Delta-induced Notch Signaling Mediated by RBP-J Inhibits MyoD Expression and Myogenesis*

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Cell fate determination by local cell-cell contact plays a pivotal role in precise pattern formation during development of multicellular organisms (1). The Notch receptors and their ligands are both cell surface molecules conserved from worm through man, and involved in cell fate determination of various cell lineages (1–3).

The mouse Notch1 (mNotch1) receptor is a transmembrane protein composed of the 180-kDa extracellular and 90-kDa intracellular regions (4). Its extracellular region contains 36 EGF repeats for ligand binding and three lin-12/Notch repeats for Notch signaling was shown by using RAMIC (25) and suppress neurogenesis in mammalian cultured cells (26, 27). More recently, the entire intracellular region (IC) of mNotch can induce transactivation through the HES1 promoter carrying the RBP-J binding motif (25) and suppress neurogenesis and myogenesis in mammalian cultured cells (26, 27).

The muscle cell differentiation is determined by the MyoD family of myogenic transcriptional regulators (MyoD, Myf-5, myf-6, mRF-2, and Mrf-4). The muscle cell commitment is determined by a MyoD-receptor containing the RAM domain which interacts with a DNA-binding protein RBP-J (mammalian homologue of Droso phila Suppressor of Hairless (Su(H)) (5–7), six cdc10/ankyrin repeats, nuclear localization signals, OPA region, and PEST sequence (4). Notch signal is triggered by interaction with its ligand, the DSL family protein which includes Delta and Jagged/Serrate in vertebrates (1, 8). All of them contain a DSL motif for binding to Notch and tandem EGF repeats, and a short cytoplasmic domain (1, 8). There are two subfamilies of ligands: Delta and Serrate which are expressed at different sites and time points during Drosophila embryogenesis (9–13). Although Serrate can compensate loss-of-function mutations of Delta at least in part (14), it is not clear whether Delta and Serrate have identical functions in Notch signaling. Fibroblasts expressing the mammalian homologue (Jagged) of Serrate can inhibit differentiation of C2C12 myoblast cells that express either endogenous Notch alone or both endogenous and transgene-derived Notch (15, 16). However, the molecular mechanism underlying this phenomenon is completely unknown. In addition, it is not known whether mammalian Delta has a similar function.

The mechanism of signal transduction through the Notch receptor is still unclear. Genetic studies of Drosophila have shown that Notch interacts functionally with Su(H) (17). Su(H) and RBP-J is shown to physically interact with the RAM domain of Notch (7, 18–20). In addition, knockout mice of Delta−/− (21), Notch−/− (22, 23), and RBP-J−/− (24) showed somewhat similar phenotypes including defects in somite formation. Expression of the truncated intracellular region (IC) of mNotch can induce transactivation through the HES1 promoter carrying the RBP-J binding motif (25) and suppress neurogenesis and myogenesis in mammalian cultured cells (26, 27).
Notch/RBP-J Signaling Inhibits MyoD Expression

Delta1 Inhibits Myogenesis and Expression of Myo1 mRNA of C2C12 Cells—To examine the role of mouse Delta1 in differentiation regulation, we first constructed a Delta1 transfectant (D10) of the mouse X63 myeloma cell line which expresses a full-length mouse Delta1-tagged C-terminal with the gene 10 epitope sequence. We also produced a fusion protein consisting of EGF repeats 11 and 12 of mNotch1 followed by the Fc portion of human IgG1 (MNE-Rg4) to monitor surface expression of the Delta1 protein on D10 cells (43, 53). Flow cytometric analysis showed that MNE-Rg4 bound strongly to the cell surface of D10 cells but not to parental X63 cells (Fig. 1A), indicating that D10 cells express a large number of Delta1 molecules which can bind to mNotch1.

When C2C12 cells were cultured in differentiation media in the presence or absence of X63 cells, C2C12 cells differentiated normally and fused into multinucleated myotubes (Fig. 1, C and E). In contrast, C2C12 cells co-cultured with D10 cells showed drastic reduction of myotube formation (Fig. 1G). C2C12 myoblasts have been shown to express endogenous Notch receptors (16, 2).

To further explore the molecular mechanism for differentiation suppression of C2C12 cells co-cultured with D10 cells, we examined expression of muscle lineage-specific genes such as MyoD, myogenin, and myosin light chain 2 (MLC2) before and 24 h after differentiation induction. When C2C12 cells were co-cultured with X63 cells, high level expression of MyoD mRNA was maintained before and after differentiation induction. Myf-5 was not detected in this particular C2C12 cell line (data not shown). Expression of mRNAs for myogenin and MLC2 was induced after differentiation induction (Fig. 2). C2C12 cells co-cultured with D10 cells markedly decreased expression of MyoD mRNA in concomitant inhibition of myogenin and MLC2 mRNAs induction. These results indicate that the mouse Delta1 serves as the functional ligand for Notch and their interaction inhibits myogenic differentiation of C2C12 cells by blocking expression of MyoD mRNA.

Delta1 Inhibits Expression of Myo1 mRNA by Up-regulating HES1 mRNA Expression in C2C12 Cells—HES1 was suggested to be a direct target of Notch signaling by co-transfection experiments (25) and reported to inhibit MyoD-induced myogenesis of 10T1/2 cells (38). We therefore tested whether HES1 mRNA was up-regulated in differentiation-induced C2C12 cells by co-culture with D10 cells. HES1 mRNA was rapidly up-regulated about 2.5 times by co-culture with D10 cells, followed by down-modulation of Myo1 mRNA (Fig. 3, A-C). By contrast,

Hybridizations were done under standard conditions (46).
the HES1 mRNA level was not changed by co-culture with X63 cells (Fig. 3, A-C). A slight down-regulation of MyoD mRNA by X63 cells appears to be caused by serum deprivation (54). These experiments indicate that Delta1 inhibits myogenesis of C2C12 cells by suppressing MyoD mRNA expression with up-regulating expression of HES1 mRNA.

As the Delta1-induced decrease of MyoD mRNA rapidly followed the up-regulation of HES1 mRNA, MyoD could be regulated by HES1 in C2C12 cells as previously suggested (38). To determine whether induction of HES1 mRNA expression requires protein synthesis, C2C12 cells were co-cultured with D10 cells in differentiation medium in the presence of 10 μM cycloheximide. The cycloheximide treatment blocked the decrease of MyoD mRNA expression but not the increase of HES1 mRNA in C2C12 cells by co-culture with D10 cells (Fig. 4). In fact, HES1 mRNA expression was augmented about 16 times by co-culture with D10 cells (Fig. 4). These results indicate that the HES1 gene is regulated not only directly by activated Notch and RBP-J without synthesis of intermediate regulatory molecules, but also negatively by the HES1 protein per se in agreement with the previous report (55).

Delta1 Up-regulates RBP-J-mediated Transcriptional Activity—Although Notch signaling by overexpression of Notch IC or RAMIC has been shown to activate transcription mediated...
by RBP-J, it has not been shown whether ligand-induced Notch signaling also transactivates RBP-J. We therefore examined whether Notch signaling in C2C12 cells triggered by co-culture with D10 cells up-regulates RBP-J-mediated transcription. To monitor RBP-J-mediated transcription, we used the Tp1 and HES1 promoters that carry RBP-J-binding sites and are transactivated by overexpression of the mNotch1 RAMIC (25, 28, 56, 57). C2C12 cells were transfected with Tp1-luciferase (pGa981–6) or the HES1-luciferase reporter plasmid (ptk-HES1) and co-cultured with either X63 or D10 cells. C2C12 cells co-cultured with D10 cells showed that transcription from the HES1 promoter was severalfold enhanced as compared with that of X63 cells (Fig. 5A). A small level enhancement of the HES1 promoter activity may be due to negative autoregulation by endogenous HES1 in C2C12 cells, because the HES1 promoter contains the N-box for HES1 binding (55). To avoid this complication, we used the Tp1 promoter which contained only the RBP-J-binding site. C2C12 cells co-cultured with increasing numbers of D10 cells showed markedly enhanced transcriptional activities through the Tp1 promoter in parallel with the number of the D10 cells added (Fig. 5B). By contrast, C2C12 cells co-cultured with X63 cells showed negligible levels of transcriptional activity through the Tp1 promoter. Essentially the same results were obtained regardless of the culture media used, i.e. for differentiation or growth.

To further confirm that RBP-J is involved in transactivation through the Tp1 promoter by ligand-induced Notch signaling, excess amounts of RBP-J constructs were co-transfected with the Tp1 reporter plasmid into C2C12 cells because excess amounts of RBP-J have been shown to inhibit transcriptional activity through the Tp1 promoter by RAMIC (28, 57). In fact, excess RBP-J significantly reduced the transcriptional activity through the Tp1 promoter in C2C12 cells co-cultured with D10 cells (Fig. 5C). Excess RBP-J reduced marginally the basal transcriptional activity of the Tp1 promoter in C2C12 cells co-cultured with X63 cells. These results indicate that interaction of Delta1 on D10 cells with the receptor Notch on C2C12 cells leads to RBP-J-mediated transactivation of the HES1 and Tp1 promoters.

Activated RBP-J Decreases MyoD Expression in C2C12 Cells—A fusion protein of human RBP-J with a viral transactivation domain VP16, hRBP-J-VP16 (58), has been shown to markedly suppress myogenesis of C2C12 cells (28). We showed above that ligand-dependent Notch signaling suppressed MyoD mRNA expression and enhanced RBP-J-dependent transcription of HES1 mRNA in C2C12 cells (Figs. 1, 2, and 5). To examine whether inhibition of MyoD expression by Notch-Delta interaction is mediated through RBP-J, we transfected the active form of mouse RBP-J, VP16-mRBP-J into C2C12 cells, and measured expression of MyoD (green) and VP16-mRBP-J (red) by two-color immunocytostaining (Fig. 6). There are few overlaps of green (MyoD) and red (VP16-mRBP-J) staining (Fig. 6, I–K), showing that MyoD expression was suppressed in VP16-mRBP-J positive cells. The frequency of MyoD+ cells was markedly reduced by VP16-mRBP-J expression as compared with control cells transfected with the vector alone (Table I). In contrast, there are many overlaps of green and red staining (Fig. 6, E–G) and no reduction of MyoD expressing cells by mRBP-J expression, showing that MyoD expression was not inhibited in wild type mRBP-J positive cells (Table I). These results indicate that the activated RBP-J also suppresses MyoD expression in C2C12 cells.

To further confirm that Delta1-induced inhibition of MyoD expression is involved in myogenic suppression, we constitutively expressed MyoD in C2C12 cells and co-cultured with D10 cells. To monitor rescue of myogenic suppression, two-color immunocytostaining experiments that detect expression of both MyoD and myoglobin were carried out (Fig. 7). C2C12 cells expressing MyoD differentiated into myoglobin expressing cells despite co-culture with D10 cells (Fig. 7, E–G). These data indicate that down-regulation of MyoD is the major cause in Delta1-induced myogenic suppression of C2C12 cells.

**DISCUSSION**

The skeletal muscle cell differentiation is controlled by basic helix loop helix transcriptional regulators (MyoD, Myf-5, myogenin, and MRF4) that belong to the MyoD family (32). Previous experiments showed that truncated mNotch1 (IC) inhibits MyoD- or Myf-5-dependent transcriptional activity of E-box-containing promoters (26) and that ligand-induced Notch signaling down-regulates expression of myogenin (15, 31). However, a direct target of ligand-induced Notch signaling was unknown. In this study, we have demonstrated that Delta1-induced Notch signaling mediated by RBP-J induces directly expression of HES1. Furthermore, the Delta1-induced Notch signaling or the activated form of mRBP-J (VP16-mRBP-J) inhibited expression of MyoD in C2C12 cells and their myogenic differentiation. Taken together with the previous report that HES1 down-regulates MyoD-dependent transcriptional activity of E-box-containing promoters and inhibits the MyoD-
induced myogenic conversion of 10T1/2 cells (38), Delta-Notch interaction is likely to induce HES1 which then down-regulates MyoD, resulting in inhibition of myogenesis.

The mammalian ligands of Notch can be divided into two groups, Delta and Serrate/Jagged (1, 8). Previously only Jagged was shown to function as a ligand of Notch (15, 16). This is the first report that Delta1 can interact with Notch and deliver a differentiation suppression signal in mammalian cultured cells. We have shown that Delta1 can bind to Notch1 (Fig. 1A), but this observation does not exclude the possibility that Delta1 interacts also with other Notch family members (Notch2, Notch3, and Notch4). It is not clear that Delta and Jagged have the identical function including preference among the Notch family members.

Su(H) has been shown to be involved in Notch signaling by extensive genetic studies in Drosophila. RBP-J has been shown to mediate differentiation suppression activity of RAMIC and IC (28). Furthermore, Epstein-Barr virus nuclear antigen 2, which physically associates with RBP-J, can also suppress differentiation of C2C12 cells (57). However, involvement of RBP-J in ligand-induced Notch signaling has not been demonstrated. In this study we showed that RBP-J is involved in Delta1-induced Notch signaling because (a) it activated the transactivation activity of the HES1 and Tp1 promoters containing RBP-J-binding motifs, (b) this activity was blocked by excess amounts of RBP-J, and finally (c) the activated form of RBP-J inhibited both MyoD transcription and differentiation of MyoD expression in C2C12 cells. C2C12 cells were transected with pCMX-N (mock vector) (A-D), pCMX-mRBP-J (E-H), or pCMX-VP16-mRBP-J (I-L). After transfection, C2C12 cells were cultured in differentiation medium for 24 h and then immunocytostaining was carried out to monitor expression of the MyoD (green, A, E, and I), and mRBP-J or VP16-mRBP-J (red, B, F, and J). The two images were superimposed (C, G, and K). Nuclei were stained with Hoechst 33342 (blue, D, H, and L). MyoD expression is inhibited by VP16-mRBP-J (I-K) but not by mRBP-J (E-G).

### Table I

| Transfected constructs | Number of nuclei | RBP-J+ MyoD | RBP-J+ MyoD |
|-----------------------|-----------------|-------------|-------------|
| pCMX-N                | 254             | 37.0a       | 63.0a       |
| mRBP-J                | 123             | 40.3        | 59.3        |
| VP16-mRBP-J           | 120             | 12.5        | 87.5        |

a Only MyoD expression was scored.
Delta1-induced myogenic suppression is rescued by the constitutive expression of MyoD (E-G). MyoD expression is unaltered in mouse embryos with the HES1 signaling does not agree with the previous findings that HES1 can strongly down-regulate MyoD expression because negatively autoregulated (55). A small and transient up-regulation followed by the reduction of MyoD mRNA expression (Figs. 3 and 4). We also confirmed that co-culture with mouse Jagged1- and HES1 belongs to the basic helix loop helix protein family whose regulators of myogenesis, such as the Id and HES families, can be induced by Notch/RBP-J signaling in C2C12 cells. Negative regulators of myogenesis, such as the Id and HES families, inhibit the transcriptional activity of the MyoD family (38, 61). HES1 is established on the basic helix loop helix protein family whose members have been shown to function in other basic helix loop helix proteins such as MyoD (38). Since RAC1 or IC of mNotch1 acts as a transcriptional activator of the HES1 promoter that contains the RBP-J-binding sites in HeLa (25), and COST (28) and C2C12 (data not shown) cells, HES1 is assumed to be responsible for blocking myogenesis by Notch1 signaling (25, 62, 63). Ligand-induced Notch signaling enhances HES1 promoter activity and up-regulates HES1 mRNA expression quickly and transiently in C2C12 cells, which is followed by the reduction of MyoD mRNA expression (Figs. 3 and 4). We also confirmed that co-culture with mouse Jagged1-expressing cells up-regulates HES1 mRNA expression and subsequently reduces MyoD mRNA expression in C2C12 cells.3

The conclusion that HES1 is the direct target of Notch/RBP-J signaling does not agree with the previous findings that HES1 expression is unaltered in mouse embryos with the HES1 genotype (65) and that knockout mice with the HES1 genotype are affected in neurogenesis but not in myogenesis (66). An explanation to reconcile these results would be that direct target genes other than HES1 may be involved in the Notch/RBP-J signaling pathway. Finally, the RBP-J protein is ubiquitously expressed (67), commonly used by the Notch family members (56), and directly and uniquely targeted by Notch signaling (24, 65). Thus RBP-J may function as a master protein in cell fate determination by Notch signaling.

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Fig. 7. The constitutive expression of MyoD rescues the Delta1-induced myogenic suppression of C2C12 cells. C2C12 cells were transplanted with pHAP1-R (mock vector) (A-D) or pHAPa-D + (mouse MyoD) (E-H) (45, 50). After transfection, C2C12 cells were co-cultured with D10 cells in differentiation medium for 4 days and then immunostaining was carried out to monitor expression of the MyoD (green, A and E) and myoglobin (red, B and F) (28). The two images were superimposed (C and G). Nuclei were stained with Hoechst 33342 (blue, D and H). The Delta1-induced myogenic suppression is rescued by the constitutive expression of MyoD (E-G).
