

Notch signaling imposes two distinct blocks in the differentiation of C2C12 myoblasts

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SUMMARY

Notch signal transduction regulates expression of downstream genes through the activation of the DNA-binding protein Su(H)/CBF1. In *Drosophila* most of Notch signaling requires Su(H); however, some Notch-dependent processes occur in the absence of Su(H) suggesting that Notch signaling does not always involve activation of this factor. Using constitutively active forms of Notch lacking CBF1-interacting sequences we identified a Notch signaling pathway that inhibits myogenic differentiation of C2C12 myoblasts in the absence of CBF1 activation. Here we show that ligand-induced Notch signaling suppresses myogenesis in C2C12 myoblasts that express a dominant negative form of CBF1, providing additional evidence for CBF1-independent Notch signal transduction. Surprisingly

mutant forms of Notch deficient in CBF1 activation are unable to antagonize MyoD activity, despite the fact that they inhibit myogenesis. Moreover, Notch-induced antagonism of MyoD requires CBF1 suggesting that the CBF1-dependent pathway mediates a cell-type-specific block in the myogenic program. However, Notch signaling in the absence of CBF1 activation blocks both myogenesis and osteogenesis, indicative of a general block in cellular differentiation. Taken together our data provide evidence for two distinct Notch signaling pathways that function to block differentiation at separate steps during the process of myogenesis in C2C12 myoblasts.

Key words: Notch, CBF1, MyoD, Myogenesis, Osteogenesis

INTRODUCTION

During the development of multicellular organisms, numerous local cell-cell interactions are required for proper cell fate specification of many different cell types and tissues. A family of molecules involved in these types of localized interactions is the Notch family of cell surface receptors (Artavanis-Tsakonas et al., 1995; Weinmaster, 1997). Notch activity is required in a wide variety of biological processes in animals ranging from worms to humans. Although the Notch neurogenic phenotype first described in *Drosophila* has been well characterized, both loss-of-function and gain-of-function studies have indicated roles for Notch in a number of developmental processes in addition to neurogenesis. As with invertebrates, the mutant phenotypes and expression patterns of the related vertebrate Notch genes imply that they are essential for a diverse array of developmental events. In fact, Notch signaling has been implicated in various processes from cell fate decisions, tissue patterning and morphogenesis, to inherited human diseases and cancer (Gridley, 1997). This observed pleiotropic activity of Notch raises the question: How does Notch signaling regulate the development of so many different cell types, tissues and structures?

In *Drosophila*, lateral inhibition mediated by the Notch

signaling pathway regulates both the selection of muscle progenitors from the mesoderm and neural progenitors from the neural ectoderm (Jan and Jan, 1993). Overexpression studies using constitutively active forms of Notch have shown that Notch signaling can inhibit cellular differentiation during myogenesis (Kato et al., 1997; Kopan et al., 1994; Luo et al., 1997; Shawber et al., 1996) and neurogenesis (Chitnis, 1995; Dorsky et al., 1997; Henrique et al., 1997; Lardelli et al., 1996; Nye et al., 1994), suggesting that the molecular mechanisms of Notch-mediated inhibition during these two different processes may be similar. While the effects of Notch signal transduction on cell fate decisions have been well studied both in vitro and in vivo, the molecular mechanisms of Notch-mediated inhibition of cellular differentiation are not well understood. Genetic and molecular studies have implicated several downstream components in the Notch signaling pathway, such as Suppressor of Hairless [Su(H)] in *Drosophila* (Fortini and Artavanis-Tsakonas, 1994) and its homologs in *Xenopus* [Su(H)] (Wettstein et al., 1997), mammals (RBPJk/CBF1/KBF2; hereafter referred to as CBF1) (Honjo, 1996) and *C. elegans* (LAG-1) (Christensen et al., 1996). These proteins share striking sequence conservation and are collectively known as the CSL proteins for CBF1, Su(H), and LAG-1 (Christensen et al., 1996). The CSL proteins bind DNA

and function as transcriptional repressors, but through direct interactions with the cytoplasmic domain of Notch these proteins are converted into transcriptional activators providing a molecular mechanism for CSL-mediated Notch signal transduction (Hsieh and Hayward, 1995; Hsieh et al., 1996).

Activation of CSL proteins by Notch signaling results in the positive regulation of the bHLH proteins encoded by the *Drosophila* Enhancer of split [E(spl)] gene complex and its homologous vertebrate genes ESR1, HES-1 and HES-5, which contain CBF1/Su(H)-binding sites in their regulatory regions (Bailey and Posakony, 1995; de la Pompa et al., 1997; Hsieh et al., 1996, 1997; Jarriault et al., 1995; Kageyama and Nakanishi, 1997; Lecourtois and Schweisguth, 1995; Wettstein et al., 1997). Notch signaling is thought to inhibit neurogenesis through activation of CSL proteins and the subsequent upregulation of genes within the E(spl)-C, and the homologs in vertebrates, HES-1 and HES-5 (Artavanis-Tsakonas et al., 1995; Kageyama and Nakanishi, 1997). The E(spl)/HES proteins can antagonize neural bHLH activators, which may account for the observed suppression of neurogenic differentiation (Kageyama and Nakanishi, 1997; Lewis, 1996). Whether this same molecular pathway involving CSL family members also functions in Notch-mediated inhibition of bHLH myogenic factors required for myogenesis remains to be determined.

We and others have shown that Notch signaling can prevent muscle cell differentiation (Kato et al., 1997; Kopan et al., 1994; Lindsell et al., 1995; Shawber et al., 1996). Members of the MyoD family of myogenic factors, such as MyoD, Myf-5, MRF4 and myogenin, are bHLH proteins that specify muscle cell fate by inducing the expression of muscle specific genes (Weintraub, 1993). Since both HES-1 and activated forms of Notch containing CBF1-interacting sequences can block MyoD-induced myogenesis it has been proposed that, like neurogenesis (Lewis, 1996), Notch signaling inhibits muscle cell differentiation through activation of CBF1 and the subsequent upregulation of HES-1 (Jarriault et al., 1995). However, even though expression of certain constitutively active forms of Notch activate CBF1 to transactivate HES-1 (Hsieh et al., 1997), we have previously reported that Notch activation of CBF1 is not necessary for Notch-induced repression of myogenesis in C2C12 myoblasts (Shawber et al., 1996). In addition, we showed that overexpression of HES-1 in C2C12 cells does not prevent muscle cell differentiation. To confirm and extend these studies we report here that, even in the presence of a dominant negative form of X-Su(H) that functions to repress Notch activation of CBF1, Jagged1-induced Notch signaling inhibits muscle differentiation. These data provide additional support that Notch signal transduction can inhibit myogenesis through a CBF1-independent pathway. Importantly, our studies have suggested that activation of Notch induces two distinct blocks in muscle differentiation, indicating that Notch signal transduction is more complex than previously thought (Honjo, 1996). The first Notch-induced block occurs independently of CBF1 activation and inhibition of MyoD function. However, the second block induced through Notch signaling appears cell-type specific, functioning in the case of myogenesis to inhibit MyoD activity and this inhibition of MyoD requires CBF1 activity.

In summary, our data identify a role for CBF1-independent

signaling in a general block in cellular differentiation, while the CBF1-dependent Notch signaling pathway appears to impose a cell-type-specific block. Thus as Notch activation blocks the activity of neural-specific transcription factors during neurogenesis (Lewis, 1996), Notch signaling through the CBF1-dependent pathway would function to inhibit MyoD during myogenesis. However, it is important to note that the CBF1-dependent antagonism of MyoD induced by Notch signaling in C2C12 myoblasts is not mediated by HES-1. Although the cellular targets of CBF1-independent Notch signaling are unknown, this pathway blocks both myogenesis and osteogenesis which may account for the observed Notch-mediated repression in differentiation of many different cell types. The second CBF1-dependent block would then function in a cell-type-specific manner to reinforce the initial Notch-induced block in cellular differentiation.

MATERIALS AND METHODS

Constructs, transfections and cell culture

The following wild-type and mutant Notch1 and Notch2 cDNA sequences were engineered in the mammalian expression vector pEF1 α -BOS (Mizushima and Nagata, 1990) to encode the amino acids indicated in Fig. 1A (GenBank accession numbers for rat Notch1, X57405 and rat Notch2, M93661). All constructs were confirmed by DNA sequencing and the details of these constructions are available upon request.

C2C12 mouse myoblasts (ATCC) were cotransfected with the constructs described above and the neomycin resistance gene. Stable expressing cell lines were selected with 400 μ g/ml G418 (GIBCO/BRL) and expression levels were determined by immunofluorescence, northern and western analyses as previously described (Shawber et al., 1996). The Jagged1-expressing Ltk⁻ fibroblast cell line (J1) has been previously reported (Lindsell et al., 1995). To control for clonal variation, a number of independent cell lines were developed and examined in this study; data from at least one representative clone is presented for each different expressing cell type.

For expression of the dominant negative X-Su(H) the various clonal C2C12 cells were transfected using Lipofectamine (GIBCO/BRL) containing 3 μ g of either parental pCS2 or pCS2-DBM X-Su(H) plasmids (a generous gift from D. Wettstein and C. Kintner, Salk Institute) as described below for the transactivation assays.

Cells were cultured in growth medium consisting of Dulbecco's Modified Eagle Medium (DMEM; GIBCO/BRL) supplemented with 10% fetal bovine serum (FBS) and 5% Cosmic Calf serum (CCS; HyClone). The C2C12 cell fusion and coculture assays have been described in detail previously (Lindsell et al., 1995; Shawber et al., 1996). Muscle cell differentiation was induced by culturing cells in DMEM containing 10% horse serum (HS).

Northern blot analysis

RNA isolation and northern blot analysis was performed as previously described (Lindsell et al., 1995; Shawber et al., 1996). After electrophoresis and transfer to nylon membrane (MSI), RNA (10 μ g) was stained with methylene blue to verify equal transfer of RNA. The myogenin probe corresponds to the 3' untranslated region of the mRNA from nucleotides 791-1486, the MLC2 probe was a 700 bp *EcoRI* fragment released from pV2LC2, the osteocalcin probe encodes a *HaeIII/EcoRI* 357 bp fragment of mouse osteocalcin cDNA (kindly provided by A. J. Celeste, Genetics Institute), the Id-1 probe contained a 900 bp *XbaI* fragment excised from PBK/RSV-ID1, and the DN X-Su(H) probe contained the *NotI* 2.5 kb fragment released from pCS2-DBM X-Su(H) (Wettstein et al., 1997).

Western blot analysis

C2C12 cells and stable Notch-expressing C2C12 cells (N1, nCDN1, CDN1, CDCN1T, N2 and CDN2) were grown in 100 mm dishes, washed twice with PBS and lysed in 500 μ l hot SDS sample buffer (Shawber et al., 1996). Specific proteins were identified following SDS/PAGE, transfer to Immobilon-P (Millipore), probing with 5261 (1: 5000), 93-4 (1: 5000) or 12CA5 (1: 1000) and detection using ECLTM (Amersham). Membranes were exposed to BIOMAX film (Kodak) and the resulting images were scanned using ScanMaker III (Microtek) and reproduced for publication using Photoshop (Adobe) software. Expression of the dominant negative X-Su(H) in transfected cells was detected using the 9E10 monoclonal antibody that recognizes the myc-tagged protein as previously described (Wettstein et al., 1997).

CBF1 transactivation assays

The different cell types analyzed were plated in 60 mm dishes at 70% confluency and cotransfected the following day using Lipofectamine (GIBCO/BRL) containing 3 μ g of the indicated construct plus 3 μ g of 4xwtCBF1Luc plasmid. In addition, 100 ng β -gal expression vector was included in the transfection mix to control for differences in transfection efficiency. DNA in a total volume of 300 μ l per plate of DMEM (no serum or penicillin/streptomycin) was combined with 10 μ l Lipofectamine and incubated for 45 minutes at room temperature. Cell monolayers were washed in DMEM and the DNA/Lipofectamine mix was added to the cells which were then incubated at 37°C in 5% CO₂ for an additional 5 hours. Following this incubation, 3 ml DMEM 20% FBS (no penicillin/streptomycin) was added to the monolayers, and 48 hours post-transfection the cells were lysed and collected in 250 μ l of reporter lysis buffer (Promega) and 20 μ l of lysate was assayed in a luminometer (Analytical Luminescence Lab).

Retroviral infections

Mouse MyoD cDNA sequences were subcloned into pSR α MSVtkneo at the *Eco*RI site to allow production of infectious virus (Muller et al., 1991) and BMP-2 was expressed from the pBABE retrovirus (Morgenstern and Land, 1990). To generate infectious virus, HEK-293T cells plated in 100 mm dishes were cotransfected with 10 μ g of the particular viral construct and 10 μ g of the ecotropic helper virus template pSV- Ψ E-MLV, by calcium phosphate transfection. 24 hours post-transfection, the cell medium was changed to 5 ml DMEM 10% FBS and the cells were incubated overnight. The next day conditioned medium was collected 3 times, each time replacing the medium with 3 ml DMEM 10% FBS. The viral supernatant was pooled, cleared by low speed centrifugation and stored in aliquots at -80°C. The viral supernatant titers were determined using infection of Rat-2 cells followed by selection in G418 for pSR α MSVtkneo encoding viruses and puromycin for pBABE encoding viruses, to determine the amount of virus to use to achieve a multiplicity of infection (MOI) of 1.0 to the cell monolayers. 24 hours post-infection the C2C12 cells were induced to differentiate for 2 or 3 days and the cells were then harvested for RNA and protein analyses.

RESULTS

Identification of mutant forms of Notch that block muscle cell differentiation but do not activate CBF1 either transiently in 3T3 fibroblasts or in stable C2C12 cell lines

Mutant forms of the different Notch genes, which lack most or all of the extracellular and transmembrane domains induce Notch signal transduction both in vertebrate and invertebrate species (Weinmaster, 1997). It is thought that ligand-independent activation of Notch signaling in cells involves the

derepression and activation of the CSL proteins. Further clues as to how Notch activates CBF1 have come from a yeast-two-hybrid screen for proteins that interact with mouse CBF1, in which a cDNA clone (mRAM23) encoding a short intracellular region of mouse Notch1 protein was isolated (Tamura et al., 1995). This CBF1 binding domain, designated the RAM domain, consists of approximately 118 amino acids immediately downstream of the transmembrane domain and does not contain the ankyrin repeats (ANK) or any other known motifs (Fig. 1A). CSL activation appears to require direct interactions between the particular CSL protein and Notch RAM domain sequences (Honjo, 1996). Although other sequences in Notch may facilitate its interaction with CSL proteins, it is the RAM domain, and specifically the 1a sequences that promote the strongest interaction between Notch and CBF1 (Hsieh et al., 1997). Even though a strong requirement for the CSL protein, Su(H), has been demonstrated during neurogenesis both in *Drosophila* and *Xenopus* (Artavanis-Tsakonas et al., 1995; Lewis, 1996; Weinmaster, 1997), we and others have reported Notch signaling in the absence of, or activation of, CSL proteins (Christensen et al., 1996; Lecourtois and Schweisguth, 1995; Ligoxygakis et al., 1998; Shawber et al., 1996; Wang et al., 1997). However, despite these reports, Notch signaling independently of CSL proteins has remained controversial. Therefore, we have reexamined the role of CBF1 in Notch-induced inhibition of muscle cell differentiation using cDNA constructs encoding various forms of Notch1 or Notch2 that were specifically designed to contain different portions of the RAM domain.

Truncated versions of Notch1 (FCDN1, nCDN1, CDN1 and CDCN1T) and Notch2 (CDN2) encode mutant cytoplasmic forms that lack the extracellular and the transmembrane domains (Fig. 1A). These constructs were engineered to encode either the complete RAM domain (FCDN1) or in the case of nCDN1 and CDN2 (Hsieh et al., 1997), a portion of the RAM domain, specifically domain 1b, while lacking domain 1a (Fig. 1A). Domain 1b has been shown to interact with CBF1, however, this interaction is weaker than that observed between domain 1a and CBF1 (Hsieh et al., 1996, 1997). The CDN1 construct initiates 17 amino acids upstream of the ANK domain and therefore lacks most of the RAM sequences necessary for productive CBF1 interactions (Shawber et al., 1996). The CDCN1T construct encodes only the six cdc10/ankyrin repeats fused to a triple tandem repeat of the HA epitope tag and thus produces a protein that is completely devoid of RAM domain sequences (Shawber et al., 1996).

We have previously reported that stable C2C12 cell lines expressing either CDN1, CDCN1T or CDN2 are inhibited in muscle cell differentiation (Hsieh et al., 1997; Shawber et al., 1996) and here we show that C2C12 myoblasts expressing nCDN1 are also unable to fuse and do not express the muscle structural gene encoding myosin light chain2 (MLC2) when induced to differentiate (Figs 3 and 5). These data suggest that the RAM domain is not required for Notch-induced inhibition of myogenesis. Moreover, loss of the RAM domain disrupts Notch-induced activation of CBF1 in HeLa cells suggesting that Notch signaling independently of CBF1 activation can prevent myogenesis (Shawber et al., 1996). It has been suggested by Honjo and coworkers that the lack of transactivation detected in this study may be due to the

particular cell line used to assay Notch-induced activation of CBF1 (Kato et al., 1997). Therefore, to further investigate the relationship between Notch-induced inhibition of muscle cell

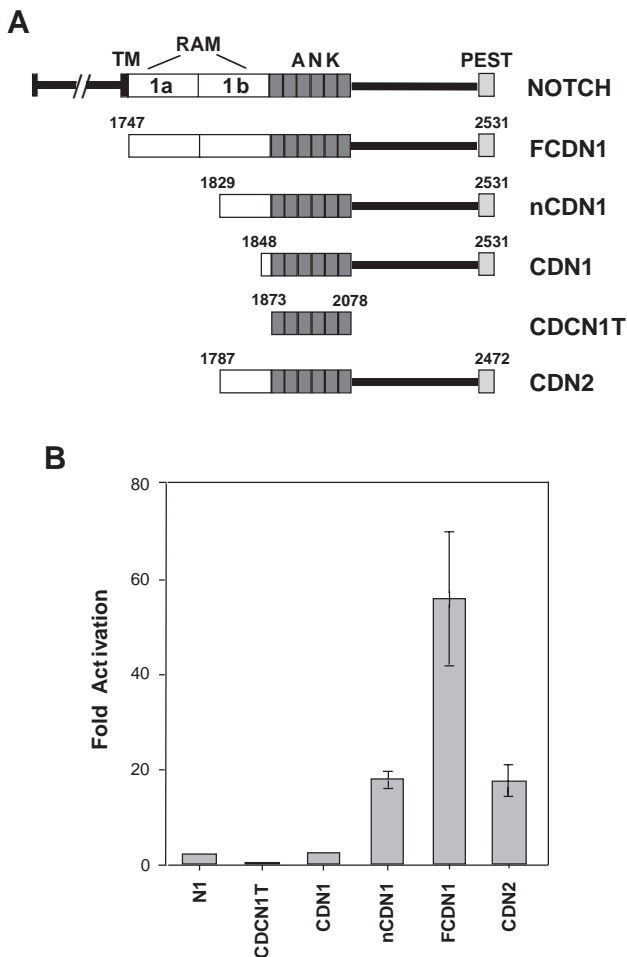


Fig. 1. Constructs encoding structurally different cytoplasmic domains of Notch1 and Notch2 differ in their ability to activate endogenous CBF1 in 3T3 fibroblasts. (A) Schematic representation of mutant Notch1 and Notch2 proteins indicating the structural motifs encoded by the cytoplasmic domains of the different constructs used in this study. Interactions between CBF1 and Notch1 require sequences (RAM; 1a and 1b) located between the transmembrane domain (TM) and the ankyrin repeats (ANK). The Notch proteins terminate with a PEST sequence rich in proline, glutamic acid, serine and threonine. The different structural domains have not been drawn to scale to indicate more clearly the sequence differences between the forms of Notch1 and Notch2 examined. The specific amino acids encoded by these Notch1 and Notch2 constructs are indicated. (B) Transient expression assays with 3T3 fibroblasts were performed to determine if N1, CDCN1T, CDN1, nCDN1, FCDN1 or CDN2 expressed in pBOS-EF1 α could activate endogenous CBF1 to transactivate a luciferase reporter construct carrying four wild-type CBF1 binding sites (Hsieh et al., 1996). 3T3 fibroblasts were cotransfected with either 3 μ g of pBOS-EF1 α (vector) or 3 μ g of the pBOS-EF1 α Notch1 and Notch2 constructs along with 3 μ g of 4XwtCBF1Luc plasmids using Lipofectamine. Cell lysates were harvested 48 hours post-transfection and assayed for luciferase activity. The activity measured is expressed as the fold increase relative to the activity detected with vector DNA and the mean and standard deviation from three independent experiments are presented.

differentiation and transactivation of reporter constructs by CBF1, we have assayed the degree of transactivation induced by the structurally different constitutively active Notch proteins in two additional cell lines: (1) 3T3 fibroblasts and (2) stable C2C12 cell lines expressing the Notch proteins depicted in Fig. 1A. These cell types were examined because previous studies have shown that constitutively active forms of Notch encoding the entire RAM domain activate endogenous 3T3 CBF1 as well as inhibit MyoD-induced myogenic conversion of 3T3 fibroblasts (Kopan et al., 1994; Schroeter, 1998). More importantly, we wanted to determine the level of CBF1 activity in the cells that actually display Notch-mediated inhibition of cellular differentiation.

To assay CBF1 transactivation in 3T3 cells, truncated, cytoplasmic forms of Notch1 or Notch2 were transiently coexpressed with a luciferase reporter construct containing four upstream copies of CBF1 binding sites (4xwtCBF1Luc) (Hsieh et al., 1996, 1997; Shawber et al., 1996). The FCDN1 construct, which encodes the entire RAM domain induced approximately 60-fold activation of this reporter construct (Fig. 1B) consistent with previous reports that structurally similar forms of Notch interact with CBF1 (Honjo, 1996). When either nCDN1 or CDN2 were transiently coexpressed with the CBF1 reporter construct approximately 20-fold transactivation was detected (Fig. 1B). The intermediate level of transactivation measured for nCDN1 or CDN2, which both contain only domain 1b of the RAM domain, is consistent with previous reports that domain 1b has a weaker, though significant, interaction with CBF1 (Hsieh et al., 1996, 1997). However, as we have previously reported for CBF1 transactivation in HeLa cells (Shawber et al., 1996), CDN1 and CDCN1T, which do not contain sequences that promote strong interactions with CBF1, both transactivated the CBF1-reporter to the same background level produced by full-length Notch (N1) (Fig. 1B). These results with 3T3 fibroblasts confirm our previous reported data with HeLa cells, and support reports that the RAM domain is necessary for productive interactions between the cytoplasmic domain of Notch and CBF1 (Honjo, 1996).

In order to determine the level of Notch-induced activation of CBF1 in cells that are inhibited in differentiation, C2C12 cells expressing the various activated forms of Notch1 or Notch2 were transfected with 4xwtCBF1Luc and assayed for their ability to transactivate the CBF1 reporter construct (Fig. 2A). nCDN1- and CDN2-expressing cells increased expression from the reporter construct approximately 17-fold and 26-fold respectively, compared to the activity detected in parental C2C12 cells. Again, as found with HeLa cells and 3T3 fibroblasts, CDN1 and CDCN1T did not effectively transactivate the reporter construct containing CBF1-binding sites, presumably because they do not contain the RAM domain. These results confirm our previous report that CBF1 activity is not necessary for Notch-mediated inhibition of muscle cell differentiation and that CDN1 and CDCN1T function to inhibit muscle cell differentiation in C2C12 myoblasts independently of CBF1.

A potent activator of CBF1 is functional in stable CDN1 and CDCN1T-expressing C2C12 cell lines

Since the CDN1 and CDCN1T-expressing C2C12 cell lines tested in the CBF1 transactivation assay are stable clonal isolates, we wanted to verify that the lack of CBF1 activity

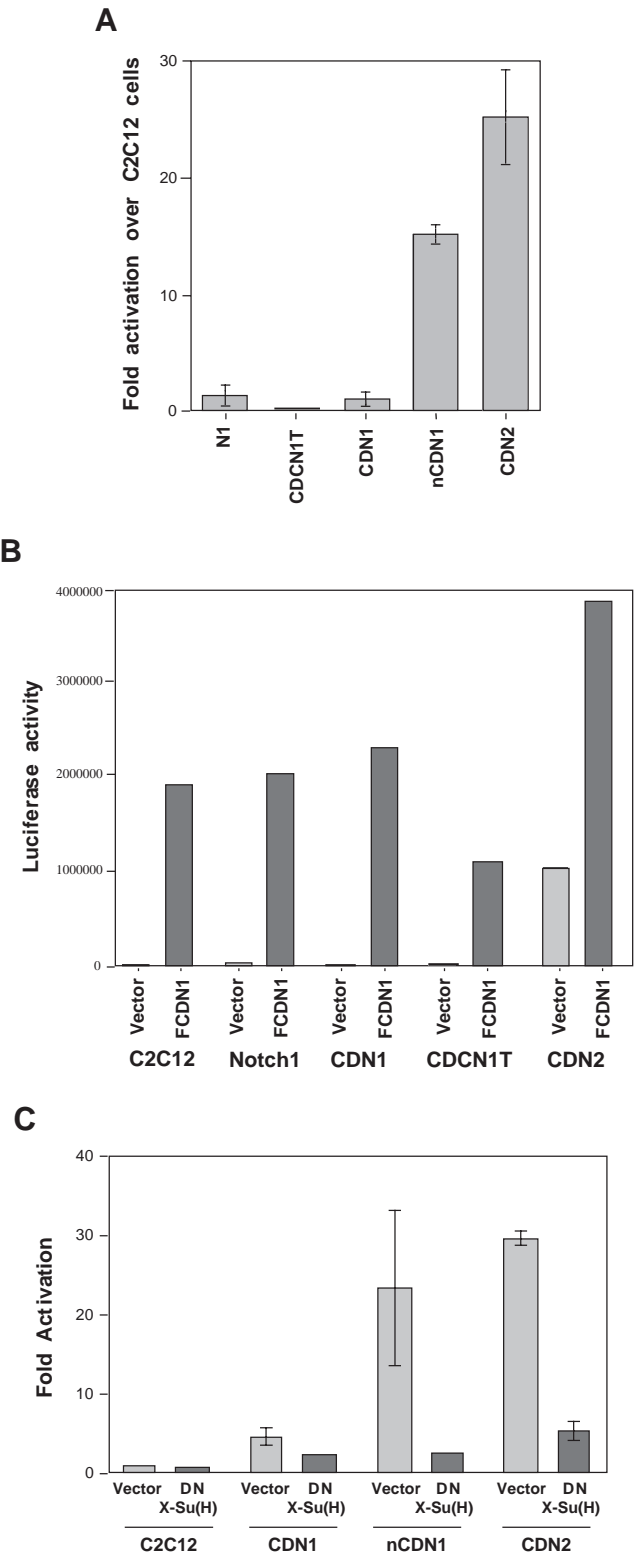
detected represents the inability of CDN1 and CDCN1T to interact with CBF1, rather than clonal defects in expression and/or function of CBF1. Therefore a potent activator of CBF1 (FCDN1) that contains the entire RAM domain (Kato et al., 1997) was transiently expressed in stable cell lines expressing the various activated forms of Notch1 or Notch2 (Fig. 1A). When FCDN1 was coexpressed with the CBF1 reporter construct in stable cell lines expressing activated forms of Notch the level of expression from the reporter construct was greatly increased (Fig. 2B). These results indicate that while CDN1- and CDCN1T-expressing cells have the ability to activate CBF1 when expressing a potent activator of CBF1 (FCDN1), CDN1 or CDCN1T on their own cannot transactivate the reporter construct. Moreover, the deficiency in CBF1 activation exhibited by these constitutively active forms of Notch1 maps to the RAM domain.

Loss of CBF1 activation does not diminish Notch-induced repression of myogenesis

Through the analysis of the constitutively active forms of Notch, CDN1 and CDCN1T, which do not activate CBF1, we were able to identify a second pathway of Notch signal transduction that functions independently of CBF1 to suppress muscle cell differentiation (Shawber et al., 1996). These forms are deficient in CBF1 activation because they do not contain the RAM sequences required for productive Notch-CBF1 interactions. To further demonstrate that Notch signaling inhibits muscle cell differentiation independently of CBF1 we sought to examine the effect of Notch signaling on myogenesis when Notch activation of CBF1 was specifically blocked in myoblasts expressing forms of Notch (nCDN1 and CDN2) that activate CBF1 (Fig. 2A). To do this we made use of a DNA-binding mutant of the *Xenopus* homolog of Su(H), called X-Su(H)^{DBM} (for DNA binding mutant) which is unable to bind DNA but still interacts with Notch (Wettstein et al., 1997). X-

Fig. 2. Activated forms of Notch1 and Notch2 expressed in C2C12 myoblasts differ in their ability to activate endogenous CBF1. (A) Stable C2C12 cell lines expressing either full-length Notch1 (N1), CDCN1T, CDN1, nCDN1 or CDN2 were tested for their ability to activate endogenous CBF1 to transactivate a luciferase reporter construct carrying four wild-type CBF1 binding sites (Hsieh et al., 1996). These stable cell lines were transfected with 3 μ g of the 4XwtCBF1Luc plasmid using Lipofectamine and cell lysates were harvested 48 hours post-transfection and assayed for luciferase activity. The activity measured is expressed as the fold activation relative to the activity detected in the parental C2C12 cells and the mean and standard deviation from three independent experiments are presented. (B) Transient expression of the FCDN1 protein in parental or stable C2C12 cell lines expressing either full-length Notch1, CDN1, CDCN1T, or CDN2 activates endogenous CBF1. The indicated C2C12 cells were cotransfected with either 3 μ g of pBOS-EF1 α (Vector) or 3 μ g of pBOS-EF1 α -FCDN1 plus 3 μ g of the 4XwtCBF1Luc plasmid using Lipofectamine and processed for luciferase activity as described above. The data points represent the average obtained from two independent experiments. (C) Expression of a dominant negative form of X-Su(H) suppresses CBF1 activity induced by constitutively active forms of Notch1 (nCDN1) and Notch2 (CDN2). Parental and stable C2C12 cell lines expressing either CDN1, nCDN1 or CDN2 were cotransfected with either 3 μ g of pCS2 (vector) or 3 μ g of pCS2-DBM X-Su(H) plus 3 μ g of the 4XwtCBF1Luc plasmid using Lipofectamine and processed as described above for luciferase activity.

Su(H)^{DBM} can block the expression of genes induced through Delta activation of Notch in *Xenopus* embryos, suggesting that X-Su(H)^{DBM} represents a dominant negative form of X-Su(H). Therefore, we used this dominant negative form of X-Su(H)^{DBM}, hereafter referred to as DN X-Su(H), to specifically block Notch-induced activation of CBF1 in C2C12 cells. To first evaluate the effectiveness of DN X-Su(H) to block Notch



activation of CBF1 we measured the activity produced from the 4xwtCBF1Luc reporter construct when it was coexpressed with DN X-Su(H) in stable CDN2- or nCDN1-expressing C2C12 cells. CDN2 and nCDN1-expressing cells showed an approximately 30-fold and 23-fold increase respectively in transactivation of the target gene as compared to parental C2C12 cells transfected with vector DNA (Fig. 2C). However when DN X-Su(H) was coexpressed in these cells, the level of luciferase activity decreased to nearly the same background level as in the parental C2C12 cells (Fig. 2C). Since DN X-Su(H) can effectively block CBF1 signaling induced by either nCDN1 or CDN2 in myoblasts we then asked if this loss in CBF1 activation affects the Notch-induced block in muscle cell differentiation. If CBF1 activation is not required for Notch signaling to block myogenesis then expression of DN X-Su(H) should not diminish the Notch-induced repression.

To directly test this idea, DN X-Su(H) was transfected into the parental cells as well as the C2C12 cell lines expressing either CDN1, nCDN1 or CDN2. One day after transfection, the cells were challenged to differentiate through exposure to differentiation medium (day 0). Northern and western blot analyses verified that the myc-tagged DN X-Su(H) was expressed when the transfected cells were induced to differentiate (Fig. 3B and C). RNA was collected at days 0, 2, and 4 in horse serum (HS) and the level of expression of the muscle-specific genes myogenin and MLC2 was determined by northern blot analysis as previously described (Shawber et

al., 1996). The expression of DN X-Su(H) showed no effect on the induction of myogenin or MLC2 in parental C2C12 cells when induced to differentiate (Fig. 3A, lanes 1-6). Even though expression of DN X-Su(H) in C2C12 cells expressing nCDN1 resulted in a loss of CBF1 activity (Fig. 2C), this constitutively active form of Notch1 still prevented the induction of muscle-specific genes in the presence of DN X-Su(H) (Fig. 3A, lanes 16-18). Inhibition of myogenesis induced by CDN2 under these same conditions was also resistant to the effects of DN X-Su(H) (data not shown). Therefore loss of CBF1 activity in cells that express forms of Notch that activate CBF1 (nCDN1 and CDN2) did not affect their ability to repress muscle cell differentiation, providing further evidence for CBF1-independent Notch-mediated inhibition of myogenesis.

Jagged1-induced Notch1 and Notch2 signaling activates CBF1 and represses myogenesis

Ligand-activation of Notch results in both the activation of the CSL proteins and the subsequent upregulation of the *Drosophila* E(spl) genes and the *Xenopus* ESR1 gene (Bailey and Posakony, 1995; Kageyama and Nakanishi, 1997; Lecourtois and Schweisguth, 1995; Wettstein et al., 1997). We have shown that ligand-independent Notch signaling in C2C12 myoblasts activates CBF1 (Fig. 2A), upregulates the expression of HES-1, and blocks muscle cell differentiation (Hsieh et al., 1997; Shawber et al., 1996). Since we have also shown that Jagged1-induced activation of full-length Notch1

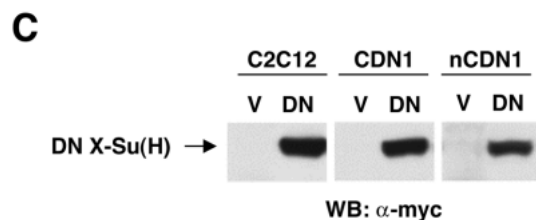
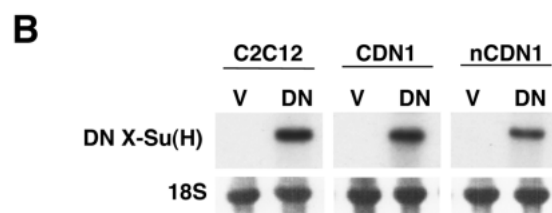
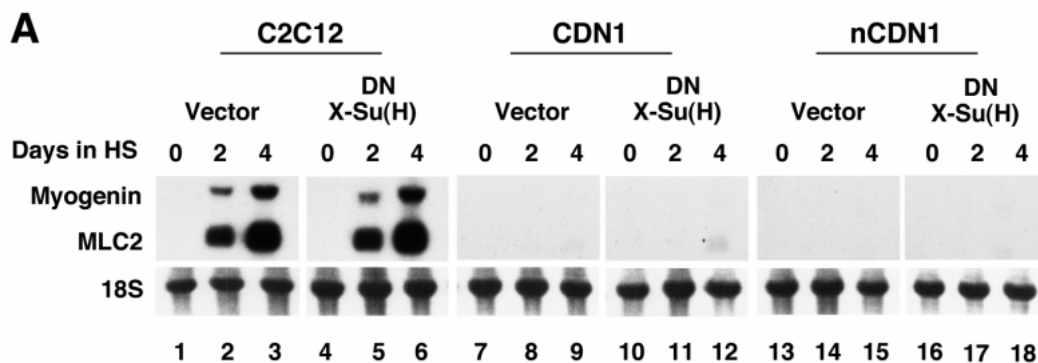


Fig. 3. Loss of CBF1 activation does not diminish Notch-induced repression of myogenesis. (A) Expression of a dominant negative (DN) form of X-Su(H) does not affect the ability of the constitutively active forms of Notch1 to inhibit muscle cell differentiation. Parental and stable C2C12 cell lines expressing either CDN1 or nCDN1 were transfected with either 6 µg of pCS2 (Vector) or 6 µg of pCS2-DBM X-Su(H) plasmid DNA [DN X-Su(H)] using Lipofectamine. 24 hours post-transfection the cells were incubated in medium containing 10% HS to induce differentiation and total RNA was collected at days 0, 2, and 4. Isolated total RNAs (10 µg) were analyzed by northern blotting using probes for myogenin and myosin light chain 2 (MLC2). Comparative loading and transfer of RNA were ascertained by methylene blue staining of 18S rRNA. (B) Expression of DN X-Su(H) mRNA in transfected myoblasts induced to differentiate into myotubes. Parental and stable C2C12 cell lines expressing either CDN1 or nCDN1 following transfection with pCS2-DBM X-Su(H) were harvested for total RNA 48 hours post-transfection and analyzed for DN X-Su(H) expression. (C) Expression of DN X-Su(H) protein in transfected myoblasts induced to differentiate into myotubes. Protein lysates were prepared from parental and stable C2C12 cell lines expressing either CDN1 or nCDN1 following transfection (48 hours) with pCS2-DBM X-Su(H) and analyzed for DN X-Su(H) expression by western blotting using anti-myc monoclonal antibody (9E10).

expressed in C2C12 myoblasts represses myogenesis (Lindsell et al., 1995) we asked if CBF1 is activated in response to the Notch ligand, Jagged1. To investigate this possibility we used an in vitro coculture assay in which Jagged1-induced activation of Notch1-expressing C2C12 cells blocks the expression of muscle-specific genes and consequently inhibits muscle cell differentiation (Lindsell et al., 1995). In addition, as found for Notch1-expressing myoblasts, when Notch2-expressing myoblasts were cocultured with Jagged1-expressing L cells (J1) and challenged to differentiate they did not fuse or express muscle-specific genes, indicating that the interaction of the Notch signaling pathway to inhibit myogenesis (Fig. 4A, lanes 6 and 10). Since both full-length Notch1 and Notch2 contain a RAM domain one would predict that ligand-induced activation of these Notch proteins would also result in CBF1 activation.

To determine if Jagged1-mediated Notch signaling activates CBF1 we transiently expressed the CBF1 reporter construct in Notch1, Notch2, or parental C2C12 cells and 24 hours post-transfection the cells were cocultured with either L cells or J1

cells for an additional 24 hours. C2C12 cells that express endogenous Notch1 and Notch2 exhibited an increase of approximately 2.5-fold transactivation of the CBF1-reporter construct when cocultured with J1 cells as compared to the parental L cells (Fig. 4B). This level of CBF1 activity detected is consistent with the low level of muscle cell inhibition previously observed when C2C12 cells are cocultured with Jagged1-expressing cells (Lindsell et al., 1995; also apparent in Fig. 4A, lane 2), which presumably reflects the low level of endogenous Notch protein expression present in parental C2C12 cells. In contrast, C2C12 cells that express high levels of either Notch1 or Notch2, through ectopic expression of these Notch proteins, when cocultured with J1 cells showed increased levels in reporter activity of 7-8 fold compared to cocultures containing parental L cells (Fig. 4B).

Although Jagged1-mediated Notch1 and Notch2 signal transduction leads to activation of CBF1, the level of activity detected with ligand-induced activation of full-length Notch1 or Notch2 was not as robust as the level of activity detected with the ligand-independent active forms of Notch1 (FCDN1 and nCDN1) or Notch2 (CDN2) (Fig. 1B and 2A). However,

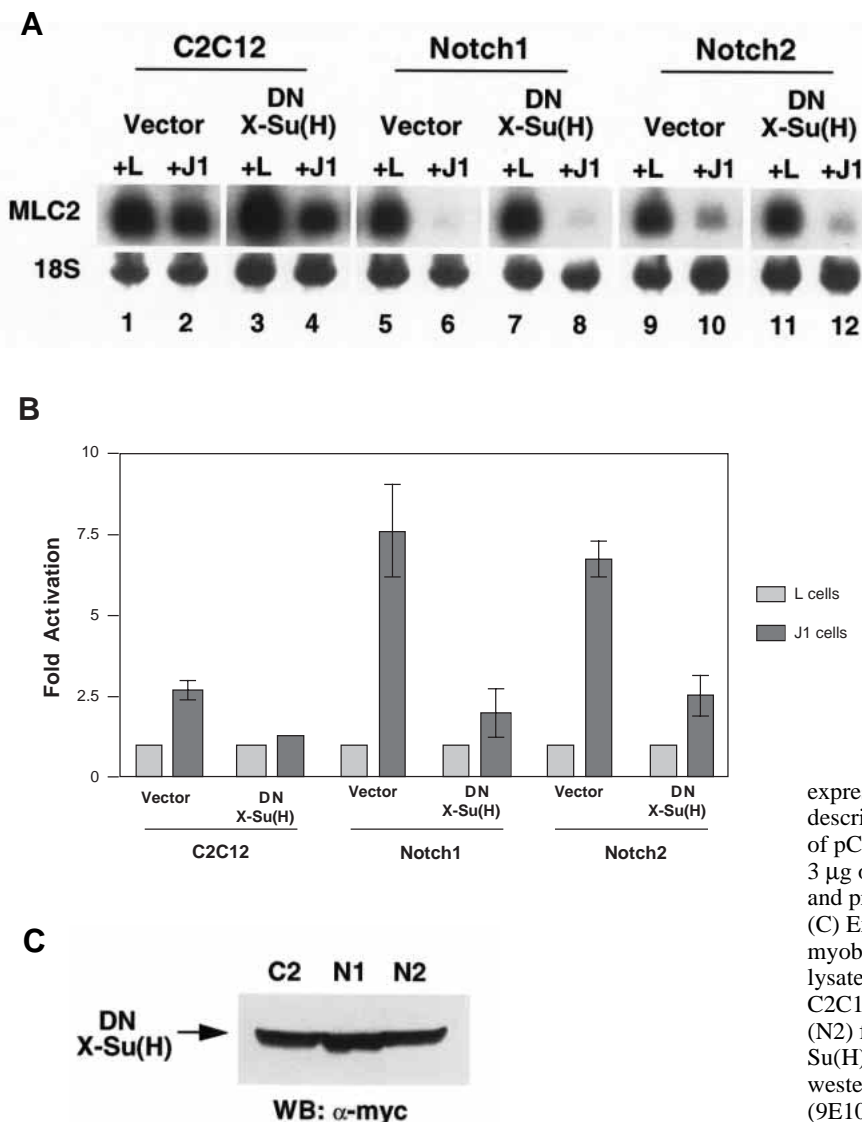


Fig. 4. Loss of CBF1 activity does not diminish inhibition of myogenesis induced by Jagged1 activation of either Notch1 or Notch2 signal transduction. (A) Expression of a dominant negative (DN) form of X-Su(H) does not perturb the repression in myogenesis induced by Jagged1-mediated Notch1 or Notch2 signaling in C2C12 myoblasts. Parental and stable C2C12 cell lines expressing either full-length Notch1 or Notch2 were transfected with either 6 μ g of pCS2 (vector) or 6 μ g of pCS2-DBM X-Su(H) plasmid DNA [DN X-Su(H)] using Lipofectamine. 24 hours post-transfection the cells were cocultured with either parental L cells (L) or Jagged1-expressing L cells (J1) and the cocultures were induced to differentiate by changing the medium to that containing 10% HS for 4 days as previously described (Lindsell et al., 1995). Isolated total RNAs (10 μ g) were analyzed by northern blotting using a myosin light chain 2 (MLC2) probe. Comparative loading and transfer of RNA were ascertained by methylene blue staining of 18S rRNA. (B) Jagged1 induced Notch1 or Notch2 signaling activates endogenous CBF1 activity that is compromised by the expression of DN X-Su(H). Parental and stable C2C12 cell lines expressing either Notch1 or Notch2 were transfected as described above with either 3 μ g of pCS2 (vector) or 3 μ g of pCS2-DBM X-Su(H) plasmid DNA [DN X-Su(H)] plus 3 μ g of the 4XwtCBF1Luc plasmid using Lipofectamine and processed as described above for luciferase activity. (C) Expression of DN X-Su(H) protein in transfected myoblasts induced to differentiate into myotubes. Protein lysates were prepared from parental (C2) and stable C2C12 cell lines expressing either Notch1 (N1) or Notch2 (N2) following transfection (48 hour) with pCS2-DBM X-Su(H) and analyzed for DN X-Su(H) expression by western blotting using anti-myc monoclonal antibody (9E10).

the increases in luciferase activity detected in the cocultures were specific for Notch-mediated activation of CBF1 since coexpression of DN X-Su(H) with the CBF1-reporter construct decreased the level of CBF1 activity in all three cell lines in the presence of Jagged1 (Fig. 4B). These data demonstrate that both Notch1 and Notch2 signaling induced by Jagged1 leads to activation of CBF1.

Loss of CBF1 activity does not diminish inhibition of myogenesis induced by Jagged1 activation of Notch1 or Notch2

Inhibition of myogenesis by constitutively active forms of Notch (CDN1 and CDCN1T) that do not activate CBF1 provided the first evidence for CBF1-independent Notch signaling in myoblasts. Further support for the existence of CBF1-independent Notch signaling comes from experiments in which CBF1 activation was blocked through the use of DN X-Su(H). Specifically, the loss of CBF1 activation in cells expressing constitutively active Notch (nCDN1 or CDN2) did not diminish the Notch-induced repression of muscle cell differentiation (Fig. 3A). Since Jagged1 can induce repression of myogenesis and stimulate activation of CBF1 in either Notch1- or Notch2-expressing myoblasts (Fig. 4A,B), we asked if CBF1 activity is required for ligand-induced Notch signaling to block myogenesis. Therefore, CBF1 activation was blocked through transient expression of DN X-Su(H) in the different myoblast lines and the ability of Jagged1-expressing cells to inhibit the differentiation of these transfected cells was determined (Fig. 4A). C2C12 cells, Notch1, or Notch2 cells transfected with either vector DNA or DN X-Su(H) DNA were subsequently cocultured with either L or J1 cells and after 3 days in differentiation medium total RNA was collected and northern analysis was performed to determine the extent of muscle cell differentiation through measuring the levels of MLC2 expression. MLC2 levels in parental, Notch1, and Notch2 cells expressing DN X-Su(H), and cocultured with J1 cells were similar to the cells expressing the vector controls (Fig. 4A). Western blot analysis of the transfected cells confirmed that DN X-Su(H) was expressed in the cocultured myoblasts (Fig. 4C). Thus, even though high expression of DN X-Su(H) in myoblasts expressing Notch1 or Notch2 resulted in a loss of ligand-induced CBF1 activity (Fig. 4B), this loss in CBF1 activity did not diminish Jagged1-activated Notch1 or Notch2 signaling that functions to suppress myogenesis (Fig. 4A, lanes 8, 12). Thus through expression of DN X-Su(H) we have shown that, in both a ligand-independent and -dependent manner, Notch signaling can inhibit muscle cell differentiation in the absence of CBF1 activation, identifying a Notch signaling pathway that functions independently of CBF1 to block myogenesis.

Notch-induced CBF1 activation correlates with Notch-induced MyoD antagonism

Previous work has suggested that Notch signaling inhibits muscle cell differentiation through the loss of MyoD expression and activity (Kopan et al., 1994; Lindsell et al., 1995; Shawber et al., 1996). If the loss in MyoD expression and function is the source of the Notch-mediated block in myogenesis induced by constitutive Notch signaling in cells, then one might expect that forced expression of MyoD would

relieve the Notch imposed block. To determine if ectopic expression of MyoD could override the block in differentiation induced by Notch signaling retroviral gene transduction was used to introduce MyoD into C2C12 cells expressing activated forms of Notch. The existence of at least two Notch signaling pathways raises the question of whether the CBF1-dependent and/or CBF1-independent Notch signaling pathways participate in the Notch-induced antagonism of MyoD. To address these questions, parental cells, as well as CDN1-, CDCN1T-, nCDN1- and CDN2-expressing C2C12 cells, were infected with retroviruses encoding either vector or MyoD sequences and 24 hours post-infection the medium was changed to differentiation medium.

After 2 days in differentiation medium, C2C12 cells infected with vector sequences were just beginning to show signs of muscle cell fusion (Fig. 5A). In contrast, CDN1-, nCDN1-, CDCN1T- and CDN2-expressing cells remained as flat mononucleated monolayers when infected with virus encoding vector sequences (Fig. 5A), consistent with these activated forms of Notch acting as inhibitors of muscle cell fusion. MyoD infected C2C12 cells differentiated earlier than vector expressing cells, displaying larger myotubes (Fig. 5A) and higher levels of MLC2 (Fig. 5B) within 2 days of infection, indicating that ectopic MyoD was functional in these cells. Interestingly, the phenotype of cells expressing structurally different activated forms of Notch differed in their responses to MyoD infection depending upon the presence or absence of the RAM domain. For example, when cells expressing CDN1 or CDCN1T that lack the RAM domain and do not activate CBF1 (Fig. 2) were infected with MyoD retroviruses large myotubes were readily apparent (Fig. 5A), suggesting that these forms of Notch do not antagonize ectopic MyoD activity. In contrast, when cells expressing nCDN1 or CDN2 that encode RAM sequences and activate CBF1 were infected with MyoD retroviruses, myotube formation was not detected (Fig. 5A). Therefore, CDN2 and nCDN1 appear to prevent ectopic MyoD from inducing myogenesis and indicate that in addition to differences in CBF1 activation the structurally different constitutively active forms of Notch, which are unable to signal through the CBF1-dependent pathway, also differ in their ability to antagonize MyoD.

To ensure that the morphological differences observed with the different cell lines in response to infection with MyoD retroviruses did not reflect differences in ectopic MyoD expression, total RNA was collected from the cells following 2 days in differentiation medium and the level of MyoD was determined by northern blot analysis. This analysis indicated that ectopic MyoD was expressed at relatively equal levels in all MyoD retroviral infected cell lines (Fig. 5B). Therefore, the lack of muscle cell differentiation detected in nCDN1 and CDN2 cells was not due to lower levels of ectopic MyoD expression in these cells. Ectopic expression of MyoD in CDN1 and CDCN1T cells also induced expression of endogenous MyoD and myogenin (data not shown), indicating re-activation of these genes when the block in myogenesis is relieved by overexpression of MyoD. Consistent with the observed level of muscle cell fusion in MyoD-infected cells (Fig. 5A), high MLC2 levels were observed in parental, CDN1 and CDCN1T cells that readily fused, while no or very low levels of MLC2 were detected in nCDN1- and CDN2-expressing cells that did not exhibit

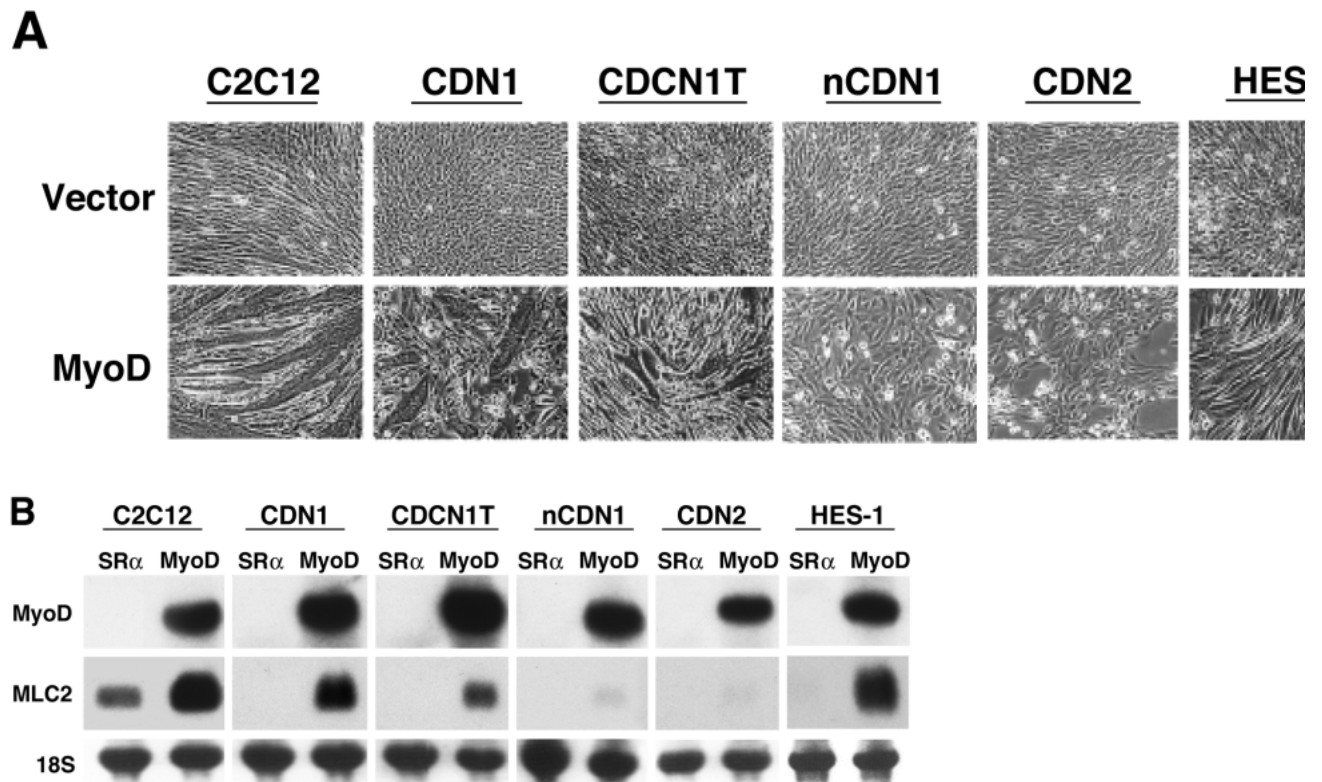


Fig. 5. MyoD rescues the block in myogenesis induced by CBF1-independent Notch signaling but not that induced by CBF1-dependent Notch signaling. (A) Parental and stable C2C12 cell lines expressing either CDN1, CDCN1T, nCDN1, CDN2 or HES-1 were infected at a MOI of 1.0 with retroviruses encoding either Vector or MyoD sequences. 24 hours post-infection the cells were induced to differentiate for 2 days and examined for the presence of myotubes. C2C12, CDN1, CDCN1T and HES-1 cells were induced to fuse and form myotubes when infected with MyoD retroviruses suggesting that ectopic MyoD was functional in these cells. In contrast, nCDN1 and CDN2 cells did not fuse but rather appeared to undergo cell death resulting in a decrease in cell density compared to the vector controls. (B) Total RNA was isolated from the indicated cells infected with retroviruses encoding either vector (SR α) or MyoD sequences and analysed by northern blotting for the level of MyoD and MLC2 expression. Equivalent levels of ectopic MyoD are expressed in all MyoD infected cells. Comparative loading and transfer of RNA were ascertained by methylene blue staining of 18S rRNA.

muscle cell fusion (Fig. 5B). Thus constitutively active forms of Notch that activate CBF1, through the presence of CBF1-interacting sequences, functionally antagonize MyoD. Surprisingly, activated forms of Notch that are unable to activate CBF1 are also unable to antagonize MyoD, despite the fact that they block myogenesis.

It has been proposed that Notch signaling represses myogenesis through Notch activation of CBF1 and the subsequent upregulation in expression of HES-1, a transcription factor that represses MyoD activity (Jarriault et al., 1995; Sasai et al., 1992). However, we have previously reported that overexpression of HES-1 in C2C12 cells does not perturb their ability to differentiate suggesting that HES-1 does not functionally inhibit endogenous MyoD expressed by C2C12 cells (Shawber et al., 1996). In support of this, HES-1-expressing C2C12 cells infected with MyoD retroviruses also fused (Fig. 5A) and expressed high levels of MLC2 (Fig. 5B) suggesting that HES-1 does not antagonize ectopic MyoD. Therefore in contrast to previous reports with fibroblasts (Jarriault et al., 1995; Sasai et al., 1992), the Notch-induced block in myogenesis does not appear to involve HES-1-mediated antagonism of MyoD activity in C2C12 myoblasts.

Loss of CBF1 activity results in a loss of MyoD antagonism induced by Notch signaling

The correlation between activation of CBF1 and antagonism of MyoD induced by constitutively active forms of Notch suggested that CBF1 is required for the Notch-induced inhibition of MyoD. Constitutively active Notch interferes with the ability of MyoD to activate MyoD reporter constructs in transient transfection assays (Kopan et al., 1994). To determine if CBF1 activity was required for Notch signaling to antagonize MyoD-mediated activation of a reporter containing MyoD-binding sites within the muscle creatine kinase (MCK) promoter enhancer region, situated upstream of the luciferase gene (MCK-luc), we coexpressed MyoD and the reporter in nCDN1 or CDN2-expressing cells either in the presence of vector or DN X-Su(H) DNA. Since the MyoD-mediated luciferase activity detected in parental C2C12 cells in the presence of either vector or DN X-Su(H) DNA was equivalent, the fold luciferase activation calculated as the ratio between luciferase activity detected for DN X-Su(H) and that detected for vector (DN-X-Su(H)/vector), was approximately 1 (Fig. 6). In contrast, when Notch-induced CBF1 activity was suppressed through the expression of DN X-Su(H) the fold activation of MyoD-mediated luciferase activity detected with

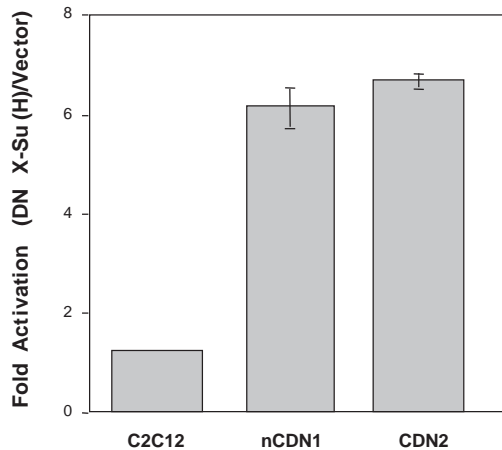


Fig. 6. Loss of CBF1 activity results in a loss of MyoD antagonism induced by constitutively active forms of Notch. Stable nCDN1- and CDN2-expressing C2C12 cell lines were cotransfected with 0.25 μ g of pSR α -MyoD plus 2 μ g of MCK-luc reporter construct either in the background of 2 μ g transfected vector pCS2 DNA or pCS2-DBM X-Su(H) DNA using Lipofectamine and processed for luciferase activity. The values represent fold activation in luciferase activity as a ratio of the luciferase activity detected in the presence of DN X-Su(H) to that detected in the presence of vector DNA (DN X-Su(H)/vector). Expression of DN X-Su(H) relieves the inhibition of MyoD activity induced by either nCDN1 or CDN2 as indicated by the approximate 6-fold activation detected.

nCDN1 or CDN2 (DN X-Su(H)/vector) was approximately 6-fold (Fig. 6). This increase in MyoD-induced luciferase activity detected in the presence of DN X-Su(H) indicates that losses in CBF1 activity lead to increases in MyoD activity consistent with the idea that CBF1 activity is required for Notch-mediated antagonism of MyoD. These data also support the idea that MyoD antagonism mediated by Notch signal transduction is restricted to the CBF1-dependent pathway. It is important to note that while forced expression of MyoD rescues the block in myogenesis induced by CDN1 and CDCN1T (Fig. 5A,B), these active forms of Notch1 are unable to activate CBF1, consistent with a requirement for CBF1 activation in Notch-induced antagonism of MyoD.

Notch signaling prevents differentiation of the osteoblast lineage

The existence of activated forms of Notch (CDN1 and CDCN1T) that inhibit muscle cell differentiation but do not antagonize MyoD activity suggested that additional target(s) of Notch-induced inhibition of myogenesis lie upstream of MyoD. This idea raised the possibility that the block imposed by Notch signaling independently of CBF1 activation may inhibit a general step in differentiation rather than a cell-type-specific one. Moreover, since Notch signaling inhibits the differentiation of many different cell types we asked if Notch signaling could also prevent bone morphogenetic protein-2 (BMP-2) induced osteogenesis of C2C12 cells and if so, does this block require CBF1 activation. BMP-2 is a member of the TGF- β superfamily that not only stimulates the maturation of committed osteoblast progenitors, but also induces the commitment of C2C12 myoblasts into osteoblasts (Rodan and Harada, 1997). Treatment of C2C12 myoblasts with

recombinant BMP-2 inhibits muscle cell differentiation and converts the differentiation pathway of these cells into that of the osteoblast lineage (Katagiri et al., 1994). To determine whether Notch signaling can prevent differentiation and respecification along the bone pathway, C2C12 cells expressing activated forms of Notch were treated with BMP-2 and northern blot analysis was used to detect bone (osteocalcin) and muscle (MLC2) specific gene expression.

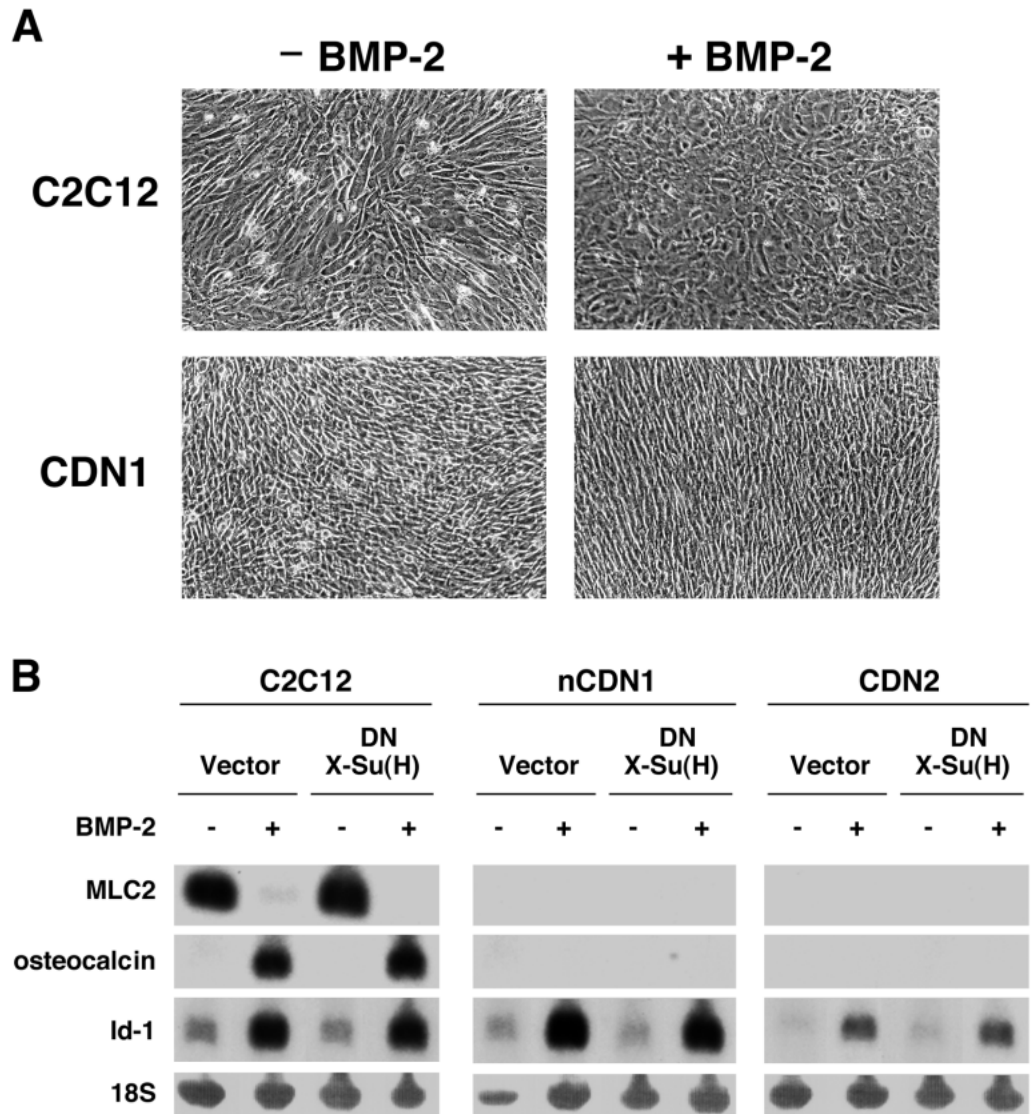
When C2C12 cells are grown in differentiation medium they undergo myogenic conversion as indicated by the formation of multinucleated myotubes (Fig. 7A). In contrast, treatment of C2C12 cells with BMP-2 completely inhibited the formation of myotubes and induced a cuboidal morphology (Fig. 7A). However, C2C12 cells expressing constitutively active forms of Notch remained as unfused, mononucleated cells both in the presence and absence of BMP-2. This block in BMP-induced osteogenesis was observed for CDCN1T, nCDN1, CDN2 and is shown in Fig. 7A for CDN1-expressing C2C12 cells. Since CDN1 and CDCN1T are unable to signal through the CBF1-dependent pathway it would appear that the Notch-induced block in osteogenesis occurs independently of CBF1. To test this idea we used the DN X-Su(H) to suppress Notch-induced CBF1 activation in CDN1, CDCN1T, nCDN1 and CDN2 expressing cells treated with BMP-2. Total RNA was collected after 3 days and northern blot analysis was used to detect changes in expression of bone- and muscle-specific genes. In the absence of BMP-2, parental C2C12 cells exhibited increased levels of the muscle structural gene MLC2 and did not express the bone-specific marker osteocalcin (Fig. 7B). Strikingly, C2C12 cells incubated in BMP-2 for 3 days did not express MLC2 but did induce expression of osteocalcin, indicating that BMP-2 converts C2C12 myoblasts into osteoblasts at the expense of the muscle cell phenotype (Fig. 7B). In contrast to the parental C2C12 cells, with or without BMP-2 treatment, C2C12 cells expressing constitutively active forms of Notch induced neither MLC2 nor osteocalcin (Fig. 7B). However, these BMP-2-treated cells did respond to factor as indicated by the upregulation in expression of the BMP-inducible gene Id-1 (Fig. 7B). Importantly, neither bone- nor muscle-specific gene expression was detected in the presence of DN X-Su(H) expression (Fig. 7B), indicating that CBF1 is not required for the Notch-induced block in bone differentiation as demonstrated for myogenesis in these same cells. Therefore, Notch signaling can inhibit both bone and muscle cell differentiation in C2C12 cells supporting the hypothesis that Notch can act as a general inhibitor of cellular differentiation.

DISCUSSION

Notch signaling can prevent muscle cell differentiation in a CBF1-independent manner

Constitutively active forms of Notch that contain the RAM domain have been shown to physically and functionally interact with CBF1 (Hsieh et al., 1996, 1997; Jarriault et al., 1995; Tamura et al., 1995). Moreover, these CBF1-activating forms of Notch1 have also been shown to inhibit the activity of MyoD and Myf-5 when expressed in fibroblasts (Kopan et al., 1994) and prevent myogenesis when transiently expressed in C2C12 cells (Hsieh et al., 1997; Kato et al., 1997). These

Fig. 7. Notch signaling prevents BMP-induced osteogenesis in C2C12 cells independently of CBF1. (A) Parental and CDN1-expressing C2C12 cells incubated either in the absence or presence of 300 ng/ml of recombinant human BMP-2 for 3 days were examined for morphological changes indicative of either myogenesis or osteogenesis. C2C12 cells in the absence of BMP (–BMP-2) fused to form myotubes; however, in the presence of BMP (+BMP-2) these cells did not fuse but rather displayed a cuboidal morphology. In contrast, both in the presence and absence of BMP-2 the CDN1-expressing myoblasts formed flat dense monolayers. (B) Total RNA was isolated from C2C12, nCDN1 and CDN2-expressing cells transfected with either vector DNA or DN X-Su(H) and infected with pBABE (–BMP-2) or pBABE-BMP-2 (+BMP-2) and cultured for 3 days. RNAs were analyzed by northern blotting using probes for MLC2, osteocalcin and Id-1. Comparative loading and transfer of RNA were ascertained by methylene blue staining of 18S rRNA.



data have led to the proposal that Notch signaling inhibits muscle cell differentiation solely through activation of CBF1 (Jarriault et al., 1995; Kato et al., 1997). In contrast to these previously reported repressors of myogenesis, both CDN1 and CDCN1T lack sequences required for Notch1 to interact with CBF1. Consequently, these two activated forms of Notch1 do not activate CBF1 in three different cell lines, HeLa (Shawber et al., 1996), 3T3, and C2C12 (data presented here), despite the fact that when expressed in myoblasts they inhibit muscle cell differentiation. Moreover, Honjo and co-workers have shown that mutant forms of Notch structurally similar to CDN1 and CDCN1T do not interact with CBF1 or Su(H); however, they did not determine whether these mutant Notch proteins inhibit myogenesis (Tamura et al., 1995). Therefore, the lack of CBF1 activity associated with CDN1 and CDCN1T supports the findings that the RAM domain is necessary for productive interactions between Notch and CBF1.

Our analysis of mutant forms of Notch1, which are constitutive repressors of myogenesis but do not activate CBF1, suggested that Notch signaling can lead to an inhibition

in muscle cell differentiation through a CBF1-independent pathway. This proposal is strengthened by the fact that expression of DN X-Su(H), a dominant negative DNA-binding mutant that perturbs CBF1 activity in C2C12 cells, does not reverse the block in muscle cell differentiation induced by either ligand-dependent or ligand-independent Notch signal transduction. Therefore, the loss of CBF1 activity does not diminish the ability of Notch signaling to inhibit myogenesis in both a ligand-dependent and ligand-independent manner. This is in contrast to studies in *Xenopus* embryos where expression of DN X-Su(H) results in an increase of primary neurons presumably through the loss of Notch signaling (Wettstein et al., 1997). Although DN X-Su(H) suppresses the activity of X-Su(H)/CBF1 in both *Xenopus* embryos and mouse myoblasts, the loss in X-Su(H) activity has a dramatic effect on primary neurogenesis in frog embryos yet has no apparent effect on Notch-induced inhibition of myogenesis in C2C12 cells. The reasons for these obvious differences are unclear; however, the possibility exists that cell types differ in their requirements for Notch signal transduction such that

developing neurons are more sensitive to changes in the CBF1-dependent pathway than myoblasts. Additional studies will identify the requirement for CBF1 in the development of other cell types and tissues regulated by Notch signaling. In fact, evidence for Su(H)-independent Notch signaling in *Drosophila* has been reported for a number of Notch-dependent processes. For example, Notch-dependent induction of the single-minded gene in midline cells during mesectoderm formation (Lecourtis and Schweisguth, 1995), the neuron/sheath cell fate decision during sense organ development (Wang et al., 1997) and proneural enhancement in the developing eye (Ligoxygakis et al., 1998) all occur in the absence of Su(H). Along with the evidence reported here and previously (Shawber et al., 1996), for CBF1-independent Notch signaling in C2C12 myoblasts, it appears that Notch activation does not always involve CBF1/Su(H).

Antagonism of MyoD by activated forms of Notch requires CBF1 activity

The previously published activated forms of Notch that antagonize MyoD activity all contain the RAM domain and consequently interact with CBF1 (Weinmaster, 1998). This led to the proposal that Notch signaling through CBF1 prevents muscle cell differentiation by antagonizing MyoD activity (Jarriault et al., 1995; Kopan et al., 1994). Consistent with these previous reports, we have found that activated forms of Notch that contain the RAM domain, nCDN1 and CDN2, antagonize ectopic MyoD function in C2C12 cells. This antagonism appears to require CBF1 since suppression of DN X-Su(H) resulted in increases in MyoD activity. However, we have been unable to determine if this CBF1-dependent, Notch-mediated antagonism of MyoD requires a direct interaction between MyoD and CBF1 (A. M. and G. W., unpublished data) or some other protein induced by CBF1. Although direct physical interactions between soluble cytoplasmic forms of Notch and CBF1 with DNA have been demonstrated (Jarriault et al., 1995), attempts to detect similar interactions between MyoD and Notch have been unsuccessful, suggesting that constitutively active forms of Notch do not directly interact with MyoD (Kopan et al., 1994).

In contrast to the RAM containing forms of Notch that activate CBF1 and antagonize MyoD, the RAM deficient forms of Notch (CDN1 and CDCN1T) do not activate CBF1 or antagonize MyoD, even though they effectively inhibit myogenesis. Therefore, the differences in Notch-induced MyoD antagonism found in this study identify an additional feature that distinguishes the two Notch signaling pathways (Shawber et al., 1996). Moreover, the second CBF1-independent Notch signaling pathway must function

upstream of MyoD since ectopic expression of MyoD overrides the block in differentiation induced by forms of Notch that are unable to signal through CBF1. Furthermore, since Notch signaling prevents induction of both myogenesis and osteogenesis in the absence of CBF1 activation, the CBF1-independent pathway appears to function as a general inhibitor of cellular differentiation. This conclusion is consistent with reports that Notch signaling blocks cellular differentiation during neurogenesis (Chitnis, 1995; Dorsky et al., 1997; Henrique et al., 1997; Lardelli et al., 1996; Nye et al., 1994), hematopoiesis (Li et al., 1998; Milner et al., 1996), and gliogenesis (Wang et al., 1998).

Two distinct blocks established by Notch signaling identify different roles for the CBF1-independent and CBF1-dependent pathways in suppression of myogenesis

The CDCN1T, CDN1, nCDN1 and CDN2 proteins all represent activated forms of Notch that when expressed in C2C12 cells prevent muscle cell differentiation. Although all these mutant Notch proteins inhibit myogenesis, they differ in their ability to activate CBF1 and antagonize MyoD suggesting that they signal differently in cells. Importantly, this analysis has indicated that Notch signaling can prevent muscle cell differentiation through two distinct pathways in C2C12 cells. The CBF1-independent pathway inhibits cellular

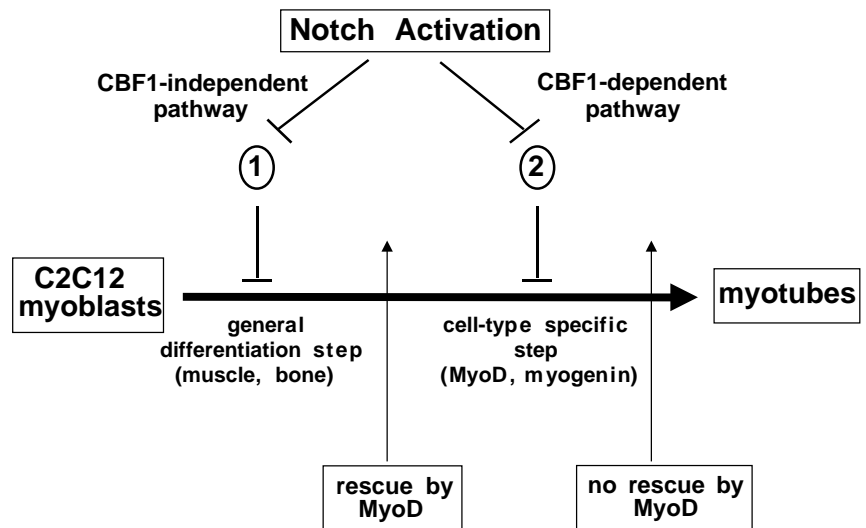


Fig. 8. Two distinct blocks established by Notch signaling identify different roles for the CBF1-independent and CBF1-dependent pathways in suppression of myogenesis. Our data indicate that there are two distinct pathways through which Notch signaling can perturb cellular differentiation and have suggested the following model for Notch-induced repression of myogenesis in C2C12 myoblasts. The first block (1) induced by Notch signal transduction, which functions in the absence of CBF1 activation, targets a general step in cellular differentiation, since both osteogenesis and myogenesis are inhibited by this pathway. The components of the CBF1-independent pathway are unknown, but they must lie upstream of MyoD since overexpression of MyoD can rescue this Notch-induced block. However, downstream of the Notch-induced CBF1-independent block there must be an additional block that directly targets MyoD activity during myogenesis, which would serve to reinforce the first block. This second block (2) functions through the CBF1-dependent pathway as CBF1 activity is required for the Notch-induced repression of MyoD activity. Since CBF1-dependent Notch signaling induced by either nCDN1 or CDN2 proteins inhibits MyoD activity, ectopic expression of MyoD did not rescue the Notch-mediated repression of myogenesis (Fig. 5).

differentiation at a target upstream of MyoD while the CBF1-dependent pathway specifically represses myogenesis through the antagonism of MyoD.

Based on our results using these various constitutively active forms of Notch, we have proposed a model for Notch-mediated inhibition of cellular differentiation in which at least two steps during the process of myogenesis are sensitive to Notch signaling (Fig. 8). In this model the CBF1-independent pathway would function to block a general step in cellular differentiation. In the case of C2C12 cells, this general step would be common to both the bone and muscle pathway. This block does not antagonize MyoD activity since ectopically expressed MyoD is functional and rescues the block in differentiation induced by Notch signaling through the CBF1-independent pathway. However secondary to this block, activated Notch induces a more cell-type-specific block, which would be muscle specific in C2C12 cells and require the CBF1-dependent pathway to antagonize MyoD-induced myogenesis. Consistent with this model, ectopic MyoD expression did not rescue the block in differentiation induced by the CBF1-dependent pathway.

A general block in differentiation could account for the Notch-mediated effects on differentiation in many different cell types. To reinforce this initial, general block in cellular differentiation Notch signaling may also regulate specific factors, such as MyoD, required for the generation and development of specific cell types. This regulation of cell-type-specific factors induced by Notch activation of CBF1 would sustain the overall Notch-induced block in cellular differentiation. Our studies with mutant forms of Notch clearly indicate that Notch signaling can inhibit myogenesis independently of CBF1; however, ligand-induced Notch activation leads to signaling through both the CBF1-independent and CBF1-dependent pathways. In *Drosophila*, many Notch-dependent processes show a strict requirement for CBF1/Su(H); however, Notch signaling independent of Su(H) has also been reported. It may be that the loss of CBF1/Su(H), which is required to both reinforce the initial CBF1-independent block as well as induce cell-type-specific determination, would also result in the loss of the general CBF1-independent block in these processes. In summary, the identification of two distinct Notch signaling pathways accounts for the detection of Notch signaling both in the presence and absence of CBF1/Su(H) and may explain the pleiotropic nature of Notch signal transduction.

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