The small G-proteins Rac1 and Cdc42 are essential for myoblast fusion in the mouse

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Rac1 and Cdc42 are small G-proteins that regulate actin dynamics and affect plasma membrane protrusion and vesicle traffic. We used conditional mutagenesis in mice to demonstrate that Rac1 and Cdc42 are essential for myoblast fusion in vivo and in vitro. The deficit in fusion of Rac1 or Cdc42 mutant myoblasts correlates with a deficit in the recruitment of actin fibers and vinculin to myoblast contact sites. Comparison of the changes observed in mutant myogenic cells indicates that Rac1 and Cdc42 function in a nonredundant and not completely overlapping manner during the fusion process. Our genetic analysis demonstrates thus that the function of Rac in myoblast fusion is evolutionarily conserved from insects to mammals and that Cdc42, a molecule hitherto not implicated in myoblast fusion, is essential for the fusion of murine myoblasts.

actin cytoskeleton | muscle development | myotube formation

Skeletal muscle fibers are syncytia that arise by the fusion of myogenic cells. Mononucleated myogenic cells, the myoblasts, fuse with each other to form multinucleated myotubes. In mammals, myoblast fusion occurs during development and in the adult, allowing generation, growth, and repair of muscle fibers (1). Myoblast fusion controls myofiber length as well as contractile capacity and muscle function (1, 2). On a cellular level, it is characterized by an alignment of myoblast and/or myotube membranes, followed by rearrangements of the actin cytoskeleton at the contact sites and membrane fusion (3–7). The current knowledge on myoblast fusion was greatly furthered by genetic analyses performed in *Drosophila*, which revealed a molecular cascade controlling fusion (8, 9). More recently, data attained through in vivo experiments in vertebrates demonstrated that many aspects of the molecular mechanisms of myoblast fusion are conserved between vertebrates and invertebrates (10, 11).

In *Drosophila*, “founder” myoblasts determine the position, the orientation, and the identity of individual muscle fibers. “Fusion-competent” myoblasts fuse with founders, forming the multinucleated fibers (12–14). Interactions between cell adhesion molecules of the Ig superfamily mediate the initial contact between founder and fusion competent cell (15–17). These interactions result in cytoskeletal rearrangements and finally fusion (reviewed in refs. 8, 9). Key intracellular components that act downstream of cell adhesion molecules to control cytoskeletal and particularly actin rearrangements are the Rac GTPases, dRac1, and dRac2, and a Rac regulator, the dimeric guanine nucleotide exchange factor encoded by dElmo and myoblast city, a homolog of vertebrate DOCK1 (18–21). The Arp2/3 complex is directly involved in cytoskeletal remodeling and actin polymerization. Kette and WAVE/Scar can control Arp2/3. In addition, WASP and Ver/Wip/Slit activate the Arp2/3 complex. Both pathways that control Arp2/3 activity are essential for fusion (22–26). These actin-rich structures are believed to position Golgi-derived prefusion vesicles to the site of membrane fusion (27) and/or to control the size of the fusion pore (23, 26).

Fusion of mammalian myoblasts was previously extensively analyzed in cultured cells, which had revealed surprisingly little overlap with the molecules important for fusion with those in invertebrates. Only recently, the first in vivo analyses in zebrafish and mice were reported, which began to reveal an extensive

conservation of the molecular mechanisms of myoblast fusion in the animal kingdom. In particular, downregulation of zebrafish Kirrel, a homolog of the Ig adhesion receptors that organize myoblast fusion in *Drosophila*, or downregulation of zebrafish Rac1 were found to interfere with myoblast fusion (28). Furthermore, downregulation of the Rac1 regulators Dock1 and Dock5 in zebrafish, or mutation of Dock1/5 in the mouse demonstrated the importance of these components in myoblast fusion (29, 30). Crk and Crkl, 2 adaptor molecules known to interact with Dock1 and to control Rac1 activation, were recently identified in zebrafish as novel regulators of myoblast fusion (30).

We report here the use of conditional mutagenesis in mice to demonstrate that Rac1 and Cdc42 are essential for myoblast fusion in vivo and in vitro. Our analysis demonstrates thus that this function of Rac1 is evolutionarily conserved from insects to mammals and that Rac1 is essential for the recruitment of actin at contact sites of fusing myoblasts, as are Rac1/Rac2 in *Drosophila*. Cdc42, like Rac1, is known to control rearrangements of the actin cytoskeleton and was previously not implicated in myoblast fusion. We show that Rac1 and Cdc42 act in a nonredundant and apparently not completely overlapping manner to recruit vinculin and cytoskeletal proteins to contact sites of fusing myoblast and to allow the fusion process to occur.

Results

Rac1 and Cdc42 are essential for early mouse development (31, 32). To analyze functions of Rac1 and Cdc42 in muscle development, we used floxed Rac1 and Cdc42 alleles (33, 34). Conditional mutations were introduced using an Lbx1cre BAC transgene that expresses cre-recombinase under the control of Lbx1 regulatory elements, resulting in recombination in myogenic precursor cells that migrate to targets like the limbs, diaphragm, and tongue (35, 36).

Analysis of the distribution of myogenic precursor cells in the limbs of conditional Rac1 or Cdc42 mutant mice revealed normal numbers and distribution of such cells (Fig. S1). Thus, Lbx1+ myogenic precursor cells migrated correctly to the limb buds in the conditional mutant mice. In addition, proliferation or differentiation, as assessed by Ki67, MyoD, desmin, and Myogenin expression, appeared unaffected at E11.5 and E12.5 (Fig. S1). However, we observed striking differences in muscle development at subsequent stages. In longitudinal sections, muscle fibers identified by staining for desmin or skeletal muscle-specific myosin were short and thin in the limbs of Rac1 and Cdc42 conditional mutants compared to control mice, indicating that fusion of myogenic cells was impaired (Fig. 1A–C and J–L). In transverse sections, myofibers appeared thin and disorganized in the conditional mutant mice (Fig. 1D–F).

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Fig. 1. Impaired myoblast fusion in conditional *Rac1* and *Cdc42* mutant mice. (A–F) Longitudinal (A–C) and transverse (D–F) sections of muscle fibers in the proximal forelimb of control (A and D), conditional *Rac1* (B and E), and conditional *Cdc42* (C and F) mice at E13.5 were analyzed by immunohistochemistry using antibodies against desmin and MyoD (A–C), or anti-desmin and the nucleic acid stain SYBR (D–F). (G–I) Distribution of myogenic cells in the forelimb of control (G), conditional *Rac1* (H), and conditional *Cdc42* (I) mice at E13.5, as revealed by immunohistochemistry using anti-desmin and anti-MyoD antibodies. (J–L) Longitudinal sections of muscle fibers in the proximal forelimb of control (J), conditional *Rac1* (K), and conditional *Cdc42* (L) mice at E18.5 were analyzed by immunohistochemistry using antibodies against myosin and the nucleic acid stain SYBR [Scale bars, (A–F, J–L) 50 μ m, (G–I) 400 μ m].

Nevertheless, MyoD⁺ and desmin⁺ cell groups were located at appropriate positions in the limbs of conditional mutant mice (Fig. 1 G–I). Similar changes were also observed in the tongue and diaphragm muscles of the conditional *Rac1* and *Cdc42* mutant mice, which are also colonized by myogenic precursor cells of the *Lbx1*-lineage (Fig. S2 A–F). The size of muscle groups was little affected at E13.5 or E14.5, Fig. 1 G–I, but significantly reduced at E18.5. Staining for cleaved caspase-3 demonstrated an increase in apoptosis in limb muscle at E14.5 in the conditional mutants, indicating that cell death contributes to the change in overall muscle size (Fig. S2 G–I). In addition, we used the *Pax3cre* allele to introduce conditional loss-of-function *Rac1* mutations in all myogenic precursors and observed short and thin muscle fibers at E14.5 in trunk and limb muscle. *Pax3cre* induced mutation of *Cdc42* interfered with embryonic survival beyond E12. We therefore restricted our further analysis to mice in which recombination was introduced by *Lbx1cre*.

To quantify the effect on myoblast fusion, limbs of control and mutant mice were dissected at E14.5, the early time point at which small muscle fibers had been observed *in vivo*. Cytoplasm and nuclei of myogenic cells were visualized using antibodies directed against desmin and MyoD, and the nuclear dye SYBR. This revealed striking changes in relative proportions of myotubes and myoblasts obtained from control and conditional mutant mice (Fig. 2). In particular, multinucleated myofibers were more frequent in preparations from control than from conditional *Rac1* or *Cdc42*

mutant mice (Fig. 2 A–C). To quantify this, we determined the fusion index, i.e., the percentage of myogenic nuclei present in multinucleated cells. In control preparations, 52% of the MyoD⁺ nuclei were present in myotubes, but only 8% and 26% of the MyoD⁺ nuclei had fused in the preparations from conditional *Rac1* and *Cdc42* mutants, respectively (Fig. 2D). Not only the fusion index was altered, but also the average number of nuclei in multinucleated cells. For instance, 32% of the myogenic nuclei were present in myotubes contained 3 or more nuclei in the control preparations, but only 2% or 5% of the nuclei in preparations from *Rac1* and *Cdc42* mutants, respectively (Fig. 2E). Even more pronounced effects were observed at E18.5 (Fig. 2 F–J). At this stage, the majority of myotubes isolated from control mice were very large and contained 11 or more nuclei. In contrast, myotubes from conditional mutant mice were small, and we observed none that contained 11 or more nuclei in preparations from *Rac1* or *CDC42* mutants. Instead, most myogenic cells had remained mononuclear (Fig. 2J).

We next assessed the fusion of control and mutant myoblasts in culture. For this, limb cells were isolated at E12.5, plated in growth medium, and subsequently cultured in differentiation medium for 72 h. In control cultures, 35% of the myogenic nuclei were present in myotubes containing 3 or more nuclei, but only 2% and 7% in cultures of *Rac1* and *Cdc42* mutant myogenic cells (Fig. 3 A–E). To assess if *Rac1* or *Cdc42* have essential roles in both fusion partners, we used mixed cultures containing control and *Rac1* or *Cdc42*

Fig. 3. Impaired myoblast fusion of cultured myogenic cells from conditional Rac1 and Cdc42 mutant mice. (A–C) Myogenic cells from the forelimb of control (A), conditional Rac1 (B), and conditional Cdc42 (C) mice were isolated, cultured for 72 h in differentiation medium, and analyzed by immunohistochemistry using antibodies directed against desmin and MyoD. (D and E) Quantification of the fusion index and of the number of myogenic nuclei present in mono- or multinucleated cells. (F–H) Mixed cultures of control cells and of myogenic cells of different genotypes that are marked genetically by expression of alkaline phosphatase (AP). AP-positive cells were derived from (F) control, (G) conditional Rac1, and (H) conditional Cdc42 mice. (I and J) The fusion index of AP-positive cells and the number of AP-positive myogenic cells containing various nuclei number were determined [Scale bars, (A–H) 50 μ m].

suffices to interfere strongly with fusion. Mutation of the murine Cdc42 gene also interferes with myoblast fusion, whereas available evidence had not revealed such a role of *Drosophila* Cdc42 (18, 25).

Rac1 and Cdc42 Are Required in both Fusion Partners. In *Drosophila* myogenesis, the fusion partners are nonequivalent, and 2 distinct cell types participate in fusion, founder and fusion-competent cells (12–14). We therefore tested if Rac1 and Cdc42 are required in both fusion partners, and mixed myogenic cells from control and conditional Rac1 or Cdc42 mutant mice in culture. Fusion was impaired to a similar extent in mixed cultures containing control and Rac1 mutant cells, as in the cultures that only contain Rac1 mutant myogenic cells. In the equivalent experiment using Cdc42 mutant cells, we observed a similar reduction in fusion in the mixed cultures as in the cultures that contain mutant myogenic cells

only. Thus, these results indicate that both fusion partners depend on Rac1 and Cdc42.

Rac1 and Cdc42 Control Filamentous Actin Assembly and Fusion in Murine Myoblasts. In *Drosophila* Rac, Rac regulators, and molecules controlling actin polymerization are essential for myoblast fusion (18–20, 22–26). These proteins function in a regulatory cascade that organizes actin at the site of myoblast adhesion and fusion, which is thought to be essential for targeted exocytosis (27) and/or for an enlargement of the fusion pore (23, 26).

Various proteins assemble at the adhesion sites of fusion-competent murine myoblasts. A high density of the cell adhesion molecules N-Cadherin and M-Cadherin, and of Cadherin-interacting proteins like α - and β -catenin can be observed at the adhesion sites. Furthermore, Arp2/3, Ena-Vasp that control actin

Fig. 4. Contact sites of cultured Rac1 and Cdc42 mutant myogenic cells. Myogenic cells from the forelimb of control, conditional Rac1 and conditional Cdc42 mice were isolated, cultured for 36 h in differentiation medium, and analyzed by immunohistochemistry using antibodies directed against (A–C) β -catenin and α -catenin, (E–G) β -catenin and vinculin, (I–K) β -catenin and F-actin, (M–O) β -catenin and Vasp, and (Q–S) β -catenin and Arp2/3. (D, H, L, P, T) Quantification of the signal intensities obtained by immunohistological analysis. Contact sites were identified by β -catenin recruitment; for quantification of the recruitment of the second protein, the ratio of the signal intensity observed at the contact sites/signal intensity observed in the cytoplasm was determined. Signal intensity differences of more than a factor of two were scored as positive for the recruitment of a particular protein. The numbers of β -catenin+ contact sites that recruited the indicated second protein are displayed [Scale bar, (A–S) 10 μ m].

polymerization, vinculin that links adhesion molecules to the cytoskeleton, and filamentous actin are found at the adhesion sites (38–41) (see also Fig. 4). Analysis of Rac1 and Cdc42 mutant myoblasts indicated that the initial adhesion process occurred correctly, as α - and β -catenin were efficiently recruited to the contact sites. In contrast, accumulation of vinculin, Ena-Vasp, and polymerized actin at the contact sites were markedly reduced. Interestingly, divergent effects on the recruitment of the Arp2/3 to contact sites were noted: Arp2/3 accumulated efficiently in the Cdc42 mutant myoblasts, but Arp2/3 recruitment in Rac1 mutant cells was reduced in a pronounced manner. This might indicate that Rac1 and Cdc42 function in nonequivalent manners, as was observed previously for instance in the epidermis (34). The changed Arp2/3 recruitment in Rac1 but not in Cdc42 mutant cells might support such a model. Alternatively, Rac1 and Cdc42 might function in a linear cascade. Rac1 and Cdc42 share many effectors, and Cdc42 is important for a significant part of Rac1 activation, at least

in fibroblasts (42). In general, we observed a somewhat weaker fusion phenotype in Cdc42 compared to Rac1 mutant myoblast, and it is thus possible that the Cdc42 phenotype reflects a greatly reduced but not completely absent Rac1 activity. In fibroblasts, Rac1 and Cdc42 control lamellipodia and filopodia formation, respectively, structures that were studied extensively because of their roles in cell migration (43, 44). However, we did not observe changes in migratory properties of Rac1 or Cdc42 mutant myogenic progenitor cells *in vivo*, indicating that other small GTPases might control migration of myogenic cells. Taken together, our analysis provides further support for the evolutionary conservation of the mechanisms of myoblast fusion in the animal kingdom. In addition, we find that Cdc42, a molecule hitherto not implicated in myoblast fusion, is also essential for the fusion process in mice. Additional work will be needed to assess if this function during myoblast fusion is conserved in other species or if it reflects a mammalian-specific role of Cdc42.

Materials and Methods

Animals. The generation of the Rac1flox, Cdc42flox, and transgenic Lbx1cre strains were previously described (33–35). Embryos with genotypes Rac1flox/flox;Lbx1cre (conditional Rac1 mutant) and Cdc42flox/flox;Lbx1cre (conditional Cdc42 mutant) were used for analysis, and Rac1flox/+;Lbx1cre or Cdc42flox/+;Lbx1cre embryos were used as controls.

Immunofluorescence Staining. Immunofluorescence staining of cryosections and cultured cells was performed after 2 h and 15 min fixation with 4% paraformaldehyde, respectively. The following antibodies were used: Anti-skeletal fast myosin, anti-desmin, anti- α -catenin, and anti-vinculin (Sigma); anti-desmin, anti-MyoD, and anti- β -catenin (Santa Cruz); anti-Myogenin and anti-Ki67 (DAKO); anti-Lbx1 (45), anti-Vasp (46), and anti-Arp2/3 (47). Secondary antibodies conjugated with Cy2, Cy3, or Cy5 were from Jackson ImmunoResearch Laboratories. SYBR (Molecular Probes) was used as a nuclear stain, and filamentous actin (F-actin) was visualized using a phalloidin-rhodamine conjugate (Molecular Probes).

To quantify the numbers of nuclei in myotubes, forelimbs of E14.5 or E18.5 embryos were dissected and digested for 40 min with collagenase I (Sigma). Cell suspensions were plated on glass coverslips coated with 10% Matrigel (BD Biosciences) and cultured in growth medium (10% FBS, Sigma; DMEM, Invitrogen) for 20 h to allow cell attachment and spreading. Myogenic cells and nuclei were visualized using antibodies against desmin, MyoD, and SYBR.

In Vitro Fusion Assays and Analysis of Contact Sites of Fusing Myoblasts.

Forelimb tissue of E12.5 embryos was digested as described above, followed by filtering through 25- μ m cell strainers (Partec) to obtain single-cell suspensions. Isolated cells were plated overnight in growth medium. Myogenic differentiation was promoted by culturing in differentiation medium containing 5% horse serum (Sigma) in DMEM for 72 h for in vitro fusion assays and for 36 h for the analysis of contact sites of fusing myoblasts. The fusion index was calculated as the number of nuclei in myotubes divided by the total number of myogenic nuclei. To quantify the recruitment of various proteins to the contact sites of myoblasts, contacting myoblasts were identified by membrane-localized β -catenin. For a quantification, the signal intensity at the contact site obtained by the staining with a specific antibody was measured using Fovea Pro 4 software and normalized to the signal intensity observed in the cytoplasm.

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