A naturally occurring calcineurin variant inhibits FoxO activity and enhances skeletal muscle regeneration

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Introduction

The serine/threonine phosphatase calcineurin (Cn) plays a main role in a wide variety of physiological and pathological processes, including the immune response, neuronal plasticity, and cardiac development and hypertrophy (Schulz and Yutzey, 2004). In response to calcium increase, Cn induces dephosphorylation, and activation of the NFAT transcription factors, a process sensitive to the action of the immunosuppressive drug cyclosporine-A (CsA). Cn enzymatic activity requires a catalytic (CnA) and a regulatory (CnB) subunit, variants of which are encoded by alternatively spliced transcripts (Guerini and Klee, 1989). The typical autoinhibitory domain present in CnAβ2 and other CnA isoforms is absent from CnAβ1, in which an unpaired C-terminal region is preserved in the CnAβ1 orthologues from different species (Fig. S1 B), especially in higher vertebrates, suggesting an evolutionarily conserved role for this Cn variant.

In skeletal muscle, the Cn/NFAT pathway mediates myotube differentiation, enhances myoblast recruitment, controls muscle fiber type specification, and ameliorates injury to dystrophic muscles (Friday et al., 2000; Naya et al., 2000; Horsley et al., 2001, 2003; Parsons et al., 2003; Stupka et al., 2006). The function of the CnAβ1 isoform has not been explored because no obvious phenotype was reported in a germline knockout of the CnAβ1 gene (Bueno et al., 2002). Notably, in that study the knockout scheme involved deletion of catalytic domain encoded by exon 2, which would still allow transcription of in-frame described: CnAα and CnAβ are ubiquitously expressed, whereas CnAγ is restricted to brain and testis. Two CnAβ isoforms, CnAβ1 and CnAβ2, which differ in their C-terminal domain, are encoded by alternatively spliced transcripts (Guerini and Klee, 1989). The typical autoinhibitory domain present in CnAβ2 and other CnA isoforms is absent from CnAβ1, in which an unrelated C-terminal domain is generated by the translation of intronic sequences (Fig. 1 A; and Fig. S1 A, available at http://www.jcb.org/cgi/content/full/jcb.200704179/DC1). This novel domain is conserved in the CnAβ1 orthologues from different species (Fig. S1 B), especially in higher vertebrates, suggesting an evolutionarily conserved role for this Cn variant.

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transcripts encoding phosphatase-dead CnAβ2 or CnAβ1 protein. Interestingly, increased CnAβ1 expression was noted in response to the muscle-restricted overexpression of a local IGF-1 isoform (mIGF-1), which preserved muscle integrity and enhanced the response to chronic degeneration in a mouse model of amyotrophic lateral sclerosis (ALS) (Dobrowolny et al., 2005), suggesting a role for CnAβ1 in satellite cell activation.

To establish a function for CnAβ1, we overexpressed the protein or blocked its production in muscle cell culture and found that, unlike other CnA variants, CnAβ1 inhibits FoxO transcriptional activity independently of its phosphatase function, regulates myoblast proliferation, and prevents myotube atrophy under starvation conditions. In addition, CnAβ1 overexpression in post-mitotic muscle enhances regeneration, reducing scar formation and accelerating the resolution of inflammation. The unique properties of the CnAβ1 isoform expand the spectrum of biological functions for calcineurins and provide new avenues for prevention of muscle atrophy.

Results

CnAβ1 is a constitutively active Cn isoform

To determine whether the absence of an autoinhibitory domain rendered the CnAβ1 isoform constitutively active without high intracellular calcium, we analyzed Cn phosphatase activity in HEK293 cells transfected with expression plasmids encoding CnAβ1, CnAβ2, or CnAβ* in which the autoinhibitory domain has been artificially deleted. Phosphatase activity in CnAβ1-transfected cells was as high as those expressing constitutively active CnAβ*, compared with cells transfected with CnAβ2 (Fig. 1 B). Cell fractionation and subsequent analysis by Western blot showed that CnAβ1 is localized both in the cytoplasm and the nucleus in proliferating C2C12 cells, with most of the protein mainly localized in the cytoplasm (Fig. 1 C). Co-transfection of C2C12 myoblasts with HA-NFATc2 and CnAβ1 or empty pcDNA3.1 (Fig. 1 D). The unique properties of the CnAβ1 isoform expand the spectrum of biological functions for calcineurins and provide new avenues for prevention of muscle atrophy.
NFAT by all Cn forms (Fig. 1 F). Together, these results show that CnAβ1 is a physiological Cn form that is constitutively active and is partially insensitive to the action of CsA.

**CnAβ1 expression is increased during skeletal muscle regeneration**

We analyzed the CnA expression patterns in differentiating myoblasts as well as different healthy and cardiotoxin-injured muscles by Northern blot and quantitative RT-PCR (qRT-PCR). All uninjured muscles showed high expression levels of CnAα and CnAβ2 and weaker expression of CnAβ1 (Fig. 2 A). Differentiation of primary and C2C12 myoblasts, both of which expressed high CnAβ1 levels, was accompanied by a progressive increase in CnAβ2 and a decline in CnAβ1 levels (Fig. 2, B and C). In vivo, skeletal muscle regeneration in response to injury induced a transient rise in CnAβ1 transcripts and a concomitant decrease in CnAβ2 transcripts (Fig. 2, D–F). IGF-1 was able to induce CnAβ1 expression both in vivo, in MLC/mIGF-1 transgenic mice especially during recovery from muscle atrophy induced by sciatic denervation (Fig. 2 G), and in vitro in starving myotubes.
To determine whether CnAβ1 was expressed by muscle precursors and regenerating fibers or by the immune infiltrate, we analyzed its tissue distribution during skeletal muscle regeneration in the tibialis anterior of wild-type mice 2 and 6 d after cardiotoxin injection. CnAβ1 was strongly expressed by muscle precursors and regenerating muscle fibers, showing a pattern similar to that of Myf5, which labels satellite cells and early regenerating fibers (Cooper et al., 1999), whereas uninjured fibers or the immune infiltrate (marked by anti-CD45) expressed little or no CnAβ1 (Fig. 3). These results implicate the predominant CnAβ2 isoform in skeletal muscle differentiation and maturation, whereas CnAβ1 might play a role during myoblast proliferation and muscle regeneration.

CnAβ1 regulates myoblast proliferation and differentiation

To characterize the function of CnAβ1, we knocked down CnAβ1 expression by transfecting proliferating C2C12 myoblasts with two different siRNAs specific for mouse CnAβ1 (termed 132 and 167) or a luciferase siRNA as a control (Fig. 4 A). After 48 h in growth medium (GM), oligonucleotide-based microarray analysis showed an induction of muscle differentiation-specific gene expression in cells transfected with the CnAβ1 siRNAs (Fig. 4 B). The induction of the muscle differentiation program was confirmed by qRT-PCR and was found to be similar to that obtained with cells in differentiation medium (DM) for 1 d (Fig. 4 C). Although the response obtained with the siRNA 167 was weaker than that obtained with the 132 siRNA, similarities in the profiles of genes up-regulated by each of the CnAβ1 siRNAs (P < 4.63 × 10^{-18}, Table S1 and S2, available at http://www.jcb.org/cgi/content/full/jcb.200704179/DC1) and down-regulated genes (P < 4.39 × 10^{-60}, Table S3 and S4) indicated that the observed effects were due to specific knockdown of CnAβ1 rather than to off-target effects. Correspondingly, an increase in myogenin expression was detected by Western blot after transfection of C2C12 cells with the CnAβ1 siRNA (Fig. 4 D).

Given that CnAβ1 knockdown results in decreased myoblast proliferation and increased differentiation and because its expression declines during myotube formation, we studied whether CnAβ1 could actually interfere with myoblast differentiation. We generated C2C12 stable transfectants in which the expression of the different CnAβ isoforms is driven by the CMV promoter (pcDNA3.1 vector) and thus is not affected by differentiation. When induced to differentiate, C2C12 cells stably overexpressing CnAβ1 showed a mild significant increase in differentiation markers only at early time points (Fig. S2, B and C) compared with the mock transfectants. These data suggest that CnAβ1 may slightly accelerate the initial differentiation steps when artificially overexpressed. It is noteworthy that this effect was only detected after switching C2C12 cells to differentiation medium and was not observed under growth conditions. A similar dual action has been previously reported for IGF-1, which is able to both enhance myoblast proliferation and up-regulate differentiation markers once the cells have been induced to differentiate (Engert et al., 1996; Musarò and Rosenthal, 1999). On the contrary, continuous overexpression of CnAβ1 had no impact on differentiation markers 5 d after shifting the cells to differentiation medium. In addition, 8 d after differentiation, myotubes overexpressing CnAβ1 showed a similar diameter to that of myotubes bearing the expression vectors for CnAβ*, CnAβ2, or the parental vector alone (see Fig. 6 H; Fig. S3 C, available at http://www.jcb.org/cgi/content/full/jcb.200704179/DC1).
Figure 4. **CnAβ1 knock-down results in myoblast differentiation.**

(A) C2C12 myoblasts were transfected with CnAβ1-specific siRNAs 132 (black bars) and 167 (gray bars) or a control siRNA, grown for 48 h in growth medium (GM), and the expression of CnAβ1 was assayed by microarray and qRT-PCR. (B and C) RNA was extracted from myoblasts transfected with control or CnAβ1 siRNAs and subjected to microarray (B) or qRT-PCR (C) analysis. Comparison with untransfected cells differentiated for 24 h (DM 1d) is shown. Gene expression is represented as fold induction ±SD (note Log. relative units) of values from cells transfected with each CnAβ1 siRNA over those transfected with control siRNA. (D) C2C12 myoblasts were transfected with control or CnAβ1 siRNA and the expression of CnAβ1, CnAβ2, and myogenin was analyzed by Western blot. *, P < 0.05; **, P < 0.005; ***, P < 0.0005.

http://www.jcb.org/cgi/content/full/jcb.200704179/DC1), indicating that although CnAβ1 expression is required to prevent myoblast differentiation, overexpression of this Cn isoform does not interfere with myotube differentiation.

Interestingly, CnAβ1 knockdown induced an increase in negative cell cycle regulators and a decrease in genes involved in cell cycle progression (Fig. 5, A and B). A corresponding decrease in BrdU incorporation (Fig. 5 C) and cell proliferation (Fig. 5 D) was observed after CnAβ1, but not CnAβ2, knock-down, accompanied by a decrease in the percentage of cells in the G2/M phase of the cell cycle and an increase in G1 (Fig. 5 E), indicating that the CnAβ1 isoform is necessary for cell cycle progression. C2C12 stable transfectants overexpressing CnAβ1 showed only a modest increase in BrdU incorporation (Fig. 5 F). Together, these results suggest that CnAβ1 is required for myoblast proliferation and that CnAβ1 knockdown results in exit from the cell cycle and subsequent myoblast differentiation.

**CnAβ1 inhibits FoxO transcription factors.**

Many of the negative cell cycle regulators induced by CnAβ1 inhibition, such as p21, Rbl2, and Gadd45a, are known to be regulated by the FoxO family of transcription factors (Furukawa-Hibi et al., 2002; Kops et al., 2002; Tran et al., 2002; Seoane et al., 2004). We observed that additional FoxO target genes were induced after CnAβ1 knockdown as observed by microarray (Fig. 6 A) and qRT-PCR analysis (Fig. 6 B), including increased expression of atrogenes Murf1 and MAFbx/Atrogin that are known to mediate FoxO-induced muscle atrophy (Sandri et al., 2004; Stitt et al., 2004). Inhibition of CnAβ1 by siRNA abrogated FoxO3α phosphorylation at Ser253, (Fig. 6 C) and also resulted in defective PDK1 and Akt phosphorylation, without affecting mTOR or Erk, suggesting that CnAβ1 is required for the activation of the PDK1-Akt-FoxO phosphorylation cascade that leads to FoxO inhibition. An increase in p38 phosphorylation was observed that is likely to be a direct consequence of C2C12 differentiation (Perdigueru et al., 2007), rather than a direct target of CnAβ1 knockdown. Moreover, overexpression of CnAβ1, but not CnAβ2 or the constitutively active CnAβ*, in C2C12 cells inhibited FoxO nuclear localization (Fig. 6 D; Fig. S3 A) and the activity of a FoxO-dependent luciferase reporter (Fig. 6 F), whereas transfection of C2C12 myoblasts with the CnAβ1 siRNAs 132 or 167 activated the reporter (Fig. 6 E). This activation was prevented by cotransfection with an expression plasmid for human CnAβ1, confirming the specificity of the response.

To determine whether FoxO inhibition by CnAβ1 can prevent myotube atrophy, C2C12 stable transfectants overexpressing CnAβ*, CnAβ1, or CnAβ2 were differentiated into myotubes for 8 d and then starved for 16 h. As shown in Fig. 6 H and Fig. S3 C, starvation caused a 35–40% decrease in myotube diameter that was prevented by CnAβ1, but not by CnAβ* or CnAβ2. Accordingly, CnAβ1 overexpression repressed FoxO in starving cells (Fig. 6 F) and CnAβ1 knockdown blocked repression of FoxO by IGF-1 (Fig. 6 G). However, CnAβ1 had no effect on a constitutively active FoxO3α-A3 mutant lacking the three Akt/SGK phosphorylation sites (Nakae et al., 2000) (Fig. 6 F), which also renders the transcription factor insensitive to IGF-1 (Zheng et al., 2000; Brunet et al., 2001). Because IGF-1 inhibits FoxO-regulated atrogenes (Sandri et al., 2004; Stitt et al., 2004; and Fig. S3 B) and CnAβ1 expression is induced by IGF-1 in atrophied and regenerating muscle (Dobrowolny et al., 2005; Fig. 2 G, Fig. S2 A), IGF-1 and CnAβ1 may share common pathways for the inhibition of FoxO and FoxO-regulated processes. Interestingly, inhibition of FoxO activity and nuclear localization by CnAβ1 did not require a functional phosphatase domain because a phosphatase-dead CnAβ1 mutant (CnAβ1mut) also reduced FoxO nuclear localization and activation (Fig. 6, D and F). In addition, NFAT-regulated genes were only slightly affected by CnAβ1 knockdown in myoblasts (Fig. 6 B). These results suggest that the main role of CnAβ1 in proliferating myoblasts is to inhibit FoxO, allowing cell proliferation and preventing differentiation, rather than to activate NFAT, which is involved in myoblast differentiation (Delling et al., 2000) after CnAβ1 expression has been shut down.
CnAβ1 enhances skeletal muscle regeneration

Given that CnAβ1 expression is induced by mIGF-1 during skeletal muscle regeneration (Fig. 2 G; Dobrowolny et al., 2005) and is necessary for the activation of the PDK1/Akt signaling pathway that leads to FoxO inhibition, we explored whether CnAβ1 shared some of the regenerative abilities of mIGF-1. We generated MLC/CnAβ1 transgenic mice, expressing CnAβ1 in a skeletal muscle-specific, post-mitotic restricted fashion using a myosin light chain (MLC)1/3 expression cassette (Fig. 7 A), and MLC/CnAα transgenic mice expressing a full-length CnAα construct, including the autoinhibitory domain, as control. DNA microarray-based transcript expression profiling of the quadriceps muscles of wild-type, MLC/CnAα, and MLC/CnAβ1 uninjured mice at 2 mo of age revealed an induction of slow fiber-associated genes including β-myosin heavy chain (Myh7), myosin light chains (Myl3, Myl2), troponins (Tnni1, Tnnt1), tropomyosins (Tpm3), and Serca2a in MLC/CnAβ1 mice compared with wild-type and MLC/CnAα mice (Table S4). Quantitative RT-PCR corroborated the up-regulation of TnI1 (slow) and slight decrease of TnI2 (fast) expression in MLC/CnAβ1 mice compared with wild-type and MLC/CnAα (Fig. 7 B). Moreover, NADH-tetrazolium reductase staining of EDL sections from wild-type and MLC/CnAβ1 mice showed a significant increase in the number of slow fibers in mice overexpressing CnAβ1 (Fig. 7, C and D), and similar results were obtained when the amount of slow MHC-expressing fibers was determined by immunohistochemistry (Fig. 7, E and F).

To determine whether supplemental CnAβ1 transgene expression contributed to muscle regeneration, we injected cardiotoxin into the tibialis anterior (TA) and quadriceps muscles of wild-type (WT), MLC/CnAα, and MLC/CnAβ1 mice and allowed regeneration to proceed for 2, 6, or 12 d (Musarò et al., 2001). Both wild-type and MLC/CnAβ1 mice presented similar muscle damage 2 d after the injury, suggesting analogous susceptibility to the toxin (Fig. 8 A). In contrast, regenerating TA muscle from MLC/CnAβ1 mice 12 d after CTX injury contained larger myotubes with centralized nuclei and less extracellular matrix accumulation when compared with regenerating wild-type muscle (Fig. 8 A). The enhanced regenerative capacity induced by CnAβ1 was also reflected in the increased mean cross-sectional area (CSA) of the MLC/CnAβ1 myofibers 12 d after CTX injection, compared with those of wild-type mice (2.1-fold increase; Fig. 8 B). Interestingly, an increase in the satellite cell markers Myf5 and Pax7 was observed 6 d after the injury in MLC/CnAβ1 mice compared with wild type (Fig. 8, C and D), paralleling the response obtained in response to IGF-1 in a murine model of ALS (Dobrowolny et al., 2005). Unlike mIGF-1 (Musarò et al., 2001), CnAβ1 overexpression did not result in muscle hypertrophy, indicating that the regenerative and hypertrophic responses induced by mIGF-1 in skeletal muscle may be driven by different mechanisms.

CnAβ1 knock-down reduces myoblast proliferation

Figure 5. CnAβ1 knock-down reduces myoblast proliferation. (A and B) C2C12 myoblasts were transfected with control or CnAβ1 siRNAs 132 and 167, grown for 48 h, and mRNA expression was analyzed by microarray (A) and qRT-PCR (B). Gene expression is shown as fold induction ± SD (note log. relative units) of values from cells transfected with each CnAβ1 siRNA over those transfected with control siRNA. (C, D, and F) C2C12 cells transfected with the different siRNAs (C and D) or stably transfected with CnAβ expression plasmids (F) were allowed to grow for 48 h and their proliferative capacity was determined by BrdU incorporation (C and F) and MTT (D) assays. (E) C2C12 cells were transfected as in A, with control or CnAβ1 siRNA, grown for 48 h, and the percentage of cells in the G0/G1 and G2/M phases of the cell cycle was determined by flow cytometry after staining the cells with propidium iodide. *, P < 0.05; **, P < 0.005; ***, P < 0.0005.
To elucidate the mechanisms underlying the enhanced skeletal muscle regeneration observed in MLC/CnAβ1 mice, we analyzed the microarray transcription profiles of wild-type, MLC/CnAβ1, and MLC/CnAα quadriceps during the regeneration process. In CTX-injured wild-type muscle, induction of genes involved in the immune response included chemotaxis and macrophage-specific genes, and extracellular matrix production, especially collagen, paralleled by a strong decrease in the expression of genes associated with the mitochondria and energy production, likely reflecting myocyte necrosis (Fig. 9A; Table S5, available at http://www.jcb.org/cgi/content/full/jcb.200704179/DC1). In contrast, MLC/CnAβ1 muscle significantly decreased the expression of genes associated with both immune response and scar formation 12 d after the muscle injury compared with wild-type mice and showed a faster recovery of mitochondrial gene expression (Fig. 9A). Quantitative RT-PCR analysis of the chemokine macrophage chemoattractant protein (MCP-1), the profibrotic factor TGF-β1, and collagen I confirmed the results seen with the microarrays, with a significant decrease of these factors in MLC/CnAβ1 mice at both 6 and 12 d after the injury, and showed no differences between the two genotypes 2 d after CTX injection (Fig. 9B), confirming that wild-type and MLC/CnAβ1 mice were equally susceptible to the action of cardiotoxin. We quantified the presence of different leukocyte populations during muscle regeneration by qRT-PCR and found decreased expression of the monocytic/macrophage marker CD14 in MLC/CnAβ1 mice compared with wild type 6 and 12 d after the injury (Fig. S4A, available at
This effect was paralleled by a lower degree of endothelial activation (Selectin P expression) and decreased expression of chemokines Ccl2/MCP-1 and Ccl5/Rantes in MLC/CnAβ1 mice (Fig. S4 A and Fig. 9), together with increased expression of the anti-inflammatory cytokine IL-10, an immunosuppressive mediator involved in the resolution of inflammation and the regulation of extracellular matrix. No significant differences were observed in CD4 and CD8 lymphocyte subpopulations between wild-type and transgenic mice, and we did not detect neutrophil markers by microarray or qRT-PCR at the analyzed time points. Together, our results suggest that the negative regulation of macrophage recruitment through Ccl2/MCP-1 and Ccl5/Rantes among others, combined with the secretion of anti-inflammatory mediators such as IL-10, may be responsible for the accelerated resolution of inflammation observed in MLC/CnAβ1 mice.

Notably, regenerating MLC/CnAβ1 muscle showed increased matrix deposition, with small, separated myotubes and persistent presence of infiltrating inflammatory cells (Fig. 10 A) that was reflected in the smaller cross-sectional area presented by the regenerating myofibers (Fig. 10 B) and the increased collagen staining (Fig. 10, C and D). Microarray analysis of MLC/CnAα mice 12 d after CTX injection revealed increased expression levels of genes associated with macrophage presence and extracellular matrix production (Fig. S5, available at http://www.jcb.org/cgi/content/full/jcb.200704179/DC1), indicating enhanced inflammation and fibrosis and supporting the impaired muscle regeneration displayed by these mice at this stage. In summary, the improved muscle regeneration associated with CnAβ1 transgene expression was characterized by enhanced muscle precursor expansion, decreased fibrosis and inflammation, and faster muscle recovery, whereas supplemental expression of CnAα exacerbated rather than improved the regenerative capacity of skeletal muscle.

**Discussion**

This study establishes CnAβ1 as an evolutionarily conserved, partially CsA-insensitive Cn splicing variant that regulates cell...
proliferation and improves muscle regeneration, inhibiting FoxO independently of its phosphatase activity. The unexpected connection between FoxO and calcineurin pathways, specific for the CnAβ1 isoform, may have important implications for the regulation of cell proliferation and for treatment of muscle wasting.

Cn activity is tightly regulated by calcium through CnB and calmodulin. In response to changes in intracellular calcium levels, the autoinhibitory domain of the enzyme is removed and the catalytic site is exposed (Ke and Huai, 2003). The lack of an autoinhibitory domain confers constitutive phosphatase activity and NFAT activation on the CnAβ1 variant in the absence of calcium increase. Although constitutive enzymatic activity has been previously described for artificially truncated CnA forms (Molkentin et al., 1998), CnAβ1 is, to our knowledge, the first naturally occurring CnA isoform with this capacity. Contrary to artificially truncated CnA isoforms, CnAβ1 is partially insensitive to the action of the immunosuppressant CsA, suggesting that the alternative C-terminal domain in CnAβ1 may interfere with the binding of the CsA-calciphilin complex to the enzyme and thus prevents its inactivation. Together, these findings indicate that the Cn/NFAT pathway can be activated without the need for a rise in intracellular calcium levels by alternative splicing of the CnAβ gene, and that this activation is partially resistant to the action of CsA.

The inhibition of FoxO by CnAβ1 has important implications. The family of FoxO transcription factors control key physiological processes, including cell proliferation, growth, atrophy, metabolism, and longevity (Greer and Brunet, 2005). In myoblasts, FoxO is sufficient to induce Gadd45 expression and prevent cell cycle progression (Furukawa-Hibi et al., 2002). The abundant expression of CnAβ1 in proliferating myoblasts is necessary to prevent FoxO activation. Indeed, CnAβ1 knockdown results in the induction of negative cell cycle regulators, blockade of cell proliferation, and subsequent myoblast differentiation. It is noteworthy that C5C12 cells overexpressing CnAβ1 fuse normally into myotubes (Fig. 6 H and Fig. S4 B), suggesting that CnAβ1 expression is necessary for myoblast proliferation rather than for inhibition of differentiation. Interestingly, when overexpressed in transgenic mice, CnAβ1 induces an increase in slow muscle fibers, an effect compatible with both FoxO inhibition and NFAT activation (Naya et al., 2000; Kamei et al., 2004; McCullagh et al., 2004).

FoxO factors play a crucial role in skeletal muscle atrophy through the induction of MAFbx/Atrogin and Murf1.
In response to IGF-1, Akt phosphorylates and inhibits FoxO, preventing Atrogin expression and muscle atrophy (Stitt et al., 2004; Schulze et al., 2005). Interestingly, CnAβ1 is induced by IGF-1 in regenerating muscle and is necessary for the inhibition of FoxO activity by IGF-1 (Fig. 2 G, Fig. 6 G, Fig. S3 A; Dobrowolny et al., 2005). In addition, CnAβ1 itself is able to inhibit FoxO and prevent myotube atrophy induced by starvation. FoxO induction of MAFbx/Atrogin has recently been shown to inhibit Cn activity (Li et al., 2004; Ni et al., 2006); thus, the inhibition of FoxO by CnAβ1 might indirectly contribute to the increased Cn activity in CnAβ1-expressing cells. Like IGF-1, CnAβ1 needs the three Akt target Ser/Thr residues in FoxO in order to inhibit its activity and furthermore, CnAβ1 expression is necessary for PDK1 and Akt activation in both myoblasts and tumor cells (Fig. 4 C and Fig. 6 D), suggesting that IGF-1 and CnAβ1 share a common signaling pathway.

Supplemental expression of CnAβ1 in post-mitotic muscle leads to enhanced muscle regeneration. Our results suggest that the quicker expansion of muscle precursors combined with a faster resolution of both the inflammatory response and subsequent extracellular matrix deposition allows the regenerating fibers to grow more rapidly in response to CnAβ1, whereas mice overexpressing the full-length CnAα isoform show delayed regeneration, with persistent inflammation and increased deposition of extracellular matrix. Interestingly, mIGF-1 enhances muscle regeneration in various models of muscle injury and disease by reducing scar formation and resolving inflammation faster, facilitating the recruitment of stem and progenitor cells (Musarò et al., 2001, 2004; Barton et al., 2002; Dobrowolny et al., 2005). Thus, although the role of Cn in skeletal muscle regeneration remains controversial and both pro- and anti-regenerative effects have been reported depending on the mouse model used (Stupka et al., 2004; Parsons et al., 2007), the present study shows that...
CnAβ1 is able to recapitulate the skeletal muscle regenerative response induced by mIGF-1, probably sharing a common mechanism, suggesting that this naturally occurring Cn isoform could prove a valuable tool in therapies aiming to counteract muscle atrophy and enhance regeneration.

Materials and methods

Cells, plasmids, and transfection

Cells. The murine myoblast cell line C2C12 was propagated in growth medium (GM) [Dulbecco's modified Eagle's medium (DMEM) 4.5 g/liter glucose, 10% FCS, 2 mM L-glutamine, and 5 mM penicillin/streptomycin] and induced to differentiate in differentiation medium (DM) [growth medium, 0% FCS, and 2% horse serum]. C2C12 cells were starved by incubating in PBS, 1 mM CaCl2, and 1 mM MgCl2 for 16 h as described (Sandri et al., 2004). Muscle satellite cells were isolated from rat EDL muscles using the single fiber technique as previously described (Suzuki et al., 2001). HEK293 cells were grown in DMEM, 1 g/liter glucose, 10% FCS, 2 mM L-glutamine, and 5 mM penicillin/streptomycin. Jurkat T cells were grown in RPMI, 10% FCS, 2 mM L-glutamine, and 5 mM penicillin/streptomycin.

Plasmids. The expression vectors pcDNA3-CnAα, pcDNA3-CnAβ1mut, pcDNA3-CnAβ2, pcDNA3-CnAα, pcDNA3.1-CnAα* and pcDNA3.1-CnAβ* carry cDNAs of the respective CnA isoforms under the control of a CMV promoter (CnAα* and CnAβ* represent the artificially truncated forms of CnAα and CnAβ2; CnAβ1mut carries a D130N mutation and lacks phosphatase activity). pGal4-Luc and p6xDBE-Luc express the luciferase reporter gene under the control of three tandem binding sites for the yeast transcription factor Gal4 or six tandem binding sites for the Caenorhabditis elegans FoxO homologue Daf16, respectively. pGal4-NFAT (1–415) expresses a chimerical protein containing the Gal4 DNA-binding domain linked to NFAT transcription activation and regulatory domains (amino acids 1–415) (Luo et al., 1996). pGFP-VIVIT carries the NFAT-specific inhibitory peptide VIVIT linked to GFP (15). pHA-NFAT expresses respectively, a GFP-FoxO3a fusion protein and a FoxO3a mutant in which the three Thr/Ser known to be phosphorylated by Akt and SGK are mutated into Ala, rendering FoxO3a insensitive to inhibition by Akt or IGF-1 (Dijkers et al., 2000; Patente et al., 2005).

Transfection. For NFAT activity analysis, C2C12 myoblasts were transfected for 6 h with 0.5 μg of Gal4-Luc and 1 μg of p6xDBE-Luc along with 3 μg of pcDNA3-CnA and 0.8 μg of pFoxO3a-A3 (or pcDNA3.1 as negative control), following the same protocols as for C2C12 cells. For FoxO activity analysis, C2C12 cells were transfected with 3 μg of p6xDBE-Luc and either 2.5 μg Cn expression vectors and 0.8 μg of pFoxO3a-A3 (or pcDNA3.1 as negative control).
or 100 pmol of CnAβ or CnAβ siRNA. Where indicated, 25 ng/ml IGF-1 was added to the culture for 16 h. IGF-1 and cyclosporin a (CsA) were purchased from Sigma-Aldrich. Similar expression levels of all CnA isoforms after transfection were detected by qRT-PCR (Fig. S3). Stable C2C12 transfectants bearing the pcDNA3.1-CnA construct or the empty pcDNA3.1 vector were generated by selecting the cells with the 0.5 mg/ml G418 after transfection. Two different polyclonal populations of transfectants were used in the functional assays. The transfectants were generated by using similar levels of the transcribed CnAβ isoform, as quantified by qRT-PCR (Fig. S3 B).

NADH staining, immunohistochemistry, muscle injury, and denervation
Nicotinamide adenine dinucleotide nitro-blue tetrazolium (NADH)-TR staining was used to detect atrophy, intermediate, and slow fibers. Mice were killed by cervical dislocation and muscles were removed as fast as possible, immersed in Traguecanth gum (Sigma-Aldrich), and frozen in isopentane-liquid nitrogen. Frozen muscle sections (8 μm) were incubated with 4% PFA for 10 min on ice. Sections were then incubated in PBT buffer for 15 min (0.3% Tween 20 in PBS) and blocked in 5% goat serum in PBS for 1 h. Primary antibodies were applied over night at 4°C in a 1:400 dilution (Anti-Myosin Skeletal, fast, Clone MY-32; Anti-Myosin Skeletal, slow, Clone NOG7.5.4D; both from Sigma-Aldrich). Secondary antibodies were used 200 times for 15 min in PBS buffer and incubated with goat anti-rabbit-AlexaFluor488 secondary antibody for 45 min at RT, washed three times for 15 min in PBS and once in PBS containing 1X Hoechst. Images were analyzed using a microscope (Axiovert 200; Carl Zeiss, Inc.) with an Acroplan 10×/0.25 Ph1 or –/– objective at room temperature, using 70% glycerol/Tris-HCl as imaging medium. Pictures were captured using a digital camera (DAX 1200; Nikon) and acquisition software from Nikon (ACT-1, ver. 2). Brightness/contrast enhancement was applied to the whole picture at the same time using Adobe Photoshop.

For skeletal muscle regeneration experiments, tubulin anterior (TA) and quadriceps muscles from 2-mo MLC/CnAβ, MLC/CnAα or wildtype mice were given five [TA] or eight (quadriceps) 5-μm injections of 10 μg cardiotoxin in PBS (Musrá et al., 2001). Quadriceps muscles were collected 2, 6, and 24 h after the injections. TA sections were incubated with anti-CnAβ-AlexaFluor488 secondary antibody and counterstained with hematoxylin and eosin. For collagen staining, TA sections were stained with the Trichrome Stain (Masson) kit following the manufacturer’s recommendations. All images were acquired using a microscope [Axioplan; Carl Zeiss, Inc.] and a digital camera (DAX 1200F; Nikon) and analyzed using NIH Image software (ACT-1, ver. 2.62). Brightness/contrast enhancement was applied to the whole picture at the same time using Adobe Photoshop. All experiments involving animals were approved by the EMBL Animal Ethics Committee and were performed according to local and institutional guidelines.

siRNA, RNA isolation, cDNAs, Northern blot, and real-time PCR
The following stealth siRNAs (Invitrogen) were used: mouse CnAβ 132 (CGUAGGUGAAGAGUCAGACUCA), mouse CnAβ 167 (GGUGA-UUAUGAGUAUAUGCUCA), human CnAβ 116 (GGUAUAUGAUGAGCUAGUCUCA), human CnAβ 247 (GAUAUGGAGAAGGCUGUCUAUCAUA), mouse and human CnAβ 246 (GACUGGAACAUAGUGCCAGCAGCAGCAGU), and Luciferase, used as a negative control (GGCGCGGAGAGAGAUUUAUGAGUAUUAUGAGUAG). Total RNA was extracted from snap-frozen tissue or cell cultures by using Trizol (Sigma-Aldrich). 20 μg total RNA were resuspended in formamide, mixed and hybridized with CnAβ, CnAα, and CnAβ-1-specific probes. For real-time PCR analysis, cDNA was generated using a Roche kit from 2 μg of total RNA in a 40-μl volume. Human tumor cDNA (matching tumor and nontumor samples from the same patient) from five lung tumor patients and five colon tumor patients was purchased from Clontech Laboratories, Inc.

A total of 2 μl of the cDNA reaction were amplified by PCR in duplicate using either Taqman probes (Applied Biosystems) or a SYBR green master kit (Finnzymes), and the following annealing temperatures and primers:
- CnAβ 1, 57°C (fwd-GAGAAGAAGGCAAGAGTCATCA-ATCAT), CnAβ 58°C (fwd-AGGCTATGACCCGCTAGA, rev-GGATCTCA- GAAAACG), CnAα, 57°C (fwd-CAAGGCGGGTACCCGTA), Rev-GGATCGCCCTCTGTA), CnB 1 (fwd-5′ATGCCAAGAAAGGGG3′, 3′-G5′- GCAAAGTGGTGGG′-3′), ubiquitin B, 58°C (fwd-5′TGCGCTCATCTT- GGCTCAGG, rev-GGAAGCAAGCTGAGCAAAAG), Tn, slow, 55°C (fwd-5′TGCTGAGGCATCCTGCTA-G3′, rev-5′GAACACTTCTGCGA- CTTCTG3′), Tn, fast, 55°C (fwd-5′GAAGGAGGACTACCTCGTCAGA′-3′, rev-5′GGCGCATGCTAGCTAGCT3′). MAFbx/Atrogin primers have been previously described (Mourkioti et al., 2006). Data were normalized with ubiquitin B values and expressed as fold induction over a control sample.

Microarray analysis and sequence comparison
Total RNA from the different samples was hybridized in triplicate on Affymetrix mouse MG 430A2.0 or the human HGU133A2.0 oligonucleotide-based arrays. Raw data were normalized and analyzed using GeneSpring-7.2 (Silicon Genetics). After chip normalization to the 50th percentile, samples were normalized to cells transfected with control siRNA and ANOVA statistical analysis was performed. For the analysis of cardio- toxin-injured mice, statistically significant genes were subjected to gene tree hierarchical clustering and lists of up-regulated and down-regulated genes were compared with annotated gene ontology lists, choosing those lists with at least 10 genes in common and a P value < 10−11 (wild type) or P < 10−6 (MLC-CnAβ1). The average of the relative gene expression variation of the genes in these lists (mitochondria, 66 genes; immune response, 261 genes, macrophage-associated, 6 genes; extracellular matrix, 164 genes; collagen, 26 genes; complete gene lists available upon request) in the different experimental conditions was calculated and expressed as fold induction over the value of the uninjured wild-type mice. Intrinsic sequences obtained from Ensemble (www.ensemble.org) were translated using the Expsay translation engine (www.expsay.org) and compared using Clustal W (www.ebi.ac.uk/clustalw).

Proliferation analysis
Cell proliferation was quantified using the MIT cell proliferation assay (Sigma-Aldrich) 0 and 48 h after cell transfection. BrdU incorporation was measured 0 and 48 h after transfection with the different siRNAs by using the BrdU ELISA Assay Kit (Roche) following the manufacturer’s instructions. Cell cycle analysis was performed 48 h after transfection of C2C12 myoblasts with the different siRNAs by fixing the cells in 70% ethanol for 2 h, staining the nuclei with 20 μg/ml propidium iodide and analyzing them in a flow cytometer (Beckman Coulter).

Western blots and EUSA
Western blots were performed as previously described [Lara-Pezzi et al., 2009]. Antibodies against CnAβ1 were generated using the CGMDVGIPHSFANNSNA peptide as an immunogen. Antibodies against CnAα2, myogenin, and p53 were obtained from Santa Cruz Biotechnology, Inc., or served as a negative control (GGCGCGGA- CGAGAUUUAUGAGUAG). Total RNA was extracted from snap-frozen tissue or cell cultures by using Trizol (Sigma-Aldrich). 20 μg total RNA were resuspended in agarose gel, blotted and hybridized with CnAα2, CnAβ2, and CnAβ-1-specific probes. For real-time PCR analysis, cDNA was generated using a Roche kit from 2 μg of total RNA in a 40-μl volume. Human tumor cDNA (matching tumor and nontumor samples from the same patient) from five lung tumor patients and five colon tumor patients was purchased from Clontech Laboratories, Inc.

Fig. S1 includes a schematic representation of the functional domains of the different Cn isoforms and shows that the Cterminal region of CnAβ1 is conserved among vertebrates. It also illustrates how the Gal4 luciferase system works and shows that both CnAβ1 and the artificially truncated CnAβ* form are constitutively able to activate NFAT-dependent transcription in C2C12 myoblasts, but not in Jurkat. Fig. S2 shows that IGF-1 induces...
CnAβ1 expression in starving myotubes and shares some targets with CnAβ1. It also demonstrates that CcC12 stable transfectants overexpress the different CnAβ isoforms at similar levels and that CnAβ1 does not interfere with myotube differentiation. Fig. S3 shows GFP-FoxO3a localization in the presence of the different CnAβ isoforms and includes phase-contrast pictures corresponding to Figs. 6 H, Fig. S4 analyzes the presence of different leukocyte subpopulations and the expression of FoxO and NFκB, targets in regenerating muscle in both wild-type and MLC/CnAβ1 mice. The supplementary tables include lists of genes generated during the different microarray analyses. Online supplemental material is available at http://www.jcb.org/cgi/content/full/jcb.200704179/DC1.


References


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