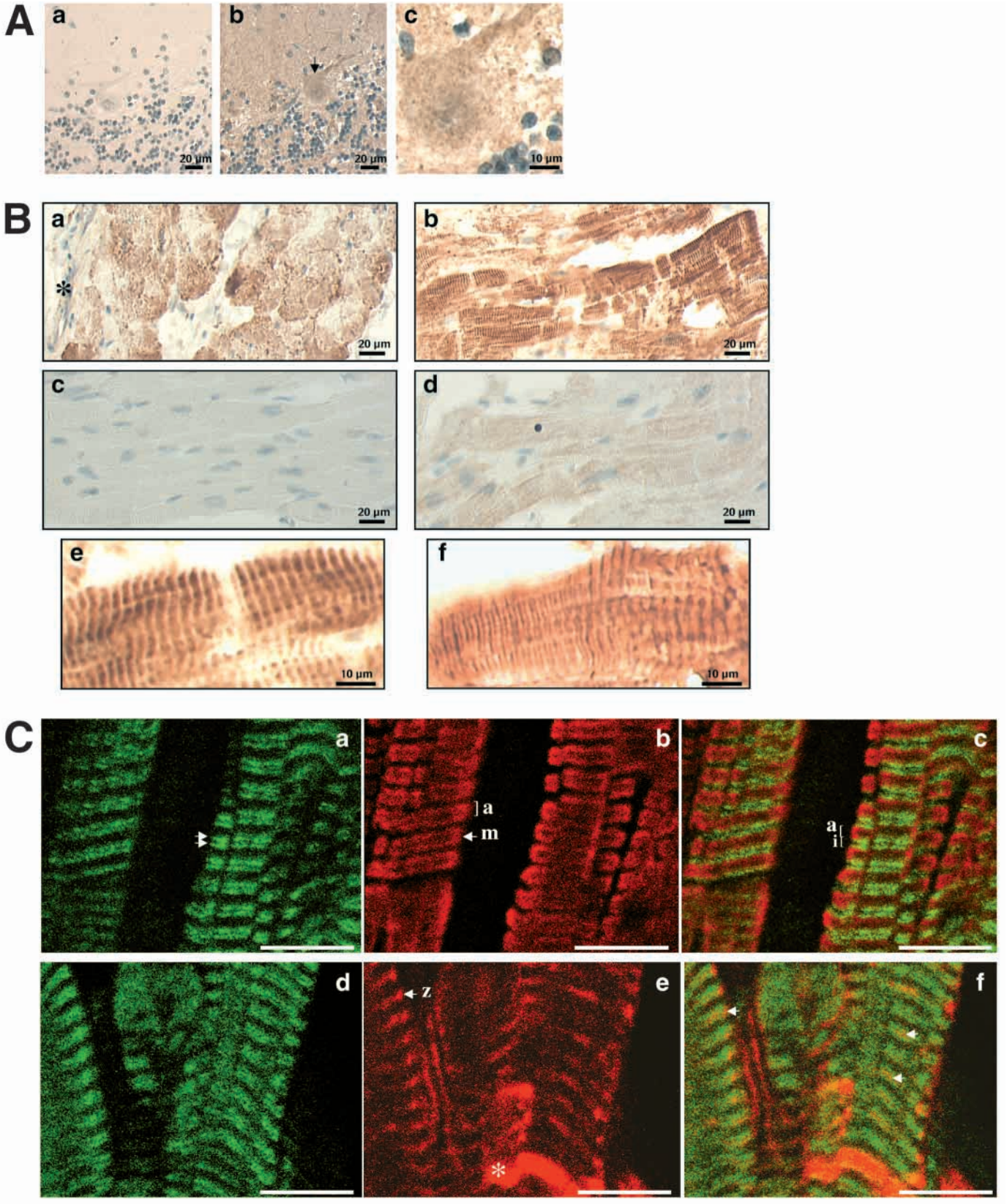
Discussion

To identify novel GEFs of the Rho small GTPases we have screened databases of mammalian sequences using a consensus profile derived from DH domains and retrieved several putative Rho GEF genes from human cardiac sources. One of these candidate genes (termed p63RhoGEF) was characterized further. Interestingly, we have retrieved from the GenBank database a mouse cDNA clone (AK010452) that corresponds to the mouse counterpart of human p63Rho-GEF (Kawai et al., 2001).

We demonstrated that p63RhoGEF is a RhoA-specific GEF by in vitro guanine nucleotide exchange assays. Moreover, p63RhoGEF activated the formation of stress fibers in fibroblasts and cardiomyocyte-derived H9C2 cells, indicating that p63RhoGEF can stimulate RhoA activity in intact cells. The inhibition of p63RhoGEF-induced stress fiber formation in fibroblasts treated by the Rho-specific inhibitor C3-exoenzyme confirmed the RhoA specificity for p63RhoGEF. This is consistent with the fact that p63RhoGEF is highly homologous to Trio2, which has been shown previously to be a RhoA-specific GEF (Debant et al., 1996). However, we cannot formally exclude the possibility that p63RhoGEF catalysed guanine nucleotide exchange activity on other C3-exoenzyme substrates such as the GTPases RhoB and RhoC.

Heart and brain-specific expression of p63RhoGEF was shown by northern blot analysis and confirmed by results from in situ hybridization and immunohistochemistry. Interestingly, the distribution pattern of the p63RhoGEF protein in heart was found to be organized in characteristic striated doublets in the sarcomere of cardiac cells. Confocal analysis of p63RhoGEF pattern with both β-MHC and vinculin patterns (specific markers of the cardiac sarcomeric structure characterizing the A-band and the Z-disk, respectively) was performed. From dual channel scan, we could conclude that p63RhoGEF is located in the sarcomeric I-band mainly constituted of cardiac α-sarcomeric actin. This result, together with the formation of F-actin stress fibres in H9C2 myoblasts and REF-53 cells, suggests that p63RhoGEF may be connected directly or indirectly to actin thin filaments. Indeed, Rho GEFs have been shown to be linked to actin or to actin-binding proteins. For example, Trio PH1 binds to the actin binding protein filamin (Bellanger et al., 2000), and the Rho GEF frabin possesses an actin-binding domain (Umikawa et al., 1999). The human Trio-like protein Duet, a close analog of human p63RhoGEF (63% identity), was localized to actin-associated cytoskeletal elements (Kawai et al., 1999). It would be of interest to investigate putative binding of p63RhoGEF via its PH domain to such structural proteins, given the fact that the p63RhoGEF PH domain is absolutely required for p63RhoGEF-mediated RhoA activation in fibroblasts and H9C2 myoblasts (data not shown). Other cytoskeletal proteins found in the Z-disk at the proximity of I-band, such as talin, vinculin, titin and α-actinin, may also contribute to the p63RhoGEF function. Interestingly, a myosin-M protein carrying a putative Rho GEF domain has been identified in *Dictyostelium* (Oishi et al., 2000). Such findings suggest that an association of a Rho GEF with a contractile protein might be required to execute appropriate function, and may be a way of regulating p63RhoGEF activity.

Indeed, it is of crucial importance to determine how the activity of these Rho-GEFs is regulated. Several Rho-GEFs have been directly involved in coupling heterotrimeric G protein to Rho. Cell surface receptors that transmit signal through heterotrimeric G protein activate Rho pathways (Sah et al., 2000) mainly by stimulating the activity of members of the Gα-12 family, which in turn, activate a GEF acting on Rho. These GEFs contain a Gα12/Gα13-binding region such as a LH and/or PDZ domains (Mao et al., 1998). These specific domains were not found in p63RhoGEF, suggesting that there is no direct evidence of its activation by Gα12/Gα13. This may indicate an alternative pathway for the regulation of p63RhoGEF activity, such as a phosphorylation event, as in the case of vav (Lopez-Lago et al., 2000) or such as a cytoskeletal targeting mediated by its PH domain. This latter hypothesis is reinforced by the observation that the PH domain of p63RhoGEF is absolutely required for its function.



Considering that overexpression of p63RhoGEF in H9C2 cardiac myoblasts resulted in the cytoskeletal reorganization of the F-actin filaments, it is tempting to postulate that p63RhoGEF is involved in the contractile process of cardiac cells. However, the precise functional role of p63RhoGEF at the sarcomeric level in cardiac cells and in other tissues such as brain remains to be determined.

Fig. 7. Immunolocalization of p63RhoGEF in human heart and brain tissue sections. (A) Detection of p63RhoGEF protein in human brain sections. (a) Normal human cerebellum section using non-specific rabbit IgG at ×40 magnification. (b) Normal human cerebellum section at ×40 magnification stained with Ab699 antiserum. (c) Enlarged and focused view of the normal human cerebellum section at ×100 magnification stained with Ab699 antiserum. The arrow shows one Purkinje cell surrounded by numerous round shaped cerebellar granular cells. (B) Detection of p63RhoGEF protein in human heart sections. (a) Transversal section of normal human heart at ×40 magnification stained with Ab699 antiserum. The asterisk indicates the location of a blood vessel. (b) Longitudinal section of normal human heart at ×40 magnification stained with Ab699 antiserum. (c) Normal human heart section using rabbit IgG at ×40 magnification. (d) Normal human heart section at ×40 magnification stained with Ab699 antiserum pre-incubated with the peptide 699 used to generate the anti-p63RhoGEF rabbit polyclonal antibody. (e) Longitudinal cardiac fibers from (b) shown at ×100 magnification stained with Ab699 antiserum. (f) Longitudinal cardiac fibers shown at ×100 magnification stained with β-MHC-specific monoclonal antibody. (C) Double immunostaining using rabbit p63RhoGEF polyclonal antibody and either mouse anti-β-MHC (a,b,c) or mouse anti-vinculin (d,e,f) in human left ventricle. The tissue sections were then analyzed by confocal microscopy. (a,d) p63RhoGEF exhibits a striated pattern of immunolabelling. Arrows in panel a indicate labelling organized in doublets. (b) β-MHC immunolabelling is found in transverse A-bands (marked as 'a') on both sides of the M-line (marked as 'm'), which is devoid of labelling. (c) Double immunostaining analysis reveals that the immunolabelled p63RhoGEF doublets are intercalated between A-bands (potentially in the I-band; marked as 'i'). (e) Vinculin immunolabelling is found in Z-disks (marked as 'z') and at the level of the intercalated disk (*). (f) Double immunostaining analysis reveals that vinculin signal is localized between immunolabelled p63RhoGEF doublet (see arrows). Bars, 10 μm.

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