FEBS 27446 FEBS Letters 547 (2003) 183–188

Overexpression of cytosolic sialidase Neu2 induces myoblast differentiation in C2C12 cells

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Received 7 May 2003; revised 5 June 2003; accepted 9 June 2003

First published online 26 June 2003

Edited by Veli-Pekka Lehto

Abstract **Cytosolic sialidase Neu2 has been implicated in myoblast differentiation. Here we observed a significant upregulation of Neu2 expression during differentiation of murine C2C12 myoblasts. This was evidenced both as an increase in Neu2 mRNA steady-state levels and in the cytosolic sialidase enzymatic activity. To understand the biological significance of Neu2 upregulation in myoblast differentiation, C2C12 cells were stably transfected with the rat cytosolic sialidase Neu2 cDNA. Neu2 overexpressing clones were characterized by a marked decrement of cell proliferation and by the capacity to undergo spontaneous myoblast differentiation also when maintained under standard growth conditions. This was evidenced by the formation of myogenin-positive myotubes and by a significant decrease in the nuclear levels of cyclin D1 protein. No differentiation was on the contrary observed in parental and mock-transfected cells under the same experimental conditions. The results indicate that Neu2 upregulation per se is sufficient to trigger myoblast differentiation in C2C12 cells.**

Key words: Sialidase; Myoblast; Differentiation; Myogenin; Ganglioside

1. Introduction

Cleavage of sialic acids from glycoconjugates is a crucial event, leading to modulation of cellular functions in several physiological and pathological processes [1,2]. Alterations in sialylation levels of glycoconjugates during malignant transformation are associated with the malignant phenotype both in terms of metastatic potential and invasiveness [3–5]. Sialidases cleave terminal $\alpha2\rightarrow3$ and $\alpha2\rightarrow6$ sialyl linkages of oligosaccharides and glycoproteins and are implicated in biological phenomena such as differentiation, proliferation, signal transduction, and cell surface interactions [6]. Three sialidase genes (*neu1–3*) have been cloned so far. They encode for different isoforms with distinct enzymatic properties and substrate specificity. Sialidases are characterized by an YRIP motif in their N-terminus that contains an arginine residue required for catalysis and several Ser-X-Asp-X-Gly-X-Thr-Trp repeated sequences (Asp-boxes) with unknown function [7]. Mammalian sialidases have been classified on the basis of their subcellular localization [8] as cytosolic, lysosomal (intralysosomal and membrane-bound), and plasma membrane-bound. Lysosomal sialidase Neul appears to play a major role in glycoprotein catabolism by collaborating with lysosomal proteases and endoglycosidases [9–11]. Plasma membrane sialidase Neu3 specifically hydrolyzes gangliosides and might play a role in cell surface events and neuronal differentiation [12–14]. The cytosolic sialidase Neu2 is highly expressed in skeletal muscle [15,16] and at different levels in liver [17] and brain [18]. Also, a murine cytosolic sialidase highly homologous to the muscle protein has been cloned from thymus [19].

Cytosolic sialidase is also capable of desialylating glycoproteins and gangliosides at neutral pH, in agreement with the presence in the cytosol of ganglioside–protein complexes [20,21]. In the past years, *Neu2* has been implicated in the myotube formation process [22].

During skeletal muscle development multipotent mesodermal precursors commit to a muscle cell fate. This is followed by myoblast proliferation, withdrawal from the cell cycle, activation of muscle-specific genes, and fusion to form multinucleated myotubes [23,24]. In this work we investigated the biological role of cytosolic sialidase Neu2 during in vitro myoblast differentiation in the murine myogenic C2C12 cell line [25], a favored model to study muscle differentiation. To this purpose, Neu2 expression was studied both at transcription and enzymatic levels during differentiation. Also, C2C12 cells were stably transfected with the rat Neu2 cDNA to determine the effect of its overexpression on myogenesis.

2. Materials and methods

2.1. Plasmid construction

The –1415/-5 fragment of the Neu2 rat promoter prior to the ATG initiation codon [26] was obtained by polymerase chain reaction (PCR) amplification of rat genomic DNA using specific primers with the KpnI adaptor in 5′ (sense, 5′-GGGGTACCCC/TTCCTGCCTCAGCTTCTCACATGC-3′; antisense, 5′-GGGGTACCCC/TGAGATCTGGGCAGAAAGAGAAGAC-3′) under the following conditions: 95°C for 2 min, 60°C for 1 min, 72°C for 2 min followed by nine cycles of 94°C for 30 s, 60°C for 30 s, 72°C for 2 min, followed by 26 cycles of 94°C for 30 s, 66°C for 30 s, 72°C for 2 min. Next, the Neu2 rat promoter was cloned into the KpnI site of the luciferase reporter expression vector pGL2-Basic (Promega), thus generating the pGL2-Basic-Neu2 rat promoter vector.

The rat Neu2 sialidase cDNA coding sequence was amplified from a PC12 cell cDNA library using specific primers (sense, 5′-CGGAATTCCG/ATGGAGACCTGCCCCGTCCTCCAGA-3′; antisense, 5′-CGGAATTCCG/TCACTGAGCACCATGTACTGTGGGA-3′)

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Abbreviations: PCR, polymerase chain reaction; 4-MUB-NANA, 4-methylumbelliferyl *N*-acetylneuraminic acid; bHLH factors, basic helix loop helix factors

0014-5793/03/$22.00
doi:10.1016/S0014-5793(03)00709-9

under the following conditions: 95°C for 2 min, 59°C for 1 min, 72°C for 3.5 min followed by nine cycles of 94°C for 30 s, 59°C for 30 s, 72°C for 3.5 min, followed by 34 cycles of 94°C for 30 s, 66°C for 30 s, 72°C for 3.5 min. Then, the rat Neu2 cDNA was cloned into the *Eco*RI site of pCDNA vector, thus generating the pCDNA-Neu2 expression vector.

2.2. Cell line, cell staining and stable DNA transfection

The mouse C2C12 myoblasts were cultured at 37°C (in an atmosphere of 5% CO_2) in Dulbecco's modified Eagle's medium with high glucose (DMEM; Sigma-Aldrich) and supplemented with 10% (v/v) fetal bovine serum (Sigma-Aldrich) and 100 μg/ml penicillin/streptomycin (Sigma-Aldrich). This growth medium was designated GM. To induce differentiation, subconfluent cells were shifted to DMEM containing 2% horse serum (or 2% horse serum plus bovine insulin, 5 ng/ml, Sigma-Aldrich) and the medium was changed every day. This differentiating medium was designated DM or DM plus insulin. To visualize myotubular structures, cells were washed three times in phosphate-buffered saline (PBS) before fixing for 10 min in 100% methanol at −20°C. Cells were stained with Giemsa reactive (Sigma-Aldrich) for 2–3 h and again washed in PBS.

To obtain stable Neu2-overexpressing C2C12 myoblasts and the corresponding mock transfectants, cells (1×10^5/60 mm dish) were transfected with pCDNA-Neu2 or pCDNA expression vectors (1 μg/dish), respectively, in Fugene reagent (Roche Molecular Biochemicals) according to the manufacturer's instructions. Stable transfectants were cloned after 10–15 days selection in G418 antibiotic (0.5 mg/ml; Promega).

2.3. Transient transfection assays

Transfections were performed by Fugene reagent. Myoblasts cells (1×10^5/60 mm dish) were transfected with a mix of 1 μg of pGL2-Basic-Neu2 promoter expression vector and 10 ng of the transfection control vector pRL-TK-Renilla luciferase (Promega). Following transfection, the medium was replaced with DM or GM. Luciferase activity was measured in the total protein lysates using the Promega Dual luciferase assay system. Data were corrected for transfection efficiency by measuring the Renilla luciferase activity according to manufacturer's instructions and normalized to 100 μg of proteins.

2.4. Sialidase assay

Cells were washed with PBS and sonicated at 4°C in nine volumes of 0.25 M sucrose containing 1 mM EDTA and a mix of protease inhibitors (Complete Mini Protease Inhibitors, Roche Molecular Biochemicals) for 10 s at an intermediate setting. The mixture was centrifuged at $600\times g$ for 10 min and the supernatant ultracentrifuged at $105\,000\times g$ for 60 min at 4°C. The supernatant was used as the cytosolic fraction and assayed for sialidase activity. The assay mixture contained 60 nmol of the substrate 4-methylumbelliferyl *N*-acetylneuraminic acid (4-MUB-NANA; Sigma-Aldrich), 100 μg of bovine serum albumin (BSA) and aliquots of enzyme fractions (50–100 μg of proteins) in a final volume of 0.2 ml of 50 mM sodium acetate buffer (pH 5.5). After incubation at 37°C for 2–3 h, the reaction was terminated by addition of 0.8 ml of 0.25 M glycine buffer (pH 10.4), and the amount of 4-methylumbelliferone released was determined fluorometrically with an excitation wavelength of 365 nm and emission of 450 nm.

2.5. RNA extraction and reverse transcription (RT)-PCR analysis

Total RNA was obtained as described by Chomczynski and Sacchi [27]. The pellet of RNA was resuspended in RNAase-free water, digested with 1 unit of DNAase (DNA-free; Ambion) for 1 h at 37°C according to the manufacturer's instructions. 2 μg of total RNA was retrotranscribed with 400 units of Moloney murine leukemia virus reverse transcriptase (Promega) for 1 h at 37°C, and the RT template was used for PCR amplification. For RT-PCR analysis of murine cytosolic Neu2 sialidase expression, primers 5′-CGAGCCAGCAAGACGGATGAG-3′ (sense) and 5′-GGCTCTACAAGCTTACTCACTACCCGG-3′ (antisense) were used under the following conditions: 95°C for 2 min, 65°C for 1 min, 72°C for 1 min followed by 32 cycles of 94°C for 30 s, 65°C for 30 s, 72°C for 1 min. For the screening of Neu2 transfectants, PCR analysis was performed using the same primers utilized to clone the rat Neu2 cDNA into its expression vector in order to avoid amplification of the endogenous murine Neu2 mRNA under the following conditions: 95°C for 2 min, 65°C for 1 min, 72°C for 1.5 min followed by 29 cycles of 94°C for 30 s, 65°C for 30 s, 72°C for 1.5 min. For RT-PCR analysis of glyceraldehyde 3-phosphate dehydrogenase expression, the primers 5′-CGTGGAGTCTCCTGGTGTCTTC-3′ (sense) and 5′-GTGAGTTGTCATATTTCTTGTGGTT-3′ (antisense) were used under the following conditions: 95°C for 2 min, 60°C for 1 min, 72°C for

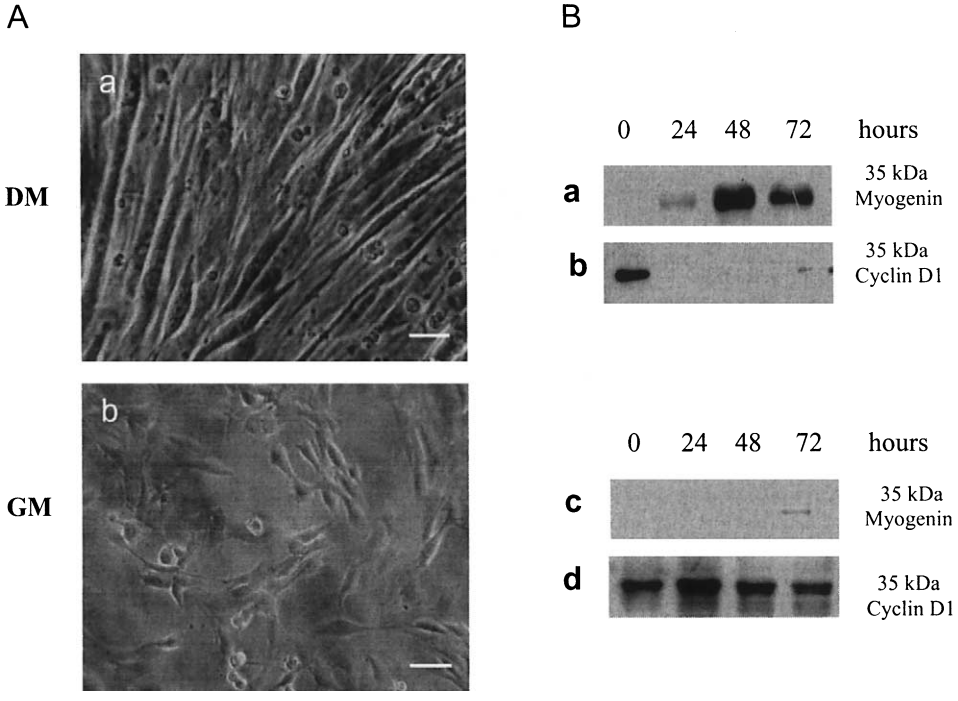

Fig. 1. C2C12 myoblast differentiation. A: Morphology of C2C12 cells differentiated in DM for 4 days (panel a) or maintained in GM (panel b). The cells were photographed under a phase contrast microscope. Note the numerous myotubes in differentiated C2C12 cells (panel a). Bars, 60 μm. B: Western blot analysis of nuclear extracts of C2C12 cells maintained in DM (panels a and b) or in GM (panels c and d) for 0–72 h. Nuclear proteins were probed with monoclonal anti-myogenin (panels a and c) and anti-cyclin Dl (panels b and d) antibodies.

1 min followed by 18 cycles of 94°C for 30 s, 60°C for 30 s, 72°C for 1 min.

2.6. Nuclear extracts and Western blot

For the nuclear extracts, cells were rinsed and harvested with ice-cold PBS. Cells were centrifuged at 4°C (800×*g* for 10 min), lysed in lysis buffer (500 μl of ice-cold 10 mM HEPES, pH 7.9, containing 10 mM KCl, 1.5 mM $MgCl_2$, 0.5 mM dithiothreitol (Sigma-Aldrich), added to protease inhibitor mix, and centrifuged at 800×*g* at 4°C for 5 min. The pellet was resuspended in 500 μl of lysis buffer with 0.5% NP-40 (Sigma-Aldrich) added, homogenized with a Dounce homogenizer, and centrifuged at 2500×*g* for 5 min at 4°C. The nuclear pellet was resuspended in 30 μl of ice-cold 20 mM HEPES, pH 7.9, containing 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 25% glycerol, 0.5 mM dithiothreitol and protease inhibitor mix, incubated on ice for 20 min, and centrifuged at 14 000×*g* for 15 min at 4°C. The supernatant containing the nuclear proteins was stored at −20°C. Protein concentration was assessed by Coomassie assay (Pierce Reagent) according to the manufacturer's instructions. Aliquots (30–50 μg) of nuclear proteins were analyzed by Western blotting using 1:500 dilution of monoclonal anti-myogenin antibody (mAb F5D, Santa Cruz Biotechnology) or of anti-cyclin Dl antibody (mAb HD11, Santa Cruz Biotechnology). Immunocomplexes were visualized using enhanced chemiluminescence reagent (Pierce) according to the manufacterer's instructions.

2.7. Immunofluorescence

C2C12 cells were plated on 12 mm glass coverslips coated with 100 ng/ml poly-L-lysine (Sigma-Aldrich), cultured until 90% confluency and then maintained in GM or shifted to DM for the following days. Cells were fixed with ice-cold methanol for 10 min at −20°C, washed with PBS and permeabilized with 0.2% Igepal (Sigma-Aldrich) in PBS containing 5% goat serum (GS) and 2% BSA for 30 min at room temperature. Cells were then incubated for 2 h in humid atmosphere with monoclonal anti-myogenin antibody (F5D clone, Santa Cruz Biotechnology) diluted 1:500 in 1% GS/PBS, washed and mounted with mowiol (Sigma-Aldrich). Fluorescent staining of cells was observed under an Axiovert S100 microscope (Zeiss). Pictures were taken with a digital camera (SensiCam) and Image-Pro Plus software version 4.5.

3. Results

3.1. Sialidase Neu2 expression during C2C12 myoblast differentiation

The murine myogenic C2C12 cell line differentiates to form typical myotube structures (Fig. 1A, panel a) when cultured at confluence in the presence of a differentiating medium (DM). In contrast, C2C12 cells retain an undifferentiated, proliferating phenotype when maintained in a growth medium (GM) (Fig. 1A, panel b). Accordingly, an increase in the nuclear levels of the transcription factor myogenin, a member of basic helix loop helix (bHLH) nuclear proteins known to play a major role during muscle cell fusion [28–30], was observed in cells grown in DM (Fig. 1B, panel a). This was paralleled by a dramatic decrease in the levels of cyclin Dl protein (Fig. 1B, panel b), a protein involved in cell cycle progression [31,32]. By contrast, no increase in the nuclear levels of myogenin was observed in C2C12 cells maintained in GM (Fig. 1B, panel c) that also retained high levels of expression of cyclin Dl protein (Fig. 1B, panel d). On this basis, Neu2 mRNA expression was investigated by semi-quantitative RT-PCR analysis during myotube formation. As shown in Fig. 2A, a rapid, long-lasting increase of Neu2 transcript steady-state levels was observed in C2C12 cells grown in DM. Accordingly, a prolonged significant increase of sialidase activity was detectable in the cytosolic fraction of differentiating C2C12 cells (Fig. 2B).

To assess whether the induction of Neu2 expression occurred, at least in part, at the transcriptional level, C2C12 cells were transiently transfected with the luciferase reporter gene under the control of the rat Neu2 promoter [26]. After 24 h, cells were given GM, DM, or DM plus insulin (a hormone growth factor that enhances myoblast differentiation

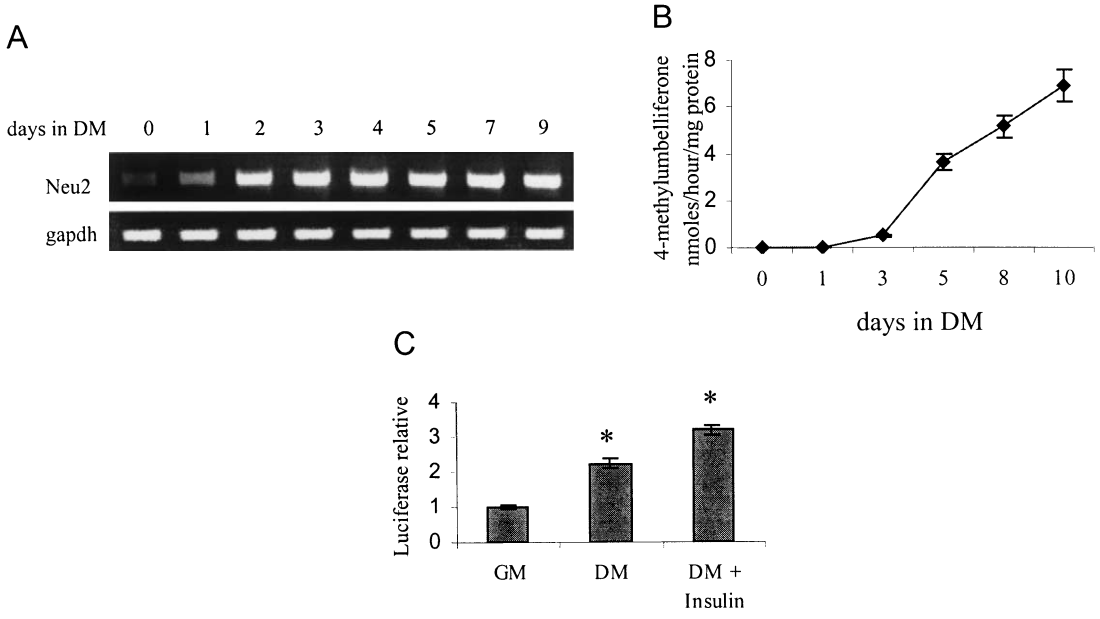

Fig. 2. Neu2 expression in differentiating C2C12 cells. A: C2C12 cells were cultured in DM for the indicated periods of time and Neu2 transcript expression was investigated by RT-PCR analysis using gapdh transcript as a control. B: Time course of enzymatic Neu2 sialidase activity in C2C12 cells maintained in DM. Cytosoluble Neu2 activity was determined using 4-MUB-NANA as substrate. The values are the average of three independent experiments. C: Transcriptional induction of Neu2 promoter in differentiating C2C12 cells. Cells were transiently transfected with the pGL2-Basic-Neu2 promoter expression vector harboring the luciferase reporter gene under the control of the rat Neu2 promoter. The day after, cells were harvested and seeded in 6 cm plates ($1.5\times1O^5$ cells/dish) in GM, DM, or DM plus insulin. After 24 h, luciferase activity in the cell extracts was measured and normalized for protein content. Statistical analysis was performed by Student's *t*-test ($n=3$ for each group). $^*P<0.05$.

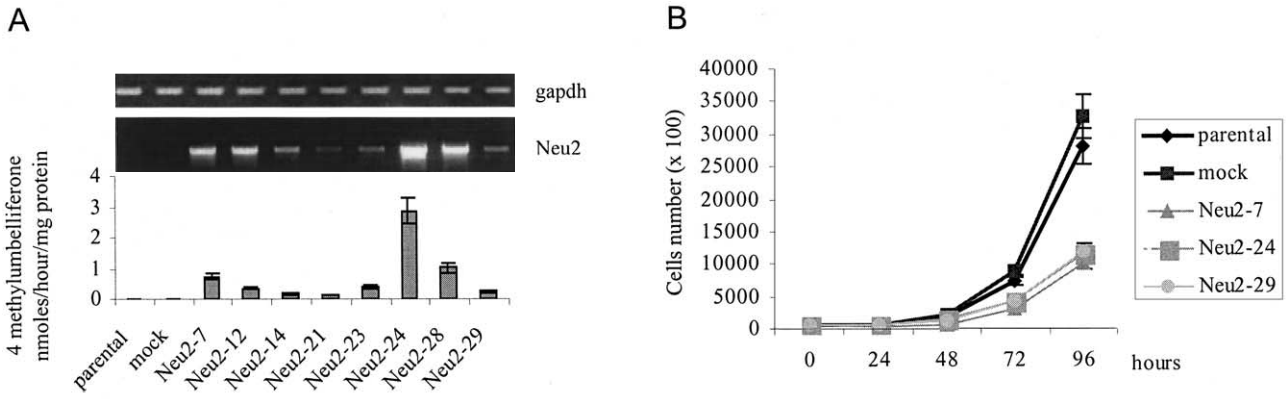

Fig. 3. Neu2 overexpression in C2C12 cells. A: Cells were stably transfected with the pCDNA-Neu2 expression vector harboring the rat Neu2 cDNA. After selection, transfectants were compared to parental and mock-transfected cells for rat Neu2 expression by RT-PCR analysis, using gapdh as a control. In parallel, cells were also tested for cytosolic Neu2 sialidase activity. B: Kinetics of proliferation of Neu2 transfectants. Parental, mock, and Neu2-7, Neu2-24, and Neu2-29 cell transfectants were seeded at 50 000 cells/60 mm-dish in GM. Cells were counted after 24–96 h. The results are the mean of three independent experiments.

[33]). The results demonstrate a ~2.2-fold induction of luciferase activity in cells grown in DM when compared to undifferentiated cells grown in GM. Such an increase was even higher (~three-fold) in cells grown in DM plus insulin (Fig. 2C).

3.2. *Neu2 overexpression induces myoblast differentiation in C2C12 cells*

To assess the biological significance of Neu2 upregulation in myoblast differentiation, C2C12 cells were stably transfected with an expression vector harboring the rat sialidase Neu2 cDNA. After selection, several resistant clones were obtained and investigated for rat Neu2 expression by semi-quantitative RT-PCR analysis, using specific primers for the transgene. Also, the clones were tested for sialidase activity in the cytosoluble fraction of cellular lysates. As shown in Fig. 3A, all the clones tested express rat Neu2 mRNA, even though to a different extent, whereas no expression was observed in parental and mock-transfected cells. The sialidase activity in the cytosolic fraction strictly correlated with the different expression levels of the corresponding rat Neu2 transcript (Fig. 3A).

All the Neu2 transfectants were characterized by a significant decrease in the growth rate when compared to parental and mock cells (Fig. 3B). Moreover, all the transfectants showed the capacity to form myotubes also when maintained in GM (Fig. 4A, panel a). This capacity was absent in mock-transfected cells (Fig. 4A, panel c). Thus, Neu2 overexpression appears to be able to induce myogenesis in C2C12 cells also under non-differentiating cell culture conditions. Accordingly, Neu2 transfectants grown in GM were characterized by high levels of myogenin protein and by the sustained downregulation of cyclin Dl protein levels when compared to mock cells, as evidenced by Western blot analysis of the nuclear extracts (Fig. 4B). The immunostaining for myogenin in Neu2 transfectant (Fig. 4A, panel b) and in mock cells (Fig. 4A, panel d) confirmed Western blot analysis.

When grown in DM, the Neu2 transfectants formed multigiant hypertrophic myotubes with an elongated morphology, significantly larger in size with respect to those observed when the cells were grown in GM or in parental C2C12 cells maintained in DM (Fig. 4C). Again, this was paralleled by a significant myogenin upregulation and cyclin Dl downregulation of the corresponding protein levels in the nuclear extracts (data not shown).

Finally, Neu2-overexpressing clones grown in GM were characterized by an increased luciferase activity driven by the rat Neu2 promoter when compared to parental or mock cells (Fig. 4D). This indicates that, as observed in differentiating parental C2C12 cells given DM (see Fig. 2C), also myoblast differentiation induced by exogenous Neu2 overexpression is paralleled by an increase in basal luciferase gene transactivation.

4. Discussion

In this study we investigated the role of cytosolic sialidase Neu2 during in vitro myoblast differentiation using murine C2C12 cells as a model. Differentiating conditions induce the withdrawal of C2C12 cells from the cell cycle, as shown by cyclin Dl downregulation, with the activation of the skeletal muscle differentiation program characterized by myogenin upregulation and multinucleated myotube formation.

This process was paralleled by the increase of expression of sialidase Neu2, as observed both at mRNA and enzymatic activity levels. The transcriptional induction of Neu2 was confirmed by transient transfection of differentiating C2C12 cells with an expression vector harboring the luciferase reporter gene under the control of the rat Neu2 [26]. Interestingly, analysis of the rat Neu2 promoter has shown the presence of several canonical and non-canonical E-box sequences that represent binding motifs for the bHLH factors involved in skeletal muscle maturation [29]. Moreover, putative sites for myogenin and MEF-2 transcription factors [34,35], both involved in the differentiation process, are also present (data not shown).

In order to better understand the importance of Neu2 upregulation in myoblast differentiation, stable Neu2 C2C12 transfectants were obtained. Analysis of the Neu2-overexpressing clones demonstrated that Neu2 upregulation was followed by the acquisition of a differentiated phenotype also when cells were maintained in the absence of differentiating conditions (DM). This was demonstrated by a decrease in the proliferation rate of the transfectants, paralleled by cyclin Dl

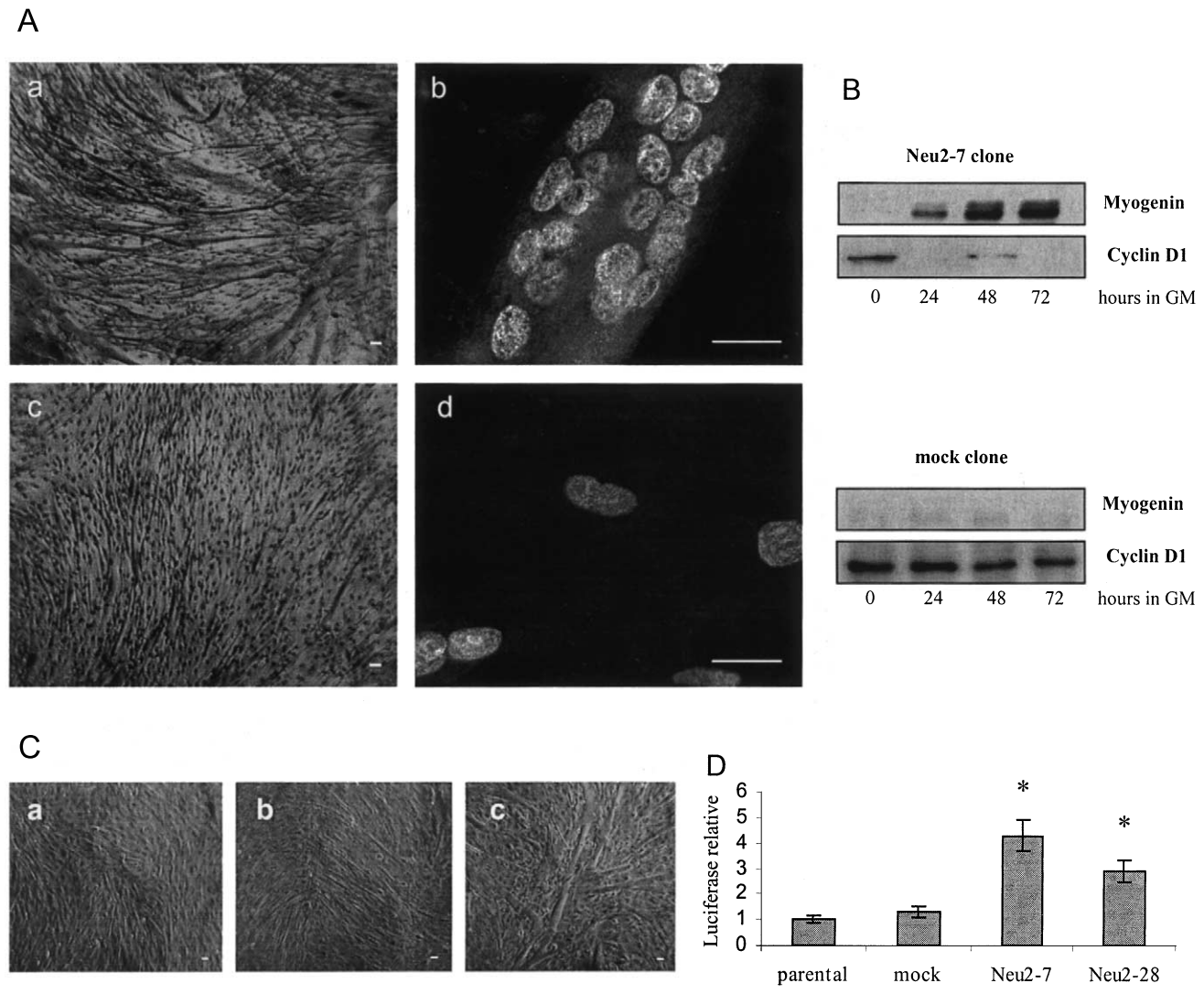

Fig. 4. Spontaneous myoblast differentiation of Neu2 transfectants. A: Differentiation of Neu2-overexpressing C2C12 cells (Neu2-7 clone) (panels a and b) and mock-transfected cells (panels c and d) after 6 days at confluence in GM. Cells were stained with Giemsa reactive (panels a and c) or immunostained with anti-myogenin antibody (panels b and d). The Neu2 transfectants form well-defined myogenin-positive myotubes, whereas the mock clone retains an undifferentiated morphology. Bars, 60 μm. B: Western blot analysis of nuclear extracts of Neu2-7 clone and mock clone maintained in GM for 0–72 h. Nuclear proteins were probed with monoclonal anti-myogenin and anti-cyclin Dl antibodies. C: Morphology of C2C12 cells grown for 4 days in GM (panel a) or DM (panel b) compared to Neu2-7 transfectants maintained in DM (panel c). The Neu2-7 transfectants form multi-giant hyperthrophic myotubes. Bars, 60 μm. D: Parental, mock and Neu2-overexpressing Neu2-7 and Neu2-28 clones were transiently transfected with the pGL2-Basic-Neu2 promoter expression vector harboring the luciferase reporter gene under the control of the rat Neu2 promoter. After 24 h in GM, luciferase activity in the cell extracts was measured and normalized for protein content ($n = 4$ for each group). *$P < 0.01$.

downregulation, and by the simultaneous expression of myogenin and myotube formation. Similar results were obtained in C2C12 cell cultures transiently transfected with the same expression vector (data not shown), thus confirming the results obtained in the stable transfection experiments. In contrast, no difference in the differentiation was observed in mock-transfected cells when compared to parental cells.

As observed in parental C2C12 cells shifted to the differentiating medium, also differentiating Neu2-overexpressing clones were characterized by an increased transcriptional activity of the transiently transfected rat Neu2 promoter. Taken together, our data suggest that Neu2 upregulation is tightly linked to the skeletal muscle maturation process and that an increase in the cytosolic sialidase activity per se is sufficient to trigger myoblast withdrawal from the cell cycle and the activation of the myotube formation program. In keeping with this hypothesis, Neu2 transfectants grown in differentiating medium formed multi-giant hypertrophic myotubes with an elongated morphology, significantly larger in size with respect to myotubes originating from parental C2C12 cells. These data extend previous observations on rat L6 myoblast in which myotube formation was prevented by treatment with Neu2 antisense oligonucleotides [22].

Previous observations had shown that in vitro myotube formation is paralleled by changes in glycosphingolipid metabolism [36]. These findings, together with our observations, raise the hypothesis that the catabolism of some endogenous gangliosides may represent an early key step in skeletal muscle maturation. Gangliosides and glycoproteins are indeed expected to be present in the cytosol [20,21] and the capacity

of Neu2 sialidase to work at neutral pH makes these cytosolic components potential targets for cytosolic sialidase activity.

Indeed, the association of glycosphingolipids to the cytoskeleton in different cell types, including muscle cells, has been demonstrated [37,38]. In particular GM3, the major ganglioside synthesized by myoblasts [39,40], might be a candidate substrate for cytosolic sialidase [41,42]. The modulation of endogenous GM3, that interacts with vimentin intermediate filaments [38], may represent an initial step in the cytoskeleton remodeling prior to cell fusion and may therefore represent a fundamental signal for triggering myoblast differentiation. In keeping with this hypothesis, Neu2 overexpression has been shown to modulate melanoma cell invasiveness and migration, two processes strictly linked to cytoskeleton functions, through a mechanism involving GM3 hydrolysis [3].

In conclusion, our data demonstrate that cytosolic sialidase Neu2 upregulation plays a crucial role in skeletal muscle maturation in vitro by controlling the decision between growth and differentiation. This sheds new light on the molecular mechanisms that control muscle cell growth and differentiation during embryonic development and regeneration through satellite cell recruitment in the adult. Also, Neu2 transduction via gene transfer may have therapeutic implications in the control of rhabdomyosarcoma tumor cells that arise from immature mesenchimal cells committed to skeletal muscle lineage with interrupted myogenesis [43,44].

Acknowledgements: This work was supported in part by grants from MIUR (Centra di Eccellenza 'IDET' and FIRB), Associazione Italiana per la Ricerca sul Cancro, and Centro per lo Studio del Trattamento dello Scompenso Cardiaco (University of Brescia).

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