A Dual Role of the GTPase Rac in Cardiac Differentiation of Stem Cells

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The function of the GTPase Rac1, a molecular switch transducing intracellular signals from growth factors, in differentiation of a specific cell type during early embryogenesis has not been investigated. To address the question, we used embryonic stem (ES) cells differentiated into cardiomyocytes, a model that faithfully recapitulates early stages of cardiogenesis. Overexpression in ES cells of a constitutively active Rac (RacV12) but not of an active mutant (RacL61D38), which does not activate the NADPH oxydase generating ROS, prevented MEF2C expression and severely compromised cardiac cell differentiation. This resulted in poor expression of ventricular myosin light chain 2 (MLC2v) and its lack of insertion into sarcomeres. Thus ES-derived cardiomyocytes featured impaired myofibrillogenesis and contractility. Overexpression of MEF2C or addition of catalase in the culture medium rescued the phenotype of racV12 cells. In contrast, RacV12 specifically expressed in ES-derived ventricular cells improved the propensity of cardioblasts to differentiate into beating cardiomyocytes. This was attributed to both a facilitation of myofibrillogenesis and a prolongation in their proliferation. The dominant negative mutant RacN17 early or lately expressed in ES-derived cells prevented myofibrillogenesis and in turn beating of cardiomyocytes. We thus suggest a stage-dependent function of the GTPase during early embryogenesis.

INTRODUCTION

The ras superfamily of monomeric GTPases includes the Rho, Ran, Rab, Rad/Gem, Arf, and the Ras families. These GTPases exert profound effects upon cell function and structure (Van Aelst and D'Souza-Schorey, 1997). Ran or Rab proteins mediate two very specific cellular events, i.e., nucleocytoplasmic shuttling and vesicular transport, respectively. In contrast, the Rho GTPases affect various cellular functions (Bishop and Hall, 2000) including cytoskeleton rearrangement, cell motility, cell growth, or cytokinesis (Takai *et al.*, 2001). Importantly, they play a role in embryogenesis (Settleman, 2001).

The Rho GTPases work as molecular switches to transduce intracellular signals from growth factors or G protein coupled receptors. The role of these GTPases in cardiac cell differentiation and heart function is not well understood. Recent in vitro studies indicate that RhoA and Rac may mediate the hypertrophic responses of agonists binding to seven transmembrane domains receptors (Sah *et al.*, 2000).

However, increased expression of RhoA in heart leads to a conduction abnormality and in turn to ventricular failure with no sign of hypertrophy (Sah *et al.*, 1999). Transgenic mice overexpressing a dominant positive mutant of Rac1 developed cardiac hypertrophy within weeks after birth, and this was associated with an alteration of focal adhesions. These animals featured a dilated cardiomyopathy accompanied with a decreased myofibril density and changes in cell adhesion structures (Sussman *et al.*, 2000). Wei *et al.* (2002) reported a crucial role of RhoGTPases in fetal cardiac cell proliferation in mice expressing RhoGDI.

Rho GTPases mediate signals from growth factors such as TGF (Mucsi *et al.*, 1996; Atfi *et al.*, 1997), a key cardiogenic factor. Yet, their specific role in early events of cardiac cell proliferation and differentiation is not known. Little information as to this issue can be obtained from transgenic mice, because the α -MHC promoter most often used to generate these genetically modified animals is mainly turned on at birth, at a time when myocytes became postmitotic cells. Similarly, RhoGDI overexpressing mice did not allow determination of the role of a specific Rho GTPase.

In this study, we investigated the role of Rac in mouse embryonic stem (ES) cell differentiation as a model of cardiac cell differentiation, which recapitulates the early stages of the complex program of mouse embryogenesis (Leahy *et al.*, 1999). We show that Rac effects depend on the stage of

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cell differentiation and that Rac GTPase activity inhibits cardiac cell differentiation at very early stages and then later promotes proliferation and myofibrillogenesis of cardiomyocytes

MATERIALS AND METHODS

Plasmid Construction, ES Cell Clone

cDNAs encoding the GTPases RhoAV14, RhoGV12, and RacV12 were subcloned in a retroviral vector. G418-resistant GP+E-86 clones expressing constitutively activated mutants of Rho GTPases were grown to collect retrovirus (Roux *et al.*, 1997). ES cells were infected with retrovirus overnight. cDNAs encoding RacV12, RacN17, and RacF28 were also subcloned in a bicistronic vector (pIRES2GFP) in which the CMV promoter sequence was excised and replaced by the 250-base pair sequence of MLC2v promoter (Meyer *et al.*, 2000). The linearized plasmid DNAs were electroporated into CGR8 ES cells according to standard protocol (Meyer *et al.*, 2000). The ES clones were propagated in the presence of LIF-conditioned medium obtained from LIF-D cells and selected for 10 d by incubation with G418 (250 μ g/ml) and further screened by PCR. Individual ES cell clones were screened by PCR for expression of GTPases. At least three independent cell clones were used for each GTPase.

cDNA encoding MLC2v was subcloned into pEGFP-N1 using the restriction sites Sal/BamHI to generate the expression vector encoding MLC2v-GFP fusion protein.

Rac Mutation

A PCR-driven amplification approach was used to generate a point mutation in the cDNA encoding RacL61 (Freeman *et al.*, 1996) to obtain a constitutively active mutant, which lost the ability to activate the membrane NADPH oxidase (Nisimoto *et al.*, 1997). Aspartate at position 38 was replaced by a lysine. A first PCR fragment of 500 base pairs was amplified using a forward primer including the mutation (5'-GCTGAAGGAGGAGAAGCTGACT-3') and a reverse primer in the 3' region of RacL61 (5'-GCTCGAGCTACAACAGGCATTTTC-3') including a BamHI restriction site. The PCR fragment was used as a reverse primer in the second PCR round together with a forward primer in the 5' region of the vector (5'-GGAATTCATGCATGCAGGCCATCAAGTG-3') including an EcoRI site. Mutation was checked by sequencing the cDNA encoding RacL61D38, which was subcloned into pIRES2-EGFP vector (Clontech, Palo Alto, CA) using the BamHI and EcoRI sites.

ES Cell Differentiation

ES cells (CGR8) were propagated in BHK21 medium supplemented with pyruvate, nonessential amino acids, mercaptoethanol, 7.5% fetal calf serum, and LIF-conditioned medium obtained from pre-confluent LIF-D cells stably transfected with a plasmid encoding LIF. The cells were trypsinized and replated every 2 d. Differentiation was carried out using the hanging drop method. Briefly, embryoid bodies (EBs) were formed in hanging drops of differentiation medium (BHK21 medium supplemented with pyruvate, nonessential amino acids, mercaptoethanol, and 20% fetal calf serum without LIF) for 2 d (D0–2). Then, the EBs were incubated for 3 d in suspension (D2–5) and for at least 7 d on gelatin-coated dishes or laminin-coated glass coverslips (D6–12; Meyer *et al.*, 2000).

Isolation of ES-derived Cardiomyocytes

To isolate ES-derived cardiomyocytes, EBs were detached from dishes at day 10 by incubation for 1 min with 0.05% trypsin at 37°C (in phosphate-buffered saline, 1 mM EDTA). Embryoid bodies were dissociated with 1 mg/ml collagenase (CLSII, Worthington, Lakewood, NJ) and 0.25 mg/ml pancreatin in a buffer containing (in

mmol/L) NaCl 117, HEPES 20, NaH₂PO₄ 1.2, KCl 5.4, MgSO₄ 1, glucose 5 (adjusted to pH 7.35). Cells were separated by centrifugation through a discontinuous two-layer Percoll gradient. Stem cell-derived cardiomyocytes were present at the interface between the two layers. They were collected and immediately plated on gelatin-coated glass dishes.

Transfection of Neonatal Rat and ES-derived Cardiomyocytes

Ventricular myocytes were isolated from 2–3-d-old rats and plated for 24 h in DMEM/M199 medium. Neonatal rat cardiomyocytes were then cotransfected with plasmids encoding RacL61D38 mutant and the red fluorescent protein (pCMVDsRed, Clontech) using Effectene (QIAGEN, Courtaboeuf, France) as previously described (Bony *et al.*, 2001). The same transfection protocol was used to transfect plasmids encoding the GTPases or MLC2vGFP in ES-derived cardiomyocytes.

Measurement of Reactive Oxygen Species in Neonatal Rat Cardiomyocytes

To monitor ROS production, myocytes were loaded for 20 min with 10 μ M 2', 5-(and-6)-chloromethyl-2',7'-dichlorodihydrofluorescein diacetate, acetyl ester (DCHF; Molecular Probes, Leiden, The Netherlands). After washing, cells then were transferred to the stage of an inverted epifluorescence Leica DMRA microscope (Rueil-Malmaison, France) equipped with a 63 $\times$ objective. Transfected cells were selected by RFP expression using a set of rhodamine filter (excitation 545 $\pm$ 10, emission 625 $\pm$ 15 nm) and fluorescence of DCHF was measured using a set of FITC filters (excitation 485 $\pm$ 10 and emission at 535 $\pm$ 20 nm) using a MicroMax 1300 Y/HS CCD camera (Princeton Instruments, Princeton, NJ) driven by MetaMorph (Universal Imaging Corporation, Roper Scientific, Evry, France).

Mouse Embryos

Mouse embryos were collected from mice at days 6, 9, and 11 postcoitum. RNA was isolated from total heart, ventricle or atria using a standard protocol (Chomczynski and Sacchi, 1987). After reverse transcription, encoding sequence of Rac1 was amplified from the cDNA using a specific set of primers (sense: 5'-TGG-GAGACGGAGCTGTGGTAAACCC-3' and antisense 5'-ACTTG-CATCAAATGCG-3').

Cell Immunofluorescence microscopy

EBs (D12–14) were fixed in 3% paraformaldehyde for 30 min and permeabilized for 30 min with 1% Triton X-100. The ES-derived isolated cardiomyocytes were fixed in 3% paraformaldehyde for 10 min and permeabilized for 10 min with 0.5% Triton X-100. Immunostaining was performed as previously described with polyclonal anti-MLC2a and anti-MLC2v antibodies (Meyer *et al.*, 2000). Monoclonal mouse antiactinin, monoclonal rat anti-Ki67, or polyclonal anti-MEF2C antibodies were purchased from Sigma (Saint Quentin Fallavier, France), Dako (Trappes, France), and Cell Signaling (Ozyme, Saint-Quentin, France) respectively.

Cell Imaging

Images of EBs or isolated cells were acquired with a Leica DMRA microscope equipped with a 40 $\times$ or 100 $\times$ objective mounted on a piezo-electric step motor. To visualize in situ immunostaining of MLC2v, optical z-sectioning of EBs was carried out using a 0.4- μ m step. To detect ECFP fluorescence, EBs or isolated cells were illuminated at 400 $\pm$ 20 nm and the CFP fluorescence recorded with a X114–2 CFP Leica filter cube that consists of a dichroic mirror DM 455 and an emission filter at 480 $\pm$ 30 nm. Images were acquired

with a MicroMax 1300 Y/HS CCD camera (Princeton) and stored as a volume file ("stack" of z-sections images) using Metamorph Digital restoration to remove noise, background, and blur of "stacks" of images was carried out using Huygens software (Huygens 2.3.9; Scientific Volume Imaging, Hilversum, The Netherlands) run on a dedicated double O 200 SGI (Silicon Graphics Industries, Mountain View, CA) and visualized using Imaris (Bitplane, Zurich, Switzerland). Beating activity was monitored by videomicroscopy using the stream acquisition mode, and beating areas were measured using the region measurement option of Metamorph. The latter were normalized to the total mesoderm area.

RT-PCR and Real-Time Quantitative PCR

Total RNA was prepared from 10 EBs after cell lysis in guanidinium thiocyanate-containing buffer using a modified phenol chloroform extraction (Chomczynski and Sacchi, 1987). After reverse transcription, 100–300 ng cDNA was used for semiquantitative PCR to stay within the linear range of amplification. PCR was carried out using a set of gene specific primers as previously described (Meyer *et al.*, 2000). Rho A and RhoG were amplified for 36 cycles using the following primers, RhoA: forward TGGAGCTGTGGTAAGACATGC and reverse AGAATCCCCAAGGAAGCTCG; RhoG: forward GTGCTTAACCCAACACC and reverse CACATCTTCTTCTCA. Rac 1 was amplified using the primers described in the embryo section. Tubulin, β MHC, GATA4, and MLC2v cDNAs were amplified for 30 cycles. Nkx2.5 and MEF2 cDNA were amplified for 30–36 cycles and the reaction run in agarose electrophoresis. The amount of PCR product was quantified for comparison by scanning photographs of ethidium bromide-fluorescent cDNA bands using a CCD camera and the NIH Image J 1.01 software.

MEF2C expression was measured using real-time quantitative PCR. After reverse transcription, 10 ng cDNA was used for real-time quantitative PCR, performed with a light cycler and the SYBR Green fast start kit (Roche, Meylan, France). The following primers were used for a real-time PCR: MEF2C forward 5'-AGATACCCACAA-CACACCACG-CGCC-3' and reverse 5'-ATCCTTCAGAGAGTCG-CATGCGCTT-3'; β -tubulin forward 5'-CGGACAGTGTGGCAAC-CAGATCGG-3' and reverse 5'-TGG CCAAAAGGACCTGACGGAAC-GG-3'. The 12- μ l reaction mix contained 1 μ l of Master SYBR Green I mix, including *Taq* DNA polymerase, buffer, deoxynucleoside triphosphate mix, SYBR Green I dye, 3 mM $MgCl_2$, and 0.5 μ M of each primer. Two microliters of 30-fold diluted cDNA was added to the mixture. Relative concentrations of mRNA were established by a standard curve using sequential dilutions of gene-specific PCR fragments. Data were normalized using RT-PCR of the β -tubulin mRNA as an index of cDNA content after reverse transcription. Amplification included initial denaturation at 95°C for 8 min, and 45 cycles of denaturation at 95°C for 3 s, annealing at 60–65°C for 8–10 s, and extension at 72°C for 7–10 s. The temperature transition rate was 20°C/s. Fluorescence was measured at the end of each extension step. After amplification, a melting curve was acquired by heating the product at 20°C/s to 95°C and then cooling it at 20°C/s to 70°C. The reaction was maintained at 70°C for 20 s followed by slow heating at 0.3°C/s to 95°C. Melting curves were used to determine the specificity of PCR products, and they were further confirmed by gel electrophoresis.

Rac GTPase Activity and Western Blot Analysis

Rac activity was measured with an assay activity kit (Upstate Biotechnology, Euromedex, Muldosheim, France) using a GST-conjugated PAK-1 protein-binding domain according to manufacturer's instructions. Proteins were extracted from EBs in NET (NaCl, EDTA, Tris) buffer containing 1% NP40 (Bony *et al.*, 2001). Proteins were assayed using the Bradford reagent, and the same amount per well was run in 12% SDS-PAGE and transferred to a nitrocellulose membrane as previously described. Blots were probed with a polyclonal anti-Rac (Santa Cruz Biotechnology, Santa Cruz, CA), anti-

Figure 1. Rac expression in embryonic hearts and ES cells. (A) RNA was extracted from embryos or embryonic hearts at different stages of development, and Rac1 mRNA was amplified by RT-PCR. (B) Western blot of proteins extracted from embryos or hearts at different stages of development using an anti-Rac antibody. (C) RNA was extracted from ES cells or embryoid bodies (day 12), and Rac1 mRNA was amplified by RT-PCR. The experiment was repeated twice with similar results. Results were normalized to tubulin expression.

gp91phox, or anti-p67phox (De Leo *et al.*, 1996) and polyclonal anti α -tubulin as indicated, and a secondary peroxidase-conjugated antibody. Immunoreactive proteins were revealed by ECL (Bony *et al.*, 2001).

RESULTS

Early Expression of Rac1 in Stem Cells and Mouse Embryos

During mouse embryogenesis, Rac1 mRNA was weakly expressed in embryos at E6, and the protein was barely detectable in Western blots. Expression was enhanced and Rac1 mRNA and protein reached their maximal level at E9 and E11, respectively. Furthermore, Rac1 was expressed in heart both in atria and ventricles, with a higher expression in ventricles (Figure 1, A and B). We further investigated expression of Rac1 in vitro in ES-derived EBs. Rac1 mRNA was found as early as in undifferentiated ES cells, and its expression remained unchanged in EBs up to day 12 (Figure 1C). Rac activity was however not detected in undifferentiated ES cells.

Constitutively Active Rac1 Impairs Cardiac Differentiation of ES Cells

To determine the role of the Rac GTPase in cardiac cell differentiation of ES cells, we generated an ES cell line expressing a constitutively active or a dominant negative mutant of Rac (RacV12 or RacN17, respectively). In RacV12 EBs, Rac activity was increased by 5 ± 0.8 -fold ($n = 3$), whereas Rac activity was abolished in RacN17 cell line (our unpublished results). The RacV12 cell line was then tested for its ability to differentiate into functional cardiomyocytes. Because the RacV12 expression vector contained an ubiquitous promoter, Rac was broadly expressed after differentiation in most cells of the three layers (i.e., ectoderm, mesoderm,

Figure 2. Beating activity of embryoid bodies (EBs) generated from ES cells expressing dominant active mutants of GTPases. ES cells expressing dominant active mutants of RhoG (RhoGV12), RhoA (RhoAV14), or Rac (RacV12) or expressing ECFP under the control of the MLC2v promoter (MLCECFP) were allowed to differentiate within EBs. Beating activity was measured each day from day 7 to day 12. The EB was considered as beating if at least three separate areas were beating. Each point is the average $\pm$ SEM of more than 250 EBs generated in five separate experiments. **Significantly different from control (MLCECFP; $p \leq 0.001$). The inset shows the overexpression of the GTPases in day 5 EBs assessed by RT-PCR.

endoderm) of the EB. The cardiac phenotype of EBs (i.e., structure and function of ES-derived cardiomyocytes) expressing RacV12 was compared with that of EBs overexpressing other constitutively active mutants of RhoGTPases, namely RhoAV14 and RhoGV12 (Figure 2, inset).

Beating activity of EBs generated from RhoAV14, RhoGV12 ES cells showed that these cells differentiated into cardiac cells as well as MLCECFP ES cells, a control cell line expressing ECFP under the control of the ventricular myosin light chain 2 (MLC2v) promoter (Meyer *et al.*, 2000; Figure 2). In contrast, although RacV12 EBs displayed the three-layer structure (our unpublished results), they lost their ability to beat, indicating a potential defect in cardiac differentiation and/or excitation-contraction coupling of ES-derived cardiomyocytes.

Mechanism Underlying RacV12-induced Impairment of Cardiac Cell Differentiation

To understand the mechanism of inhibition of beating activity in RacV12 EBs, mRNAs encoding cardiac transcription factors were amplified by PCR after reverse transcription of RNA extracted from EBs at day 5. Expression of Nkx2.5 or GATA4 mRNA in RacV12 EBs was not different from that in EBs expressing RhoGV12 or ECFP. In contrast, MEF2C expression was dramatically decreased in RacV12 EBs (Figure 3A). To confirm this result, real-time quantitative PCR was performed. This more quantitative approach revealed that

Figure 3. Expression of cardiac genes in EBs generated from ES cells expressing dominant active mutants of GTPases. (A) RNA was extracted from 5 (D5) or 7 d (D7) old embryoid bodies (EBs) expressing ECFP under the control of the MLC2v promoter (MLCECFP; lane 1), RhoA (RhoAV14; lane 2), RhoG (RhoGV12; lane 3), or Rac (RacV12; lane 4). mRNA of genes encoding cardiac transcription factors (GATA4, MEF2C, Nkx2.5) and an housekeeping gene, tubulin (tub) were amplified by RT-PCR. Both tubulin and GATA4 or MEF2C and Nkx2.5 were amplified together in the same PCR reaction. The experiment was repeated three times with similar results. (B) RNA was extracted from MLCECFP or RacV12 EBs at day 5 and expression of MEF2C mRNA was measured by real-time quantitative PCR. The bar graph represents the mean $\pm$ SEM of three experiments. **Significantly different from control (MLCECFP; $p \leq 0.001$).

MEF2C mRNA expression was eight times lower in RacV12 EBs, compared with controls (Figure 3B). Furthermore, we were also not able to detect MEF2C protein in RacV12 EBs using Western blot or immunofluorescence (our unpublished results).

Expression of genes encoding constitutive contractile proteins such as MLC2v and myosin heavy chain (β -MHC) was also determined in EBs expressing constitutively active Rho GTPases. Figure 4 shows that in RacV12 EBs but not in any other EBs, the level of expression of MLC2v gene was significantly reduced, whereas expression of β -MHC (Figure 4A) and MLC2a (our unpublished results) was not affected. MLC2v expression and incorporation into the sarcomeres was evaluated by immunofluorescence of EBs or ES-derived cardiomyocytes isolated from EBs expressing dominant active GTPase mutants. In RacV12 EBs, fewer MLC2v positive ventricular cardiomyocytes were observed compared with control (i.e., MLCECFP clone; Figure 4B, left panel). At high magnification, images of stained actinin showed a lack of sarcomeric units (Figure 4B, right panel). This was further confirmed in cardiomyocytes isolated from RacV12 EBs. RacV12 cardiomyocytes, stained with specific anti-MLC2 antibodies, revealed that the ventricular light chain of myosin was weakly expressed and not incorporated into sarcomeric units. In contrast, the atrial myosin light chain was normally expressed (Figure 4C).

The reduced expression of MEF2C in RacV12 EBs may be responsible for impaired expression of MLC2v. To test this hypothesis, we attempted to rescue the RacV12 EB phenotype by overexpressing MEF2C in the RacV12 ES cell line (Figure 5A). Differentiation of RacV12/MEF2C ES cells re-

Figure 4. Impaired cardiac differentiation and myofibrillogenesis in RacV12 embryoid bodies (EBs). (A) MLC2v and β MHC mRNA were amplified by RT-PCR from RNA extracted from day 12 EBs expressing RhoGV12 (RhoGV12), RhoAV14 (RhoAV14), RacV12 (RacV12) or expressing ECFP under the control of the MLC2v promoter (MLCECFP). (B) Anti-actinin staining of cardiomyocytes within EBs expressing ECFP under the control of the MLC2v promoter (MLCECFP), used as a control, or RacV12 (RacV12). The left images were acquired at 10 $\times$ magnification to visualize the extent of cardiac strands within the EB. The right images were acquired at 40 $\times$ magnification and results from a 3D restoration of a stack of images acquired by optical z-section of EBs. (C) Cardiomyocytes were enzymatically isolated from the EBs expressing ECFP, RhoA, RacV12, or RhoG and purified on a Percoll gradient. Cells were then stained with specific anti-MLC2v (top panels) or anti-MLC2a (bottom panels) antibodies. Images are representative of five experiments with similar results.

vealed that the level of Rac expression was comparable in RacV12 EBs and RacV12/MEF2C EBs (Figure 5B, inset). Figure 5B shows that beating activity of RacV12/MEF2C EBs was restored and was comparable to control EBs. MLC2v expression and sarcomerogenesis were also both restored in RacV12/MEF2C EBs (Figure 5, C and D).

Reactive Oxygen Species Trigger Downregulation of MEF2C Expression and Accounts for the Phenotype of RacV12 ES-derived Cardiomyocytes

We next addressed the mechanism of downregulation of the MEF2C gene in RacV12 EBs. One important and specific

effector of Rac is a membrane-associated NADPH oxydase, which generates reactive oxygen species (ROS; Babior, 1999). ROS are known to repress the activity of promoters for several genes including myoD and α -actin (Morel and Barouki, 1999). Thus, we evaluated the role of ROS in cardiac differentiation of ES cells by incubating EBs with 100 nM H_2O_2 from days 0 to 7. For these experiments, we used cells expressing ECFP under control of the α -actin promoter (Behfar *et al.*, 2002) to track the cardiomyocytes at an early stage of differentiation. Figure 6A shows that clusters of ECFP expressing cells were detected as early as day 5 in control EBs, whereas no fluorescence could be observed in EBs treated with H_2O_2 . Furthermore, H_2O_2 dramatically

Figure 5. Overexpression of MEF2C rescues the cardiac phenotype of RacV12 EBs. (A) MEF2C mRNA was amplified by real-time quantitative PCR from RNA isolated from control (MLCECFP) EBs or from embryoid bodies (EBs) expressing RacV12 or both RacV12 and MEF2C at day 7. (B) Beating activity of the EBs. The data are the mean $\pm$ SEM of at least 100 EBs generated in three separate experiments. **Significantly different from control (MLCECFP) or MEF2CRACV12 EBs ($p \leq 0.001$). The inset shows the overexpression of Rac in both RacV12 and RacV12MEF2C EBs (day 5). (C) Actinin (left images) and MLC2v (right images) immunofluorescence of control EBs (top panels) or of bodies expressing both RacV12 and MEF2C (bottom panels). Images were acquired at 10 $\times$ magnification. (D) High magnification (40 $\times$) and 3D reconstruction of a stack of images of actinin immunostaining obtained from z-sections of an EB expressing both RacV12 and MEF2C. Images of representative of three experiments with similar results.

prevented beating activity of EBs (Figure 6B) and decreased MEF2C expression (Figure 6C). This is reminiscent of the RacV12 EB phenotype. When added from days 7 to 12, H₂O₂ did not longer decrease beating activity of EBs (our unpublished results).

Essential NADPH oxydase components, namely the membrane gp91^{phox} and Rac-regulated p67^{phox}, are expressed in EBs as early as days 5–7 (Figure 6D). To further investigate the role of ROS in ES cell differentiation, we generated a stable ES cell line expressing a constitutively active Rac

Figure 6. Reactive oxygen species impair cardiac differentiation. (A) ECFP expression in ES-derived cardiomyocytes expressing the fluorescent protein under the control of the α -actin promoter (actECFP), within a control (left image) or an EB treated from days 3 to 7 with 100 nM H₂O₂. (B) Beating activity of embryoid bodies (EBs) expressing ECFP under the control of α -actin promoter (actECFP) nontreated or treated for 5 d (D3–7) with 100 nM H₂O₂. The bar graph represents the mean $\pm$ SEM of three experiments gathering at least 90 EBs. **Significantly different from control (actECFP; $p \leq 0.001$). (C) Expression of MEF2C mRNA in EBs nontreated or treated for 5 d (D3–7) with 100 nM H₂O₂ obtained in real-time quantitative PCR in three experiments. (D) Western blot analysis of expression of gp91^{phox} and p67^{phox} in EBs at different stages of differentiation. The blots are representative of two experiments.

Figure 7. Rac-mediated generation of reactive oxygen species is responsible for deficient cardiac differentiation of ES cells. (A) pcDNA 3.1 (mock), RacV12, or a dominant active double mutant of Rac, RacL61D38 (RacD38), that lost the ability to activate the NADPH oxydase were cotransfected with a plasmid encoding the red fluorescent protein (RFP; left panels) into neonatal cardiomyocytes. The myocytes were loaded the next day with DCHF (right panels) to visualize intracellular ROS generation. (B) Beating activity of control (■) or RacL61D38 EBs (▲). *Significantly different from control; $p \leq 0.001$, $n = 3$ separate experiments including at least 50 embryoid bodies (EBs). The inset shows the overexpression of RacL61D38 or Rac V12 in EBs (day 5) compared with the endogenous (wt) Rac. (C) Beating activity of wild-type (▲) RacV12 (■) EBs nontreated or treated from day 2 (◆) or 5 (▼) with 1000 U/ml catalase. *Significantly different from control; $p \leq 0.001$, $n = 3$ separate experiments including at least 50 EBs. (D) Actinin immunostaining of EBs expressing RacL61D38 or RacV12 in the presence of catalase in the medium. A single image of a RacL61D38 (a) or of a RacV12 EB cultured from day 2 in the presence of 1000U/ml catalase (c) was acquired at a 10 $\times$ magnification. A stack of images of a RacL61D38 (b) or of a RacV12 EB cultured in the presence of 1000 U/ml catalase (d) was acquired at high magnification (63 $\times$). Images were restored using Huygens software and visualized as a shadow projection using Imaris software. Images are representative of three experiments.

mutant (L61D38), which has lost the ability to activate the membrane NADPH oxydase and, therefore, the ability to generate ROS. To check that the constitutively active mutant L61D38 was deficient in NADPH oxydase activity in cardiac cells, neonatal rat cardiomyocytes were transiently transfected with RacV12 or RacL61D38. Loading the cells with DCHF, a ROS-sensitive fluorescent probe, revealed that RacV12-transfected cells generated ROS normally. In contrast, cells expressing RacL61D38 did not produce any ROS

(Figure 7A), even though the level of overexpression of RacL61D38 in EBs was similar to that of RacV12ES cells (inset). Cardiomyocytes differentiated from RacL61D38 ES cells were indistinguishable from wild-type myocytes, and MEF2C expression was not affected by expression of RacL61D38 (our unpublished results). More interestingly, the beating activity of EBs and the number of cardiomyocytes with sarcomeric actinin within the EBs was increased (Figure 7, B and D) compared with RacV12 EBs (Figure 4).

Figure 8. RacN17 prevents myofibrillogenesis of ES-derived cardiomyocytes. (A) Expression of cardiac genes in day 7 RacN17 embryoid bodies (EBs). RNA was extracted from wt or RacN17 EBs at day 7. RT-PCR was then used to amplify the genes. (B) Beating activity of wild-type EBs (■) or of EBs expressing Rac dominant negative mutant (RacN17) under the control of the CMV promoter (▲). *Significantly different from control; $p \leq 0.001$, $n = 3$ separate experiments including at least 45 EBs. (C) 10 $\times$ image of actinin stained ES-derived cardiomyocytes within an EB. (D) 63 $\times$ restored 3D image of actinin stained ES-derived cardiomyocytes within an EB. Arrows indicate areas of incomplete organization of sarcomeric units. Images were restored using Huygens software and visualized as a shadow projection using Imaris software. Images are representative of three experiments.

As an alternative approach, we reasoned that if Rac-induced activation of NADPH oxydase impaired cardiac cell differentiation, a scavenger of ROS should relieve this effect. Thus, we added in the differentiation medium of RacV12 EBs 1000 U/ml catalase. Added at day 5, catalase partially rescued beating activity of RacV12 EBs. Added as early as day 2, the ROS scavenger fully restored beating activity of EBs (Figure 7C). In the same line MEF2C mRNA content and in turn MLC2v expression were restored in racV12 EBs (MEF2C mRNA at day 5 [in a.u.], 1 ± 0.1 in wt day 5 EBs vs. 0.1 ± 0.05 in RacV12 vs. 1.2 ± 0.1 in RacV12 treated with catalase from day 2, $n = 3$). Myofibrillogenesis visualized by actinin decoration of EBs was then normal in RacV12 EBs cultured in the presence of catalase (Figure 7D).

Inhibition of Endogenous Rac Impaired Beating Activity of Embryoid Bodies

We then allowed an ES cell clone expressing a dominant negative mutant of rac (RacN17) to differentiate. Inhibition of Rac as early as in ES cells did not affect the process of cardiac differentiation as revealed by normal expression of the transcription factors Nkx2.5 and MEF2C, or of the constitutive cardiac genes, β MHC or MLC2v (Figure 8A). However, spontaneous activity of EBs was severely impaired (Figure 8B). Actinin staining of EBs revealed the presence of cardiomyocytes (Figure 8C) but a poor myofibrillogenesis. Indeed sarcomeric units of ES-derived cardiomyocytes were not fully organized (Figure 8D).

Late and Cardiac-restricted Expression of RacV12 Improves Cardioblasts Proliferation and Myofibrillogenesis

To further investigate the specific role of Rac in cardiac differentiation, we generated a DNA vector including the MLC2v promoter to exclusively express a dominant negative (RacN17), a fast cycling (RacF28; Lin *et al.*, 1999), or a

dominant active (RacV12) Rac mutant in ES-derived ventricular cells. Furthermore, this approach allowed us to bypass early expression of RacV12. Indeed, when RacV12 was expressed under the control of MLC2v promoter, the GTPase was overexpressed only in ES-derived ventricular cardiomyocytes from days 5 to 7 of differentiation when the MLC2v promoter was turned on as visualized by GFP expression in beating cells (Meyer *et al.*, 2000). Cardiac differentiation of these cells was greatly improved within the EBs, as shown by the beating activity of EBs (Figure 9A) and MEF2C expression in cardiac mesodermal area of EBs (right panel). Moreover, the percentage of the mesoderm (i.e., median layer) featuring a beating activity was significantly increased when compared with control EBs ($60 \pm 5\%$ vs. $28 \pm 4\%$, $n = 5$). A similar phenotype was obtained when RacF28, a fast cycling mutant of Rac, was expressed under control of the MLC2v promoter. In contrast, cardiac differentiation was impaired in RacN17 EBs, and only $10 \pm 3\%$ ($n = 5$) of the mesoderm was beating. Under control of the MLC2v promoter, RacV12 did not affect MEF2C expression. α -actinin and α -MLC2v staining of EBs revealed that myofibrillar strands were more abundant in MLCRacV12 than in MLCRacN17 ES-derived mesoderm (Figure 9B). In addition, RT-PCR of RNA extracted from MLC2RacV12 or MLCRacN17 EBs at days 7 or 9 did not show any significant difference in expression of cardiac transcription factors (Nkx2.5, MEF2C, or GATA4), when compared with MLC-CECFP EBs used as control (our unpublished results).

To further investigate the nontranscriptional mechanism of improvement or impairment of beating activity in MLCRacV12 and MLCRacN17 EBs, respectively, we conducted experiments designed to look at myofibrillogenesis of ES-derived cardiomyocytes. Cardiomyocytes were isolated from wild-type EBs at day 9 and transfected with plasmids encoding RacV12-GFP or RacN17-GFP, together with a DNA vector encoding the fusion protein MLC2vGFP. In wild-type or RacV12-transfected cells, MLC2vGFP

Figure 9. Overexpression of Rac in ES-derived cardioblasts favors their proliferation and myofibrillogenesis. ES cell clones expressing Rac dominant active mutant (RacV12), fast cycling (RacF28) or dominant negative mutant (RacN17) under the control of MLC2v promoter were generated and allowed to differentiate within embryoid bodies (EBs). (A) Beating activity *, E: Significantly different from control (MLC2ECFP; $p \leq 0.001$). The inset shows the nuclear staining of MEF2C in MLCRacV12 EBs. (B) MLC2v (top images) and actinin (bottom images) staining of ES-derived cardioblasts expressing RacV12 (left images) or RacN17 (right images) within EBs at day 10. The inset in the top left panel shows the regular structure of sarcomeres in RacV12 expressing ES-derived cardioblasts. (C) ES-derived cardioblasts were isolated from wild-type EBs, cultured for 2 d and transfected with a plasmid encoding the fusion protein MLC2vGFP alone (mock) or together with RacV12GFP or RacN17GFP. Arrows show the membrane localization of RacGFP in comparison to the sarcomeric distribution of MLC2vGFP. RacN17 prevents full sarcomerogenesis. The graph on the right represents mean $\pm$ SEM of at least 45 cells from two separate experiments featuring a complete myofibrillogenesis 18 h after transfection. (D) Ki67 (top panel) and BrdU (bottom panel) staining of nuclei from ES-derived cardioblasts expressing RacV12 or ECFP (used as a control) isolated from EBs and cultured for 2 d. The graph on the right represents mean $\pm$ SEM of at least 30 cells. *Significantly different from control; $p \leq 0.001$. Scale bars, 10 μ m.

formed aligned and well-defined sarcomeric units, and this process was accomplished more rapidly than in wild-type cells. In contrast, in RacN17-transfected cells, MLC2vGFP

failed to fully incorporate into sarcomeres (Figure 9C) as previously found in CMVRacN17 ES-derived cardiomyocytes (Figure 8D). Next, we isolated ES-derived cardiomyo-

cytes from MLCRacV12 or MLCECFP EBs and plated them in culture. Using both an antibody raised against Ki67, a proliferative marker expressed in any stage of cell cycle of mitotic cells but G0 (Scholzen and Gerdes, 2000), BrdU revealed that ES-derived cardiomyocytes expressing RacV12 were still proliferating at day 3 postisolation, whereas most of the cardiomyocytes expressing ECFP exited the cell cycle (Figure 9D).

DISCUSSION

Our study reveals the use of the powerful model of ES cells combined with overexpression of genes to uncover the role of Rho GTPases in cardiac early development. We show here that Rac plays an important role in early stages of differentiation of stem cells and that this GTPase acts as a suppressor of MEF2C, a process likely to be mediated by ROS.

Among the constitutively active Rho GTPase mutants expressed as early as in undifferentiated stem cells, Rac conferred a unique cardiac phenotype. Although early expression of constitutively active mutants of RhoA or RhoG improved cardiac differentiation of ES cells, the constitutively active mutant of Rac seriously impaired the process. In RacV12ES-derived cardioblasts, downregulation of the cardiac-specific transcription factor MEF2C, a factor that tightly regulates numerous cardiac-specific genes (Harvey, 1999), resulted in a poor expression of MLC2v, which compromised the organization of myofibrils. This would be expected, based on the key role of this protein in the formation of sarcomeres (Chen *et al.*, 1998). Restoration of both sarcomerogenesis and beating activity by overexpression of MEF2C in RacV12 ES-derived cardiomyocytes demonstrates that lack of expression of the transcription factor is at the origin of the defect in cardiac cell differentiation of RacV12 ES cells.

Searching for the mechanism underlying the impaired expression of MEF2C in RacV12ES cells, we reasoned that such a mechanism should be specific of RacV12 because MEF2C was expressed to the same extent in ES-derived cardiomyocytes expressing constitutively active mutants of other RhoGTPases (i.e., RhoG, RhoA) as in control MLCECFP cells. Therefore, we looked for a Rac-specific effector. The membrane NADPH oxidase is a major and specific Rac1/Rac2 effector enzyme (Babior, 1999; Archer and Bar-Sagi, 2002). The NADPH oxidase is comprised of five components (p40^{phox}, p47^{phox}, p22^{phox}, p67^{phox}, and gp91^{phox}) and generates ROS. Rac1 and Rac2 can both bind to p67^{phox} (Nisimoto *et al.*, 1997) and, thereby, activate the NADPH oxidase. Gp91^{phox}, p22^{phox}, and p67^{phox} are highly expressed in embryonic tissues, including the heart, and in early differentiation stages of ES-derived EBs (Sauer *et al.*, 2000; Cheng *et al.*, 2001; Figure 6D). Because ROS repress expression of numerous cardiac genes (Morel and Barouki, 1999), we evaluated the involvement of ROS in the repression of MEF2C expression by studying the propensity of ES cells expressing a constitutively activated mutant of Rac (RacL61D38), which lost the ability to bind p67^{phox} and in turn to activate the NADPH oxidase (Nisimoto *et al.*, 1997), to differentiate into cardiac cells (Figure 7). Such cells expressed normal levels of MEF2C, featured regular sarcomeres, and thus differentiated normally into functional beating cardiac cells. Furthermore, day-to-day addition of 100

nM H₂O₂ at early stage of differentiation (days 0–7), at a concentration expected from RacV12 activity within differentiating cells (Price *et al.*, 2002), to the EBs also severely impaired cardiac differentiation of ES cells, as shown by the lack of both transactivation of the MEF2C-dependent α -actin promoter and beating activity of EBs. On the other hand, the ROS scavenger, catalase added to the culture medium of EBs rescued MEF2C expression, myofibrillogenesis and in turn beating activity of RacV12 EBs. Together, these findings demonstrate the crucial deleterious role of Rac-induced ROS generation in early stages of cardiac cell differentiation.

Limitations of our cell model include the noncell type- or time-restricted expression of RacV12, because the mutant was driven by an ubiquitous promoter leading to a strong overexpression (Figure 5, inset) of a GTPase switched in constitutively activated and nonregulated conformation. Besides MEF2C downregulation, the cardiac phenotype of RacV12 EBs may be aggravated by ROS generation in any cell type that differentiated within EBs. Rac-generated ROS may induce a loss of cadherin-mediated cell-cell connection (van Wetering *et al.*, 2002), a process required for early cardiac development (Linask *et al.*, 1997). Therefore, to provide more insight into the role of Rac in cardiac differentiation, we used the ventricular myosin light chain 2 promoter to specifically drive expression of GTPase mutants in ES-derived ventricular cells (Meyer *et al.*, 2000). Under such cardiac- and time-restricted expression, RacV12 significantly improved or accelerated the process of cardiac differentiation, as visualized by the extent of beating areas within EBs as soon as the MLC2v promoter was turned on at day 7 (Meyer *et al.*, 2000; Figure 9). A similar effect was observed with the fast cycling mutant RacF28, a more physiological way to increase Rac activity that can still be endogenously regulated. Furthermore, such an extensive beating area within the mesoderm of MLC2RacV12 EBs is similar to the one obtained in TGF β -treated, ES-derived cardiomyocytes (Behfar *et al.*, 2002), as expected from activation of Rac by the growth factor (Atfi *et al.*, 1997). In contrast, the dominant negative mutant RacN17 prevented cardiac myofibrillogenesis. Thus when expressed only in ventricular cells and at a stage of differentiation when cardiac transcription factors has already reached maximal level of expression (Meyer *et al.*, 2000), Rac GTPase activity turns out to be required for terminal differentiation of cardiac precursors.

Two explanations are suggested to account for the great propensity of MLC2RacV12 ES cells to differentiate into contractile cardiomyocytes. First, Rac improves myofibrillogenesis of cardioblasts, as shown by a complete incorporation of MLC2vGFP into sarcomeric units of ES-derived cardiomyocytes expressing RacV12 and an inhibition of the process in cells early or lately expressing RacN17. This effect is likely to depend on lamellipodia generated at the membrane by Rac. These actin structures serve as a niche for the setting of the first contractile proteins within the z-bodies (Sanger *et al.*, 2000). Second, Rac prolongs proliferation of cardioblasts, as shown by both Ki67 and BrdU staining (Figure 9). This result is in line with the cardiac phenotype of RhoGDI-overexpressing mice (Wei *et al.*, 2002) and with the expected effect of Rac on cyclin D1 (Coleman and Marshall, 2001), E2F stimulation and pRb hyperphosphorylation (Gjoerup *et al.*, 1998).

Rac delays cardiac differentiation by repressing expression of a major cardiac transcription factor, MEF2C. At very early stages of differentiation and during gastrulation, the GTPase may thus be critical to facilitate proliferation and migration of cells, including muscle precursors (Sugihara *et al.*, 1998; Settleman, 1999, 2000, 2001; Knight *et al.*, 2000), while preventing cell differentiation of one of the earliest cell types developing in the embryo, namely, the cardioblast. This could be mediated partially by ROS and NF κ B pathways (Joneson and Bar-Sagi, 1998; Babior, 1999; Joyce *et al.*, 1999). Rac-induced ROS may also be critical for the physiological apoptotic process that occurs during early stages of embryogenesis (Pampfer, 2000; Poelmann *et al.*, 2000). At later stages, Rac activated by cardiogenic factors such as TGF β (Mucsi *et al.*, 1996; Atfi *et al.*, 1997) becomes essential in the process of cardioblast proliferation and myofibrillogenesis of cardiomyocytes.

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