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Involvement of histone H4 gene transcription factor 1 in downregulation of vimentin gene expression during skeletal muscle differentiation

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Abstract Upon in vitro myogenesis, the intermediate filament protein vimentin is replaced by desmin, the switch in gene expression occurring essentially at the transcriptional level. Trying to elucidate the molecular mechanisms of this genetic control, we show here that the vimentin promoter is specifically recognized and activated by a protein most probably identical to H4TF-1, and that this factor is present in proliferating myoblasts but disappears upon fusion of these cells into multinucleated myotubes. Our results suggest that H4TF-1 is a differentiation stage-specific factor involved in the downregulation of vimentin gene expression during myogenesis.

Key words: Cytoskeleton; Intermediate filament; Vimentin; H4TF-1; Myogenesis; C2C12 cell

1. Introduction

During vertebrate development, the synthesis of intermediate filament (IF) proteins, a superfamily of around 50 members with more or less restricted tissue specificity, undergoes a complex regulation. In the embryo, vimentin is specific of the mesoderm. In the adult organism, it is maintained in some mesodermal derivatives, while in others it is replaced by distinct IFs. In mammalian adult skeletal muscle, for instance, IFs are made of desmin [1]. Vimentin is present in human fetal skeletal muscle until 36 weeks of gestation but its proportion progressively decreases in favor of desmin during development [2]. Upon rat skeletal muscle regeneration, vimentin synthesis is observed in activated satellite cells, it increases at the beginning of regeneration, and then decreases to become undetectable in mature, regenerated fibers [3]. In myogenic cell lines in vitro, proliferating myoblasts stop producing vimentin when they are induced to fuse into multinucleated myotubes that acquire numerous characters of differentiated muscle cells.

Functional study of the human vimentin promoter has led us and others to identify positive and negative regulatory elements, some of which may be involved in the control of vimentin gene expression during cell differentiation (Fig. 1; for a review, see [4]). Two AP1 and an NF-κB binding sites activate the vimentin promoter in proliferating cells [5], and a negative element, *NE1*, counteracts the positive effect of NF-κB [6]. Both positive elements may become inactive during myogenesis to allow downregulation of vimentin synthesis, but to date it is not yet possible to say what regulatory elements are responsible for all aspects of the complex developmental control of vimentin gene transcription.

More recently, we characterized a short region of the vimentin promoter, including (i) a negative element, *NE2*, which might reinforce vimentin gene expression blockade in differentiated muscle cells [7] and (ii) a sequence we named *H4* motif, homologous to the binding site of transactivating factor H4TF-1 [8]. Here, we show that a protein, most probably H4TF-1 itself, specifically recognizes the vimentin *H4* motif, and that this factor is present in proliferating myoblasts but is not detected in differentiated myotubes. Besides, the vimentin *H4* motif has decreased transcriptional activity in myotubes, suggesting that it contributes to the negative regulation of vimentin gene expression upon muscle differentiation. Through our results, it appears that a histone gene transcription factor may be recruited to the complex vimentin promoter to participate in differentiation stage-specific gene expression, this factor being actually lost upon in vitro myogenesis.

2. Materials and methods

2.1. Cell culture

Murine C2C12 myoblasts and C3H 10T1/2 fibroblasts were obtained from the ECACC (Porton Down, UK). Myoblasts were grown in DMEM+20% fetal bovine serum (Life Technologies). Myotubes were obtained by switching confluent myoblasts to DMEM+2% horse serum (Life Technologies) for 3 days. Fibroblasts were grown in MEM+10% fetal bovine serum.

2.2. Transfection and reporter gene expression assay

pVimCAT plasmids were derived from p1757vimCAT, which includes the bacterial chloramphenicol acetyltransferase (CAT) gene under the control of the human vimentin promoter (nucleotides (nt) −1757 to +78). Two 5′-deletion mutants, p642vimCAT and p606vimCAT, were generated from p1757vimCAT by PCR (upstream borders of vimentin sequence −642 and −606, respectively). Transfection internal reference was provided by pEF1lacZ plasmid, containing the bacterial lacZ gene under the control of the human EF1α promoter.

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Abbreviations: CAT, chloramphenicol acetyltransferase; EMSA, electrophoretic mobility shift assay; HLH, helix-loop-helix; IF, intermediate filament

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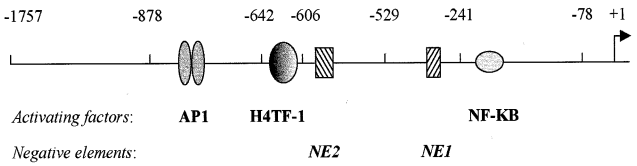

Fig. 1. Human vimentin gene promoter. Nucleotide numbering indicates the boundaries of the main regulatory subregions identified (+1: transcriptional start). Positive and negative control elements are represented by gray ovals and hatched boxes, respectively.

C2C12 myoblasts were transfected 24 h after seeding, using 7.5 μl Fugene 6 transfection reagent (Roche Diagnostics), 2.25 μg pVimCAT and 0.25 μg pEF1lacZ per 25 cm^2 flask. 48 h later, cells were either lysed (transfected myoblasts), or allowed to differentiate for 72 h before lysis (transfected myotubes). CAT and β-galactosidase were assayed by enzyme-linked immunosorbent assay, according to the manufacturer's instructions (Roche Diagnostics). Experiments were done three times, with each point in triplicate.

2.3. Preparation of whole nuclear extracts

All steps were performed at 4°C with buffers supplemented just before use with 0.15 mM spermidine, 0.15 mM spermine, 1 mM dithiothreitol (DTT), 1 mM PMSF, 2 mM benzamidine (Sigma), and Complete® protease inhibitor mix (Roche Diagnostics). Cells were washed, scraped and pelleted in phosphate-buffered saline, suspended in three volumes buffer A (20 mM HEPES pH 7, 0.15 mM EDTA, 0.15 mM EGTA, 10 mM KCl), and lysed with NP40 (1% final concentration). Released nuclei were stabilized by addition of one volume buffer S (50 mM HEPES pH 7, 0.25 mM EDTA, 10 mM KCl, 70% sucrose), pelleted, and washed in five volumes buffer B (10 mM HEPES pH 8, 0.1 mM EDTA, 0.1 M NaCl, 25% glycerol). After centrifugation, nuclei were gently suspended in one volume buffer C (10 mM HEPES pH 8, 0.1 mM EDTA, 0.4 M NaCl, 25% glycerol) and agitated 30 min at maximum speed on a Labnet Shaker 20. Nuclei were discarded after high speed centrifugation and the supernatant was stored in aliquots at −80°C. Proteins were quantified by the Bradford assay (Bio-Rad).

2.4. Electrophoretic mobility shift assay (EMSA)

Nuclear proteins (5 μg) were incubated 10 min at 25°C with 2.5 μg polydI-dC·polydI-dC and 0.25 μg calf thymus DNA in 20 mM HEPES pH 8, 40 mM NaCl, 6 mM $MgCl_2$, 7.5% glycerol. Cold, competitor oligonucleotides, when used, were added at this step (1.75 pmol). 35 fmol ^{32}P-labeled probe was added for another 20 min incubation. The mixture was loaded onto a native 4% polyacrylamide gel (29:1) and electrophoresed 2 h at 10 V/cm in 0.125× TBE. The gel was dried and analyzed by PhosphoImager.

The following double-stranded oligonucleotides were used: *vH4*, human vimentin *H4TF-1* motif (nt −641 to −612); *hH4*, H4TF-1 binding site of the human histone H4 gene (nt −109 to −80); *mvH4* and *mhH4*, same oligonucleotides as *vH4* and *hH4* with four point mutations to abolish factor interaction [8]; *SP1*, consensus SP1 target site (Promega). Probe *vH4* was 5′-end-labeled with [γ-^{32}P]ATP (3000 Ci/mmol; NEN) by T4 polynucleotide kinase (Biolabs).

2.5. Southwestern blot

Nuclear proteins (100 μg) were boiled in sample buffer (1% sodium dodecyl sulphate (SDS), 50 mM Tris–HCl pH 7.5, 140 mM β-mercaptoethanol, 10% glycerol, 0.001% pyronin Y) and electrophoresed at 25 mA on an 0.1% SDS, 8% polyacrylamide gel in 50 mM Tris, 400 mM glycine, 0.1% SDS, in the presence of prestained molecular weight standards (Sigma). They were electrotransferred to a nitrocellulose sheet 2 h at 0.5 A in 25 mM Tris, 190 mM glycine, 20% methanol, using a Bio-Rad transfer cell. The blot was blocked 1 h at 37°C in 5% non-fat dry milk, 50 mM Tris–HCl pH 7.5, 50 mM NaCl, 0.1 mM EDTA, 1 mM DTT, equilibrated 5 min in binding buffer (10 mM Tris–HCl pH 7.5, 50 mM NaCl, 0.1 mM EDTA, 1 mM DTT), incubated 1 h at 25°C with the *vH4* probe (10^6 cpm/ml) in 20 ml binding buffer, washed three times in binding buffer, dried and autoradiographed.

3. Results

3.1. Interaction of fibroblast nuclear factors with the human vimentin H4 motif

The vimentin *H4* motif bears a perfect match to the binding site of transcription factor H4TF-1: 5′-CCCTCCCCC-3′ (nt −630 to −622), which appeared strongly protected in our DNase I footprinting experiments [7]. To further characterize the factor(s) involved, we analyzed protein–DNA interactions obtained in vitro with nuclear extracts from proliferating C3H 10T1/2 fibroblasts, in EMSA with the *vH4* probe.

Two major, highly retarded complexes were produced (arrowheads in Fig. 2, lane 1), the specificity of which was tested with various competitor oligonucleotides. Formation of the faster migrating complex (filled arrowhead) was abolished by homologous competitor (*vH4*; lane 2), as well as by competitor including the human histone H4 gene H4TF-1 binding site (*hH4*; lane 5). When *vH4* and *hH4* oligonucleotides beared point mutations reported to disrupt H4TF-1 binding (5′-**TCGTACTCC**-3′ instead of 5′-CCCTCCCCC-3′ in *mvH4* and *mhH4*; [8]), no competition occurred (lanes 3 and 6, respectively). The complex remained also stable in the presence of *SP1* oligonucleotide, which we assayed because the GC-rich *H4* motif would potentially bind transcription factor SP1 (lane 4). On the other hand, formation of the slower migrating complex (open arrowhead) was reduced by the five oligonucleotides tested (lanes 2–6), and was clearly suppressed by *SP1* competitor (lane 4).

From these results we conclude that the vimentin *H4* motif specifically binds a nuclear factor which might actually be H4TF-1. This conclusion is supported by the fact that the

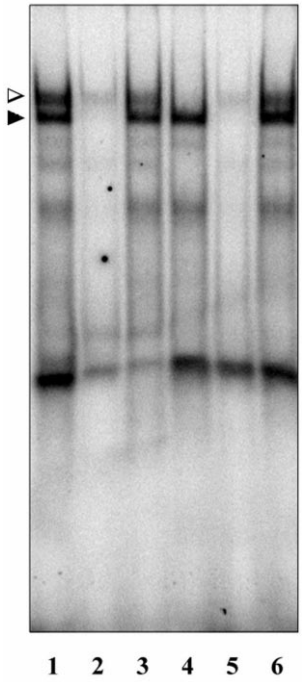

Fig. 2. Fibroblast nuclear factor binding to the human vimentin gene *H4* motif. EMSA with C3H 10T1/2 fibroblast whole nuclear extract, using ^{32}P-labeled *vH4* probe, in the absence (lane 1) or presence of cold competitor oligonucleotides: *vH4*, *mvH4*, *SP1*, *hH4* and *mhH4* (lanes 2–6, respectively). Open and filled arrowheads point to protein–DNA complexes specifically abolished by *SP1* and *H4* competitors, respectively.

faster migrating complex we obtain is abundant in the presence of 6 mM Mg^{2+} and is considerably destabilized when Mg^{2+} concentration is lowered to 1 mM (not shown), exactly as was demonstrated by Dailey et al. with H4TF-1 on the human histone H4 gene [9]. The vimentin *H4* motif also seems to bind SP1, although with slightly reduced specificity.

3.2. Fate of nuclear factors binding to the vimentin H4 motif in myogenic cells

Since the vimentin gene is active in myoblasts and becomes repressed upon in vitro myogenesis, we searched for vimentin *H4* binding activities in the myogenic cell line C2C12, in proliferation or differentiation conditions. We performed EMSAs to compare interactions of the *vH4* probe with nuclear extracts from C3H 10T1/2 fibroblasts, C2C12 myoblasts, and C2C12 myotubes (Fig. 3A). The two protein–DNA complexes described in the case of fibroblasts (lane 1) were generated with C2C12 myoblast nuclear extracts (lane 2). Anyhow, when nuclear extracts from myotubes were used, the more retarded complex remained unchanged, whereas the faster migrating complex, that probably due to H4TF-1, became barely detectable (lane 3). Disappearance of the faster migrating complex in myotube extract was identically observed in the presence of *SP1* competitor oligonucleotide (Fig. 3B, compare lanes 2 and 3).

To get more information about the factors involved, we performed Southwestern blotting analysis of our nuclear extracts. A single polypeptide was recognized by the *vH4* probe in extracts from C2C12 myoblasts (Fig. 3C, lane 2). Its molecular weight is around 110–115 kDa, consistent with that reported for H4TF-1 [10]. No signal was obtained with the myotube extract (lane 3), the integrity of which was checked with a vimentin *NE2* probe revealing the presence of the negative element binding factors [7] (not shown). We conclude that H4TF-1 (or at least a closely related polypeptide) is present in C2C12 myoblasts, that it specifically binds to the vimentin promoter, and that this interaction is lost upon muscle differentiation.

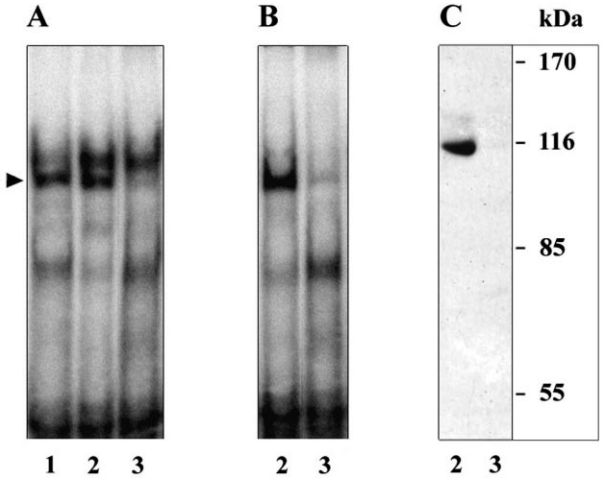

Fig. 3. Loss of binding to the vimentin *H4* motif upon myoblast differentiation. Interaction of whole nuclear extracts from C3H 10T1/2 fibroblasts (lane 1), C2C12 myoblasts (lane 2) and myotubes (lane 3), with ^{32}P-labeled *vH4* probe. (A, B) EMSA, (A) in the absence or (B) in the presence of *SP1* competitor. (C) Southwestern blot. Arrowhead in (A) points to a protein–DNA complex which disappears upon myogenic differentiation of C2C12 cells.

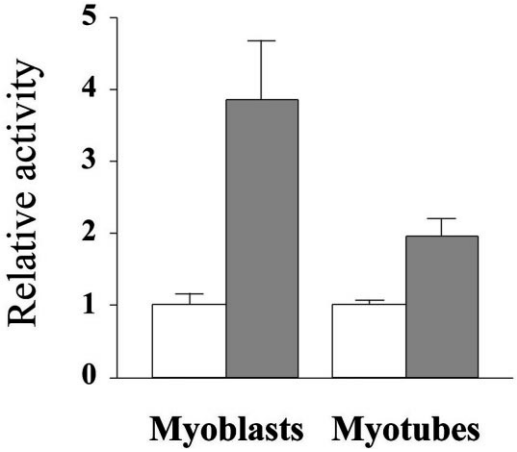

Fig. 4. Decrease in transcriptional activity of the vimentin *H4* motif in C2C12 myotubes. Normalized CAT reporter gene activity, obtained after transfection of C2C12 cells by p606vimCAT (open bars) and p642vimCAT (filled bars), is represented as mean values ± S.E.M. For clarity the mean of p606vimCAT values is set to 1, so that p642vimCAT values are directly shown as fold activation over p606vimCAT, due to the presence of the *H4* motif.

3.3. Transcriptional activity of the vimentin H4 motif in C2C12 cells

To know whether loss of binding to the vimentin *H4* motif influences transcriptional activity of this sequence in C2C12 cells, we undertook transfection experiments. To establish a good correlation between EMSA and transcription analysis, preparation of nuclear extracts and transfection were always done in parallel on the same cell cultures, in proliferation or differentiation conditions.

Transcriptional activity of the *H4* motif was estimated by comparison of normalized CAT enzyme production from p606vimCAT and from p642vimCAT, since the only difference between the two plasmids is the presence of the *H4* motif between nt −642 and −606 in the latter. We confirmed that the vimentin *H4* motif stimulates transcription in C2C12 myoblasts (3–5-fold activation over p606vimCAT). When myoblasts differentiated into myotubes, we observed, parallel to the decrease in H4TF-1-like binding activity in nuclear extracts, a 2-fold reduction of transcriptional stimulation by the *H4* motif in transfected cells (Fig. 4). From these results, we hypothesize that loss of interaction of the *H4* motif with a factor that may be H4TF-1, contributes to the downregulation of vimentin gene expression during in vitro myogenesis.

4. Discussion

Myogenesis takes place in vivo during embryonal development or in the adult organism upon muscle regeneration. It has been extensively studied in vitro using various myogenic cell lines and the molecular mechanisms which control muscle gene induction have been relatively well unraveled, involving a cascade of transcriptional activation initiated by the MyoD family of basic helix-loop-helix (HLH) regulatory proteins (for a review, see [11]). Upon serum starvation, myoblasts turn on the synthesis of myogenin, which induces irreversible withdrawal from the cell cycle and coordinate expression of

genes encoding sarcomeric proteins and muscle-specific enzymes. Then, myoblasts fuse to generate postmitotic, multinucleated myotubes with a characteristic contractile phenotype.

Several constituents of the cytoskeleton undergo rearrangements upon myogenesis. We are interested in studying IF protein synthesis during skeletal muscle differentiation in vitro. How repression of vimentin gene expression occurs in these conditions is not clearly understood yet. Among several transcription factors binding to the vimentin promoter, AP1 and NF-κB have been identified as positive regulators of vimentin gene expression in proliferating cells [5]. It has been suggested that in muscle cells, AP1 and MyoD functionally antagonize each other through physical association between Jun and MyoD [12], so that upon muscle differentiation MyoD might block AP1-dependent activation of the vimentin promoter. However, no decrease in c-jun expression or AP1 binding activity was observed in C2C12 cells [13]. NF-κB was reported to stimulate cell proliferation and to block muscle differentiation through activation of the cyclin D1 gene [14], suggesting that the positive effect of NF-κB on the vimentin promoter may be counteracted during myogenesis. In other works, NF-κB has been shown to stimulate myoblast fusion and induction of muscle-specific genes [15]. Thus, if AP1 and NF-κB are involved in the downregulation of vimentin gene expression upon myogenesis, this has not been unambiguously demonstrated yet. In the present study, our interest has focused on a third regulatory element within the vimentin promoter, namely the *H4* motif. We show that a factor specifically binding to this sequence in fibroblast and myoblast nuclear extracts exhibits apparent molecular weight and conditions of binding to DNA in agreement with its being H4TF-1. Upon myoblast fusion, binding of this factor is lost, whereas transcriptional activation through the *H4* motif decreases 2-fold. Residual activation in myotubes might rely upon SP1-dependent complex formation. From these results we hypothesize that the vimentin promoter is recognized and activated by H4TF-1 in myoblasts but not in myotubes, and that this differentiation stage-specific, functional interaction contributes to the downregulation of vimentin gene expression during in vitro myogenesis. Diverse mutated versions of the *H4* motif have been studied but none of them allowed to discriminate between H4TF-1 and SP1 binding [8,16,17]. Furthermore, both factors were shown to protect the eight G nucleotides on the complementary strand of the *H4* motif against in vitro methylation [10,17]. Taken together, these data indicate that H4TF-1 and SP1 binding sites completely overlap. In our study, involvement of H4TF-1 rather than SP1 in differentiation stage-specific activity of the vimentin *H4* motif may be deduced from the fact that binding of H4TF-1, not of SP1, is lost in myotube extracts.

H4TF-1 has been identified as a transcription factor binding to the human histone H4 gene [18], stimulating its transcription 4–5-fold [8]. It does not bind to non-H4 histone genes, and its abundance does not vary appreciably throughout the cell cycle in proliferating fibroblasts [19]. A distinct factor controls S phase-induced histone H4 gene expression but increases transcription only 2-fold [20]. Thus, H4TF-1 is not a cell cycle-dependent regulator but it is required for maximal transcription of the active histone H4 gene. It also participates in transcriptional activation of other genes, such as those encoding human and murine blood clotting factor VII [16,17].

No antibody against H4TF-1 exists since the factor has been only partially purified [10]. No candidate gene encoding H4TF-1 has been identified. Thus, characterization of H4TF-1 activity essentially relies upon indirect evidence. Our results with the vimentin promoter reproduce observations done with the histone H4 gene, that is the binding of a factor around 110 kDa, destabilized at low Mg^{2+} concentrations, lost upon mutation of 5′-CCCTCCCCC-3′ to 5′-TCGTACTCC-3′ in target sequence, not competed out by *SP1* consensus [8–10]. It is noteworthy that in the case of the human factor VII gene also, two complexes are formed with the H4TF-1 binding motif in EMSA, the more retarded one including SP1 [16]. We do not observe interaction of the vimentin *H4* motif with SP1 in Southwestern blot experiment, probably because an additional factor is required to form a stable complex with SP1, which cannot be achieved after electrophoretic separation of the proteins.

Some factors controlling muscle-specific gene induction are also found in undifferentiated myoblasts, i.e. the desmin myotube-specific enhancer binding factor identified by Gao et al. [21]. Here, by studying the vimentin promoter, we have uncovered H4TF-1 as a proliferating cell-specific factor that disappears upon fusion of growing myoblasts into differentiated myotubes. Interestingly, the H4TF-1 recognition site is present in the promoters of other genes shown to be downregulated upon myogenesis, including c-fos, Id2A and Id3. c-fos is involved in the positive control of cell proliferation and may need to be repressed when myoblasts withdraw from the cell cycle. MyoD actually downregulates transcription of the c-fos gene upon muscle cell differentiation [22]. Id proteins are HLH transcription factors that lack DNA binding domains. They inhibit myogenesis through sequestering of E proteins required for the activation of muscle-specific genes by basic HLH myogenic factors [23]. Expression of Id2A and Id3 is high in growing myoblasts and is dramatically downregulated after the cells are induced to differentiate [24,25]. Thus, H4TF-1 might play a more general role during myogenesis and contribute to the downregulation of several genes upon muscle cell differentiation, including immediate early response genes like vimentin and c-fos, as well as genes encoding members of the Id family of muscle differentiation inhibitors. Besides, within the vimentin promoter, where multiple factors potentially contribute to the regulation of gene expression, H4TF-1 might be important, as in the context of a few other genes, to achieve maximal transcription levels in conditions where expression of the gene is switched on.

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