N-cadherin Activation Substitutes for the Cell Contact Control in Cell Cycle Arrest and Myogenic Differentiation

INVolvement OF p120 AND β-catenin*

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N-cadherin is expressed throughout skeletal myogenesis and has been proposed to be involved in the differentiation program of myogenic precursors. Here, we further characterize the N-cadherin involvement and its mechanism of action at the onset of differentiation, through controlled N-cadherin activation by plating isolated C2 myoblasts on surfaces coated with a chimeric Ncad-Fc homophilic ligand (N-cadherin ectodomain fused to the immunoglobulin G Fc fragment). We show that N-cadherin activation substitutes for the cell density in myogenic differentiation by promoting myogenin and troponin T expression. In addition, N-cadherin adhesion participates to the associated cell cycle arrest through the nuclear accumulation of cyclin-dependent kinase inhibitors p21 and p27. Mouse primary myoblast cultures exhibited similar responses to N-cadherin as C2 cells. RNA interference knockdowns of the N-cadherin-associated cytoplasmic proteins p120 and β-catenin produced opposite effects on the differentiation pathway. p120 silencing resulted in a decreased myogenic differentiation, associated with a reduction in cadherin-catenin content, which may explain its action on myogenic differentiation. β-Catenin silencing led to a stimulatory effect on myogenin expression, without any effect on cell cycle. Our results demonstrate that N-cadherin adhesion may account for cell-cell contact-dependent cell cycle arrest and differentiation of myogenic cells, involving regulation through p120 and β-catenins.

Skeletal myogenesis is a multi-step process, regulated by spatiotemporal cues in the embryo. The determination of the myogenic lineage, the differentiation of precursors in myoblasts, and their terminal differentiation leading to the formation of innervated muscle fibers are regulated by environmental signals provided by diffusible factors, extracellular matrix, and intercellular contacts (1, 2). Some of these developmental events require a high degree of coordination between changes in myoblast adhesion and the progression toward myogenesis, particularly during migration, alignment, and fusion of myoblasts associated with their exit from the cell cycle and the induction of histo-differentiation genes (3). The adhesive properties of myogenic cells are modulated by the orchestrated expression of a combination of cell adhesion molecules, such as cadherins. At least four classical cadherins (R-, N-, M-, and 11) are expressed in embryonic muscles during mouse or chicken development (4, 5). Some cell adhesion molecules of the immunoglobulin superfamily have also been proposed to play a role in myogenic differentiation (6–9). However, the precise function of these cell adhesion molecules in the control of muscle differentiation remains elusive.

Cadherins are major constituents of intercellular junctions mediating cell adhesion through Ca2+-dependent homophilic interaction of their extracellular domain and anchoring of their cytoplasmic domain to the actin cytoskeleton by catenins (10, 11). They contribute to cell aggregation, segregation, and migration associated with dynamic cell-cell contact remodeling (10, 12, 13). A role for cadherins in myogenesis was first evidenced by functional perturbation of N-cadherin during Xenopus development (14). The inhibition of N-cadherin-mediated contacts by specific antibodies perturbs myoblast fusion in vitro (7) and impairs the induction of muscle-specific genes (5, 15). Conversely, forced expression of N-cadherin in myogenic behavior fibroblastic cells promotes the expression of skeletal muscle proteins in three-dimensional cultures (16, 17). When beads coated with the extracellular domain of N-cadherin were applied on subconfluent cultures of C2 myoblasts, myogenic differentiation was enhanced (18). However, myoblasts from mice bearing a null mutation of the N-cadherin gene differentiate normally in vitro and in vivo, likely because of a functional redundancy of cadherins expressed in skeletal muscle (19). This hypothesis is strengthened by the fact that cadherins R and M are expressed during myogenesis in vivo and have been shown to participate in skeletal muscle differentiation in vitro (20–25). These results mainly rely on functional perturbation or overexpression of cadherins. However, no study has investigated whether the activation of cadherins (in the manner of growth factor receptors) is able to trigger myogenic cell differentiation autonomously.

Recently, Ncad-Fc recombinant chimera (extracellular domain of cadherin fused to Fc fragment of immunoglobulin) were developed as tools to control cadherin homophilic binding and to study cellular responses triggered by cadherin activation, which resulted in significant progress in the determination of cadherin-initiated signaling pathways (26–30). We used this approach to investigate whether isolated N-cadherin activation
could control myogenic differentiation. Our work demonstrates that N-cadherin engagement, independently of other cell adhesion molecules, is sufficient to induce cell cycle arrest and myogenic differentiation of C2 cells and primary myoblasts. The p120 catenin is required for myogenic differentiation, whereas β-catenin inhibits myogenic differentiation, independent of cell cycle regulation.

EXPERIMENTAL PROCEDURES

Cell Culture and Induction of Myogenic Differentiation—The mouse myogenic C2 cell line (31) was maintained undifferentiated at low density in Dulbecco’s modified Eagle’s medium (DMEM)

1 supplemented with 10% fetal calf serum (growth medium (GM)) under 7.5% CO2 at 37°C for cell maintenance, or at 100% confluence (Fig. 1A, B). The cells were plated at high density (105 cells/cm2) in DMEM plus 2% horse serum (differentiation medium (DM)). For primary myoblast cell cultures, newborn mice (OF1 strain) were sacrificed according to guidelines of the local animal experimental ethics committee. The limbs were rinsed in PBS before dissecting muscle from the bones and cartilage. Tissues were incubated in PBS, 0.125% trypsin without EDTA for 30 min at 37°C and dissociated by several flushes. The cell pellet was resuspended in DMEM supplemented with 10% fetal calf serum and 10% horse serum and preplated for 6 h on uncoated tissue culture dishes. The floating cell population enriched in myoblasts was then allowed to attach on gelatin-coated dishes at 104 cells/cm2 (32). After 2 days (three or four divisions) allowing the formation of small colonies, the culture medium was switched to DMEM plus 10% horse serum for differentiation.

Antibodies and Reagents—The following antibodies were used for immunostaining: rabbit anti-myogenin (1:100, M225 clone; Santa Cruz, Santa Cruz, Tebu, Le Perray-en-Yvelines, France), mouse anti-Troponin T (1:50, clone JLT-12; Sigma), rabbit anti-p21 (1:200, C-19; Santa Cruz), and rabbit anti-p27 (1:100, M-197; Santa Cruz). The following antibodies were used for Western blotting: mouse anti-pan-cadherin (1:2000, clone CH19; Sigma), mouse anti-p120 (1:2000, clone 98; Transduction Laboratories, Becton Dickinson Europe, Le Pont de Claix, France), rabbit anti-β-catenin (1:5000; Sigma), mouse anti-γ-catenin/plakoglobin (1:2000, clone 15; Transduction Laboratories), mouse anti-p27 (1:1000; clone 57, Transduction Laboratories), mouse anti-troponin T (1:1000, clone JLT-12; Sigma), and mouse anti-myogenin (1:2000, clone F5D; Santa Cruz).

DNA and siRNA Transfections—C2 cells grown at 5 × 104 cells/cm2 (mid-confluence) in GM without antibiotics were transfected with the LipofectAMINE 2000 reagent according to the manufacturer's instructions (Invitrogen). 5 μl of LipofectAMINE 2000 were mixed with 2 μg of DNA expression vector or 100 μl of DNA expression vector or 100 μl of siRNA duplexes in 100 μl of Opti-MEM medium for 30 min. The cells were incubated with this mixture for 24 h in GM without antibiotics, and differentiation was induced by medium switch when cells reached confluence. The cells were either fixed for immunostaining or submitted to protein extraction for Western blotting, after 2 days in culture corresponding to the beginning of post-lipofection. The following DNA vectors were used: (control DNA) pSUPER (33) for mock transfections and pSUPER-p120 (32) for p120 silencing containing the insertion in both sense and antisense orientation of the specific p120 sequence: GATGGTTATCCAGGTGGCA, corresponding to the 653–673 amino acids of the mouse sequence (NM076165). The RNA duplex sequence used for β-catenin silencing corresponds to the 492–498 amino acids of the mouse sequence (NM076164) and was noted β-cat siRNA: rCrUrGrUrUrGrUrGrUr-GrArArGrCGrUrCrCuTT (Prologis, Paris, France). As a control RNA, we used inefficient RNA duplex corresponding to the 79–85 amino acids sequence (control dsRNA): rCrUrGrUrGrUrArUrArGrCrGrAr-GrArGr-CGrCrTT.

Preparation of Adhesion Substrates and N-cadherin Activation—Fibronectin (Fn, 1 μg/cm2; PAA Laboratories, GmbH Linz, Austria) and N-cadherin homophilic ligand, N-cadFc (NcadFc, 5 μg/cm2) (26) were coated on silanized glass coverslips or thermosterilized bacterial dishes (Falcon, Becton Dickinson Europe) as reported previously (30). Alternatively, soluble chimera was directly diluted in DM at the final concentration of 50 μg/ml in the presence of 3% bovine serum albumin. The

1 The abbreviations used are: DMEM, Dulbecco’s modified Eagle’s medium; siRNA, small interfering RNA; dsRNA, double strand RNA; DNA, deoxyribonucleic acid; GM, growth medium; GM, growth medium; ld, low density; hd, high density of cells; md, medium density of cell; Fm, fibronectin; PBS, phosphate-buffered saline; TRITC, tetramethylrhodamine isothiocyanate; BrdU, bromodeoxyuridine; β-cat, β-catenin.
tiation was assessed through the expression of myogenin and the histodifferentiation marker troponin T. Myogenin and troponin T immunolabeling were each present in around 30% of cells after 3 days of culture on Ncad-Fc in DM, similar to hd:DM conditions (Fig. 1, C and D). In contrast, the relative numbers of myogenin-positive nuclei or troponin T-positive cells were significantly lower on fibronectin (Fig. 1, C and D). The induction of troponin T expression either by hd or by Ncad-Fc was confirmed by Western blot analysis (Fig. 1 E). The time course of appearance of myogenin-positive nuclei in C2 cells cultured at low density on Ncad-Fc was indistinguishable from that observed in hd:DM conditions (Fig. 1F), demonstrating that N-cadherin activation promotes myogenin expression from day 1, as efficiently as hd. Because myogenin-positive nuclei were undetectable in GM on Ncad-Fc (data not shown), myogenin expression requires serum withdrawal in addition to N-cadherin activation. Thus, these results indicate that N-cadherin activation is sufficient to mimic the effect of cell-cell contact on the myogenic differentiation of C2 cells.

To characterize the involvement of N-cadherin in this process, we performed a dose-response analysis of N-cadherin-triggered myogenin expression. The myogenin-positive nuclei at day 3 was low for Ncad-Fc concentration inferior to 2 µg/cm² and increased with Ncad-Fc concentration to reach a plateau at 5 µg/cm² (Fig. 1G). Soluble Ncad-Fc was unable to induce myogenin expression at ld:DM with concentrations as high as 50 µg/ml (data not shown). Our data suggest that the N-cadherin adhesion receptor induces myogenin expression through a dose-dependent, solid phase homophilic binding to the N-cadherin extracellular domain (i.e. N-cadherin activation). Therefore, N-cadherin is sufficient to substitute for the cell-cell contact in the myogenic differentiation.

**N-cadherin Activation Triggers Cell Cycle Arrest Prior to Differentiation**—Previous reports show that N-cadherin overexpression leads to the up-regulation of p27 in Chinese hamster ovary fibroblastic cells (36), controlling cell cycle arrest. We wondered whether N-cadherin activation could be involved autonomously in the cell cycle arrest during differentiation.
program of myoblasts. The nuclear accumulation of the cyclin-dependent kinase (cdk) inhibitors p21 and p27 was analyzed by immunostaining for p21 and p27 after 3 days in DM. Isolated C2 cells on Ncad-Fc exhibited 57 and 76% of p21- and p27-positive nuclei, respectively (Fig. 2A and B), similar proportions of cdk inhibitor-positive nuclei (75 and 82% for p21 and p27, respectively) were observed for 3 days in DM (Fig. 2B). In contrast, C2 cells cultured on fibronectin exhibited only 30% of cdk inhibitor-positive nuclei (Fig. 2A and B). In addition to this nuclear accumulation of cdk inhibitors, Western blot analysis revealed higher levels of p27 in DM and in Ncad-Fc compared with fibronectin (Fig. 2C). Thus, the activation of N-cadherin induces p21 and p27 nuclear accumulation during myogenic differentiation, supporting the notion that N-cadherin activation mimics the cell-cell contact action on cell cycle arrest.

We also performed different approaches to directly evaluate the action of isolated N-cadherin activation on cell cycle progression. The proliferation of C2 cells on Ncad-Fc or fibronectin was estimated by counting viable cells cultured in ld:GM or ld:DM conditions (Fig. 2D). The number of cells was increased by 10-fold on fibronectin and only by 4-fold on Ncad-Fc on the third day in GM. C2 cells also proliferated on fibronectin in low serum medium (6-fold increase), whereas the combination of Ncad-Fc and low serum led to a complete abrogation of cell multiplication. The involvement of N-cadherin in the adhesion-dependent cell cycle arrest was further evaluated through BrdU incorporation and p21 coincubation after 24 h. Although 42% (DM) and 60% (GM) of cells on fibronectin displayed BrdU incorporation together with negative staining for p21 (BrdU-/p21+), only 11% (DM) and 23% (GM) on Ncad-Fc (Fig. 2E). Similar proportions of BrdU+/p21+ nuclei were observed at ld:DM (Fig. 2E). Conversely, only 26% of cells cultivated on fibronectin were negative for BrdU and positive for p21 (BrdU-/p21+), whereas 55% of cells on Ncad-Fc were BrdU+/p21+ (compared with 63% for ld:DM; Fig. 2E). Thus, our experiments demonstrate that N-cadherin activation is sufficient to diminish the proportion of cells that passes through the S phase, again suggesting that N-cadherin negatively regulates cell cycle progression during myogenic differentiation.

The link between cell cycle arrest and myogenic differentiation was then investigated through BrdU incorporation in combination with myogenin or p21 immunostaining at ld:DM on Ncad-Fc (Fig. 2F). After 1 day, 18% of cells were engaged in differentiation (BrdU+/myogenin−) whereas 50% were stopped in the cell cycle (BrdU+/p21−). By 3 days, more than 76% of the cells belonged to the BrdU+/p21+ population, but they were only 31% with BrdU-/myogenin+ staining. Moreover, less than 5% of myogenin-positive cells had incorporated BrdU (BrdU+/ myogenin+), suggesting that the induction of myogenin in cycling cells is a rare event. The uncycled and differentiated population (BrdU+/myogenin+) belongs at least partially to the population of cells stopped in cell cycle (BrdU+/p21+). Altogether, our results show that N-cadherin receptor activation regulates cell cycle arrest associated with myogenic differentiation.

**N-cadherin Activation Induces Differentiation of Mouse Primary Myoblasts**—To expand the results obtained in the C2 cell line to a more physiologically relevant system, the involvement of N-cadherin activation was examined in primary myoblasts from newborn mice. Proliferating myoblasts were allowed to adhere on gelatin-coated dishes and to divide three or four times.
During myogenic differentiation, because p120 and \( \beta \)-catenin arrest and differentiation of mouse primary myoblasts.

Myoblasts were isolated from limb muscles of newborn mice and grown in proliferating conditions for 2 days on gelatin. The cells were switched to DM for 0.5 or 1 day, and the expression of myogenin was evaluated by Western blot. B, proliferating 2-day-old myoblasts were plated at low density on Ncad-Fc or Fn and cultured for 1 additional day in the presence of DM. They were fixed and immunostained for myogenin or p21 (red), and the nuclei were counterstained with Hoechst (blue). Bars, 10 \( \mu \)m. C, quantitative analysis performed on two independent experiments of myogenin and p21-positive nuclei in Id on Ncad-Fc or Fn, in comparison with small colonies of 3-day-old myoblasts on gelatin (Gel).

Western blot analysis revealed that transfected cells exhibited a significant decrease in p120 protein after 3 days of culture either in proliferation (md:GM) or differentiation (hd:DM) conditions (Fig. 4A). Interestingly, p120 silencing led to a global decrease in the expression of cadherin and \( \beta \)-catenin, as previously reported (30, 38). For \( \beta \)-catenin, we designed a synthetic siRNA duplex in its coding region. Western blot analysis revealed that efficient \( \beta \)-catenin silencing was achieved 3 days post-transfection with concentrations of \( \beta \)-catenin siRNA duplex ranging from 100 to 500 nm in GM (Fig. 4B) and also in DM at the median concentration (200 nm) used for further experiments (Fig. 4C). In contrast to p120 silencing, \( \beta \)-catenin knockdown did not alter significantly total cadherin expression.

Interestingly, \( \beta \)-catenin siRNA led to an up-regulation of \( \gamma \)-catenin/plakoglobin (Fig. 4, B and C), which may functionally replace \( \beta \)-catenin at the plasma membrane to link N-cadherin to a-catelin and to the actin cytoskeleton (10).

\( p120 \) or \( \beta \)-Catemin Silencing Alters Myogenic Differentiation—The effect of p120 and \( \beta \)-catenin silencing on myogenic differentiation was evaluated by following myogenin expression in C2 cells in hd:DM. Immunostaining counts revealed a 40% decrease of myogenin-positive nuclei in p120 siRNA-expressing cells (Fig. 4D). Western blot analysis confirmed this decrease of myogenin expression (Fig. 4E). Thus, p120 siRNA associated with \( \beta \)-catenin and cadherin down-regulation counteracts myogenic differentiation. In contrast, \( \beta \)-catenin siRNA led to a 40% increase of myogenin-positive cells (Fig. 4D) and a global increase of myogenin expression (Fig. 4E). These results suggest that \( \beta \)-catenin silencing potentiates myogenic differentiation. This effect of \( \beta \)-catenin could be independent of its association with N-cadherin at the membrane, given the fact that \( \gamma \)-catenin level was augmented. At the opposite of \( \beta \)-catenin silencing, C2 cells were submitted to lithium treatment known to stabilize and increase cytosolic pools of \( \beta \)-catenin (39). As previously reported (40), this treatment actually led to a global increase of \( \beta \)-catenin (Fig. 4F) and counteracted myogenin expression, but only if added after 1 day in DM (Fig. 4F).

Thus, our results with silencing and stabilization of \( \beta \)-catenin converge to the hypothesis that \( \beta \)-catenin exerts a negative action on myogenic differentiation. To determine whether the negative effect of \( \beta \)-catenin on myogenic differentiation affects myogenin induction per se or through an effect on cell cycle, BrdU incorporation was followed in combination with myogenin immunostaining in \( \beta \)-catenin siRNA-treated cells in hd:DM conditions. \( \beta \)-Catenin silencing led to a 3-fold increase in the number of cells accumulating myogenin in their nuclei (Fig. 4, G and H). A small but significant percentage of nuclei accumulated myogenin in GM (6%), which has never been observed in any other condition with GM. However, \( \beta \)-catenin silencing did not significantly interfere with BrdU incorporation (Fig. 4H) or p27 level of expression (data not shown), indicating no major effect on cell cycle regulation. Our results suggest together that \( \beta \)-catenin regulates negatively the induction of myogenic differentiation during the step of myogenin expression, by a process independent of cell cycle exit.

DISCUSSION

The differentiation of skeletal myogenic cells, classically induced by serum deprivation in vitro, has been reported to depend on cell density. We demonstrate here that N-cadherin-dependent homophilic adhesion recapitulates the cell contact-dependent effect on the myogenic differentiation process: (i) N-cadherin engagement controls both cell cycle arrest and myogenin induction in C2 cells or in primary myoblasts, (ii) the catenin p120 positively affects myogenic induction likely through its stabilizing properties on the cadherin-catenin complex.
plex, and (iii) β-catenin contributes to a negative pathway during myogenic differentiation.

Previous reports based either on functional perturbation or overexpression of N-cadherin suggested a modulator role for N-cadherin in myogenic differentiation (5, 7, 15–17). The involvement of N-cadherin in myogenic differentiation was also investigated through application of N-cadherin-coated beads upon C2 cells in culture, which in this case established actual cell-cell and cell-matrix contacts (18). Our results show that cell density-dependent myogenic differentiation of C2 cells is mimicked by N-cadherin engagement, without contribution of any other adhesion molecules: N-cadherin activation is indeed sufficient to promote myogenin and troponin T expression in isolated myoblasts. A similar positive effect on myogenin expression was observed in response to N-cadherin engagement in mouse primary myoblasts, extending our data on C2 cell line. However, because N-cadherin−/− myoblasts differentiate normally in vitro (19), M- or R-cadherins, which are also expressed by myogenic precursors (4, 5), could equally regulate the cell contact-dependent differentiation. In conclusion, cadherins may act in vivo as adhesion and signaling sensors between neighboring cells for the induction of myogenic differentiation. In addition, these cadherins of different specificity may differentially control the site of differentiation, specifying myogenic cell subpopulations, as suggested for motoneurons (41).

Cadherins E, N, or VE exert a negative regulation on cell proliferation in various cell types (36, 42–44) and during tumor cell growth (45, 46). In particular, the overexpression of N-cadherin leads to the up-regulation of p27 in Chinese hamster ovary cells, modulating cell cycle arrest (36). In myogenic cells, N-cadherin activation triggers also the accumulation of p21 and p27 and diminution of DNA synthesis and cell proliferation, suggesting that N-cadherin activation regulates cell cycle exit during the course of myogenic differentiation. A main function for N-cadherin during myogenesis in vivo may be to regulate the proliferation/differentiation switch of myogenic precursors, through mechanisms corresponding in vitro to the contact inhibition of cell growth. The induction of myogenin expression by N-cadherin acti-
Cadherin Activation Controls Cell Differentiation

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