Delta-1 Activation of Notch-1 Signaling Results in 

**HES-1** Transactivation

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**The Notch receptor is involved in many cell fate determination events in vertebrates and invertebrates. It has been shown in Drosophila melanogaster that Delta-dependent Notch signaling activates the transcription factor Suppressor of Hairless, leading to an increased expression of the Enhancer of Split genes. Genetic evidence has also implicated the kuzbanian gene, which encodes a disintegrin metalloprotease, in the Notch signaling pathway. By using a two-cell coculture assay, we show here that vertebrate DI-1 activates the Notch-1 cascade. Consistent with previous data obtained with active forms of Notch-1 a HES-1-derived promoter construct is transactivated in cells expressing Notch-1 in response to DI-1 stimulation. Impairing the proteolytic maturation of the full-length receptor leads to a decrease in HES-1 transactivation, further supporting the hypothesis that only mature processed Notch is expressed at the cell surface and activated by its ligand. Furthermore, we observed that DI-1-induced HES-1 transactivation was dependent both on Kuzbanian and RBP-J activities, consistent with the involvement of these two proteins in Notch signaling in Drosophila. We also observed that exposure of Notch-1-expressing cells to DI-1 results in an increased level of endogenous HES-1 mRNA. Finally, coculture of DI-1-expressing cells with myogenic C2 cells suppresses differentiation of C2 cells into myotubes, as previously demonstrated for Jagged-1 and Jagged-2, and also leads to an increased level of endogenous HES-1 mRNA. Thus, DI-1 behaves as a functional ligand for Notch-1 and has the same ability to suppress cell differentiation as the Jagged proteins do.**

The Notch gene family encodes transmembrane receptors that have been implicated in many aspects of cell fate determination in vertebrates and invertebrates. In Drosophila melanogaster, one consequence of activation of the Notch signaling pathway is the maintenance of the receiving cells in an uncommitted state through a process called lateral inhibition. For example, during peripheral neurogenesis, this signaling pathway is involved in the choice of a single precursor among a group of equivalent proneural cells. Following cell-cell interaction, one cell differentiates into a sensory organ precursor and inhibits its neighbors from adopting a neural fate. This inhibitory signal is mediated by ligand-induced activation of the Notch pathway. Different genes have been implicated in the Notch pathway, including Delta (DI) and Serrate (Ser), encoding two structurally related transmembrane ligands of Notch; Suppressor of Hairless [Su(H)], which encodes a DNA binding protein; and genes of the Enhancer of Split complex [E(sp1)] (see references 3 and 9 for reviews). The E(sp1) gene products accumulate in response to Notch activation and were shown to be downstream targets of the Notch pathway (6, 17, 20, 40). The E(sp1) proteins would in turn repress transcription of differentiation factors, such as the proneural Achaete-Scute genes (32, 37, 48). Recently, Kuzbanian/Sup-17, a newly recognized component of the Notch cascade, has been isolated in Drosophila and Caenorhabditis elegans (49, 52, 58, 68). This gene encodes a transmembrane metalloprotease which acts cell-autonomously in the Notch-expressing cell, upstream of the activated Notch receptor (49, 52, 58, 68).

Vertebrate homologs of these genes have been identified. To date, four Notch genes are known to exist in mammals (18, 38, 63, 66, 67). Much evidence supports a role for Notch in cell fate decisions in vertebrates. In particular, intracellular forms of Notch, similar to forms producing a gain-of-function phenotype in Drosophila, are able to suppress muscle cell differentiation (34, 35, 43, 47, 57) as well as neuronal differentiation (47). In addition, injection of such an activated form of Xenopus laevis Notch into Xenopus embryos causes changes in cell determination (11). Retinal ganglion cell differentiation in chickens and Xenopus has also been shown to be inversely correlated with the level of Notch activity (5, 15).

Similarly, vertebrate counterparts of the two Drosophila genes Delta and Serrate have been isolated: Delta-(like)-1 in mice and chickens (Dll-1 and DI-1, respectively) (7, 23) and Delta-like-3 (Dll-3) in mice (16); Delta-2 in Xenopus (30); and the potential orthologs of Serrate, Jagged-1 in rats and humans (41, 43) and Jagged-2 in rats, mice, and humans (43, 56, 64). These genes encode transmembrane proteins that, like the Drosophila ligands, all include in their extracellular region EGF repeats and a DSL (for Delta-Serrate-Lag-2) domain, which could be involved in interactions with Notch (46). These genes have also been implicated in cell fate determination in vertebrates. Notably, it has been shown that both Jagged-1 and Jagged-2, when expressed at the surface of fibroblasts, are able to inhibit the differentiation of cocultivated C2 myoblast cells, whether or not they are transfected with the Notch-1 cDNA (41, 43). Suppression of muscle differentiation induced by activated forms of Notch or by presentation of a Serrate-like
ligand and are very similar in phenotype and lead to activation of identical subsets of genes (43). Since at least three Notch homologs (Notch-1, -2, and -3) are expressed in C2 cells (43), it has been postulated that inhibition of the differentiation of C2 cells by Serrate-like ligands is mediated by Notch signaling. Taken together, these data suggest that vertebrate homologs of Notch ligands can modulate cell fate through activation of a Notch receptor.

Homologs of Drosophila E(spl) genes have been identified, including HES-1 (33, 54). Retroviral infection studies leading to overexpression of HES-1 and phenotypic analysis of murine HES-1-null embryos have demonstrated a role for HES-1 in neurogenesis and retinal differentiation (26, 27, 61). Notch ligands can modulate cell fate through activation of a Notch receptor. Notch signaling in vertebrates probably involves the same components as in Drosophila. The implication of RBP-J and HES-1 downstream of Notch-1 has been suggested by experiments done with extracellularly truncated active forms of the Notch-1 receptor. RBP-J has been shown to interact with the intracellular part of Notch-1 directly (4, 29, 60). Moreover, these RBP-J-Notch-1 complexes can transactivate a HES-1 gene promoter construct by direct interaction with RBP-J binding sites (29, 34). These results suggest a model in which, following ligand activation, processing occurs, releasing the intracellular part of Notch, which associates with RBP-J and activates transcription of its target genes. How ligand binding triggers signal transduction is not known, however.

To provide evidence for ligand-dependent Notch signaling, we set up a two-cell coculture assay, and we show here that DI-1 is able to activate the Notch-1 receptor, leading to activation of a HES-1 promoter construct as well as activation of the endogenous HES-1 gene. As previously suggested (8, 42, 49), our data confirm the hypothesis that maturation of the mammalian receptor is required for its activity. Furthermore, we demonstrate that mammalian homologs of components of the Notch pathway in Drosophila are implicated in DI-1-induced Notch-1 signaling. Indeed, both Kuzbanian and RBP-J activities are required for DI-1-induced transactivation of the HES-1 promoter. Taken together, our data suggest that DI-1-induced Notch-1 activation results in HES-1 gene transactivation in the Notch-1-expressing cells, a process reminiscent of Notch signaling in Drosophila. We also show that, like Jagged-1 and Jagged-2, DI-1 suppresses myogenic differentiation of C2 cells while at the same time increasing the level of DI-1 mRNA. Thus, DI-1 behaves as a functional ligand for Notch-1 and has the same ability to suppress myogenic cell differentiation as the Jagged proteins do.

MATERIALS AND METHODS

Cells. HeLa and HeLa-N1 cells were grown in Dulbecco’s modified Eagle’s medium (DMEM) supplemented with 10% fetal calf serum. C2 cells were grown in DMEM supplemented with 20% fetal calf serum. QT6 and QT6-DI1 cells were grown in Ham F-10 medium supplemented with 10% tryptose phosphate, 5% fetal calf serum, and 1% chicken serum. HeLa cells were stably transfected by calcium phosphate precipitation with the murine Notch-1 cDNA cloned into pCDNA3 (Invitrogen). Following G418 selection (0.8 mg/ml), a clone was selected for further studies and named HeLa-N1. QT6 cells were infected by the RCAS-DI1 and RCAS-DI1dn constructs (24) as follows. The retroviral vectors were transfected into a primary culture of chick fibroblasts, as previously described (45). After 5 days of culture, when 100% of the cells were infected, the supernatant containing the viral particles was added to QT6 cells for 6 h and subsequently replaced by QT6 medium supplemented with 1% glucose. Following 7 days of culture, 100% of the QT6 cells were infected and expressed the transgene.

Transfection experiments. Transfection of HeLa-N1 cells (5 × 10^6) was performed by calcium phosphate precipitation in 6-well plates with 0.4 μg of luciferase vector (either HES-1-luc or HES-1-ΔLuc [29]) alone or in combination with 0.8 μg of pCS2-ΔE (29, 36), 1.6 μg of pCDNA3-Δ1PDX (42), or 1.6 μg of pCMV-mKuz DN (42). One day later, 2 × 10^6 QT6 or QT6-DI1 cells were added and cocultivated for 24 h in QT6 medium (补充 literature). Co-transfection with 10^6 QT6 or QT6-DI1 cells led to the same results. The final calcium concentration was adjusted to 0.08 g/liter. Luciferase activity was determined as previously described (49) in a Berthold luminometer 48 h after transfection. All transfections were performed in duplicate.

Antibodies and immunofluorescence. Expression at the cell membrane was assessed by indirect immunofluorescence, as previously described (29). Notch expression was detected with affinity-purified rabbit anti-Notch (42), used at 1/100 dilution. This antibody is directed against amino acids 1759 to 2306 of the intracellular domain of murine Notch-1. To detect Dl-1 expression, we used a mouse monoclonal troponin T antisera (catalog no. T6277; Sigma Chemical) at 1/100 dilution. Slides were mounted and viewed with a confocal laser scanning microscope (Zeiss) (magnification, ×63; numerical aperture, 1.4).

Dissociation assay. C2 cells were plated in 3.5-mm-diameter plates so that they would reach 70% confluence the following day. QT6 or QT6-DI1 cells (10^6) were then added. One day later the medium was changed to differentiation medium (DMEM supplemented with 2% fetal calf serum). After 6 days, cells were fixed, and the expression of troponin T was assessed by indirect immunofluorescence as described above and viewed with a Nikon fluorescence microscope (magnification, ×40).

Northern blot analysis. HeLa-N1 cells were plated in 10-cm-diameter plates so that they would reach 50% confluence the following day. QT6 or QT6-DI1 cells (6 × 10^6) were then added or cultured alone, and the cells were grown in QT6 medium for 25 h. Total RNA was isolated from cultured cells with Trizol (Life Technologies). C2 cells (1.9 × 10^6) were plated in 10-cm-diameter plates. Two days later, 6 × 10^6 QT6 or QT6-DI1 cells were added, and the cells were grown in DMEM supplemented with 20% fetal calf serum for 17 h. Poly(A)^+ RNA was then isolated by using the polyATtract system (Promega). Total RNA (25 μg) or 2 μg of poly(A)^+ RNA was analyzed by Northern blotting as described previously (53), except that the filters were hybridized in Church buffer. The probe used to detect HES-1 expression was the full-length rat HES-1 cDNA (a gift from R. Kagayama and S. Nakanihshi). To normalize RNA amounts, we used as a probe a rat GAPDH cDNA (65) (a gift of F. Aurade), which encodes a ribosomal protein expressed at a high and constant level in adult human tissues. In addition, this 26S probe does not hybridize with QT6 RNAs, allowing normalization of the RNA from Notch-1-expressing cells only. Other probes used to analyze ligand expression were human Jagged-1 cDNA (a gift of S. Artavanis-Tsakonas), murine Dll-1 cDNA (a gift of A. Gassler), and murine Dll-3 cDNA (a gift of R. Beddington).

RESULTS

In order to analyze the intracellular events induced by Notch activation, we attempted to reconstitute an ex vivo system where the Notch signaling pathway could be activated in a ligand-dependent manner. Therefore, we first constructed a cell line expressing the Notch-1 receptor. HeLa cells were stably transfected with the murine Notch-1 cDNA and a subclone was further characterized and named HeLa-N1. As Delta-expressing cells, we used a QT6 stability line infected with a retrovirus expressing the chicken Dll-1 cDNA (24). These cells were named QT6-DI1. As control cells, we used indifferently both wild-type QT6 cells or QT6 cells infected by RCAS retrovirus incorporating antisense Dll-1 cDNA (see Materials and Methods) (24). Expression was assessed by indirect immunofluorescence, and staining that included the membranes as well as the secretory pathway was observed for both proteins (Fig. 1). Membrane staining was confirmed by fixing the cells without permeabilization.

Interaction of DI-1-expressing cells with Notch-1-expressing cells leads to transactivation of the HES-1 promoter in the Notch-expressing cells. We and others have previously shown that active forms of the Notch receptor can activate transactivation from the HES-1 promoter (29, 34). We postulated that if active truncated forms of Notch mimic normal Notch activation, stimulation of the Notch receptor by a ligand would have the same effect on HES-1 transcription. Thus, to follow Notch activation, we transiently transfected HeLa-N1 cells with the
reporter luciferase gene under the control of the HES-1 promoter (HES-1-luc [29]). QT6-Dl1 cells or QT6 control cells (either wild-type QT6 or QT6 retroinfected with an antisense cDNA for cDl-1) were then added and cocultivated for 24 h (see Materials and Methods). As a positive control, a constitutively active membrane-tethered form of Notch, ΔE (29, 36), was cotransfected with HES-1-luc in the Notch-1-expressing cells.

Transactivation of the HES-1 promoter was observed when HeLa-N1 cells were cocultivated with QT6-Dl1 (Fig. 2A, bar 4) but not when HeLa-N1 cells were cocultivated with QT6 control cells (Fig. 2A, bar 3) nor when control HeLa cells were cocultivated with QT6-Dl1 cells (Fig. 2B, bar 4). Furthermore, the level of this Dl-1-induced transactivation was slightly higher than transactivation elicited by the ΔE construct (Fig. 2A, compare bars 2 and 4). The lower activity of ΔE in HeLa-N1 cells compared to that in HeLa cells is probably due to clonal variation. Therefore, HES-1 transactivation is dependent on the presence of Notch-1 in stimulated cells and the presence of Dl-1 in stimulating cells. We conclude that Dl-1 is a functional ligand for Notch-1.

**Inhibition of HES-1-luc transactivation by an inhibitor of Notch maturation.** It has been shown that the Notch receptor undergoes a constitutive proteolytic cleavage in the extracellular part of the protein during its transport to the cell membrane and that only this mature form is detectable at the cell surface (8, 42, 49). Logeat et al. showed that a furin-like convertase is responsible for Notch constitutive maturation and, as suggested by others (8), that the processed extracellular part remains attached to the membrane-tethered C terminus of the molecule. Further experiments showed that this proteolytic maturation step was required for Notch surface expression and that it was severely impaired in a stable cell line expressing an inhibitor of furin-like convertase, α1-antitrypsin Portland (known as α1PDX [2]) (42).

Biotinylation and immunoprecipitation experiments confirmed that the Notch-1 protein expressed in our HeLa-N1 cells was also processed and that only this mature receptor was present at the cell surface (reference 42 and data not shown). A prediction of the model presented above is that the presence of α1PDX in HeLa-N1 cells would reduce the amount of mature Notch-1 receptor at the cell surface and as a consequence would inhibit transactivation of the HES-1-luc reporter. To test this hypothesis, we cotransfected the cDNA encoding α1PDX with the HES-1-luc reporter in HeLa-N1 cells. When these transfected HeLa-N1 cells were cocultivated with the...
Activation represents the ratio between individual luciferase activity and the activity measured with the luciferase plasmid alone. The bars represent the standard errors.

One day later, QT6 control cells (lane 3) or QT6-Dl1 cells (lane 4) were added and cocultivated for 24 h with the transfected HeLa cells. Luciferase activity was measured 48 h after transfection in a luminometer (Berthold). The mean fold inductions from 11 independent experiments performed in duplicate are shown. Luciferase activation represents the ratio between individual luciferase activity and the activity measured with the luciferase plasmid alone. The bars represent the standard errors. (B) Control HeLa cells were transfected with 400 ng of HES-1-luc alone (bars 1, 3, and 4) or together with 800 ng of the active form of Notch-1 (Fig. 4, bars 7 and 8). One day later, QT6 control cells (bars 3 and 4) or QT6-Dl1 cells (bars 6 and 8) were added and cocultivated for 24 h with the transfected HeLa cells. Luciferase activity was measured 48 h after transfection, as described above. The mean fold inductions from three independent experiments performed in duplicate are shown. Luciferase activation represents the ratio between individual luciferase activity and the activity measured with the luciferase plasmid alone. The bars represent the standard errors.

QT6-Dl1 cells, a significant inhibition of HES-1-luc transactivation was observed (Fig. 3, bars 7 and 8), whereas cotransfection of the cDNA encoding a1PDX did not decrease ΔE-induced HES-1-luc transactivation (Fig. 3, bars 3 and 4) or HES-1-luc levels when HeLa-N1 cells were cocultivated with control QT6 cells (Fig. 3, bars 5 and 6). These results show that maturation of the Notch receptor is necessary for activation by its ligand and thus that only the mature form of Notch-1 is functional.

**Di1-induced transactivation of HES-1-luc is inhibited by a truncated dominant-negative form of Kuzbanian.** Genetic experiments with *Drosophila* and nematodes have suggested that a membrane metalloprotease, Kuzbanian (Kuz), is a previously unrecognized component of the Notch signaling pathway and that it is involved in Notch activation (49, 52, 58, 68). At least one ortholog of *kuzbanian* has been isolated in vertebrates (49). Deletion studies of this protein have defined a truncated form of either *Drosophila* or murine Kuz which lacks the prodomain and the metalloprotease domain of the Kuz protein and acts phenotypically in *Drosophila* as a dominant-negative molecule (Kuz DN [49]). In addition, injection of this truncated form of murine Kuz in *Xenopus* embryos resulted in overproduction of primary neurons (49). We thus postulated that if Kuz is implicated in Notch-1 activation in our system, then introducing a dominant-negative form in Notch-1-expressing cells should alter Notch-1 signaling. To test this hypothesis, we cotransfected murine Kuz DN (mKuz DN2 [49]) with the HES-1-luc reporter gene in HeLa-N1 cells prior to cocultivation with the QT6-Dl1 cells. A reproducible inhibition of Di1-induced transactivation of the HES-1 promoter could be observed (Fig. 4, bars 7 and 8), but inhibition of ΔE-induced HES-1-luc transactivation (Fig. 4, bars 3 and 4) or of the HES-1-luc activity elicited by incubation with control QT6 cells (Fig. 4, bars 5 and 6) was not observed. We conclude from these results that Kuzbanian activity is needed for transcriptional response to Di1-induced Notch-1 signaling in our system. In addition, Kuzbanian acts upstream of ΔE, suggesting that the extracellular part of Notch-1 is required in this process.

**RBP-J is implicated in Di1-induced transactivation of the HES-1 promoter.** We have previously shown that active forms of Notch transactivate the HES-1 promoter through the RBP-J binding sites. To investigate whether the presence of these sites is required for Di1-induced transactivation of the HES-1 promoter, we used a mutant form of the HES-1 reporter construct, from which the two RBP-J binding sites, A and B, had been deleted (HES-1-ΔABluc [29]). As shown in Fig. 5, when this mutant form of the HES-1 promoter was used, Di1- (Fig. 5, bars 7 and 8) as well as ΔE (Fig. 5, bars 3 and 4)-induced transactivation was abolished. We conclude that Di1 stimu-
lates Notch-1 signaling through an RBP-J-dependent pathway in HeLa-N1 cells.

**HES-1 expression is increased in Notch-1-expressing cells cocultured with Dl-1-expressing cells.** To determine if transcription of the endogenous **HES-1** gene was affected in the HeLa-N1 cells after cocultivation with the QT6-Dl1 cells, we performed Northern blot analysis. As shown in Fig. 6, no signal for **HES-1** could be detected in RNA from either QT6 cells (lane 1) or QT6-Dl1 cells (lane 2) with a probe derived from the rat **HES-1** cDNA, most likely because the probe does not cross-hybridize. Therefore, the **HES-1** signal in RNA from cocultured cells represents **HES-1** expression in the HeLa-N1 cells. HeLa-N1 cells exhibited a weak signal (Fig. 6, lane 3), which was strongly increased by cocultivation with QT6-Dl1 cells (lane 5), but not with control QT6 cells (lane 4). These results correlate with those obtained by the luciferase assay, and confirm the hypothesis that **HES-1** is a target of the Notch signaling pathway in HeLa-N1 cells.

It has been suggested that feedback loops (either negative or positive, depending on the ligand concentration) regulate the expression of Notch and its two ligands in *Drosophila* (12, 21, 25). Evidence exists for such regulation in myoblast C2 cells (41, 43). When C2 cells are grown in a low-serum medium which does not hybridize with quail RNA (bottom panel).

**Dl-1 suppresses muscle cell differentiation.** As a mean of validating the physiological relevance of our system, we decided to test whether Dl-1 was able to inhibit the differentiation of Notch-expressing cells, as it has been shown for Jagged-1 and Jagged-2, two Serrate-related mammalian proteins (41, 43). When C2 cells are grown in a low-serum medium (i.e., differentiation medium), they start to express the tropo-
blot analysis, using the same probe derived from the rat HES-1 cDNA. C2 cells cocultivated with control QT6 cells (Fig. 8, upper panel, lane 1) exhibited a weak signal, which was increased by cocultivation with QT6-Dl1 cells (Fig. 8, upper panel, lane 2). The intensities of the signals were quantified by phosphorimager and, after normalization with GADPH (Fig. 8, bottom panel, lanes 1 and 2), a reproducible increase of three- to fourfold in HES-1 transcripts was observed. Thus, as in HeLa-N1 cells, there is an increase in the level of endogenous HES-1 mRNA in C2 cells exposed to Dl-1. This result supports the hypothesis that a Notch signaling pathway is activated in the C2 cells cocultivated with Dl-1-expressing cells.

We also present evidence that mammalian counterparts of Drosophila components of the Notch pathway are indeed implicated in Dl-1-dependent Notch-1 signaling.

Previous results from our laboratory have shown that maturation of the Notch-1 receptor by a member of the furin family of convertases is required for cell surface expression (42). Here we confirm and extend these results by showing that inhibition of this proteolytic step prevents Notch signaling. In the presence of α1PDX the receptor is trapped within the cell (42) and therefore is not exposed to the ligand presented by QT6 cells. However, α1PDX is unlikely to affect ligand-dependent processing, since activation of HES-1-luc by ∆E was unaffected in these studies. Thus, the functional form of the mammalian Notch-1 receptor is the heterodimeric molecule composed of the two cleavage products, which are associated at the cell surface.

We report here the ex vivo reconstitution of a Delta-1-induced Notch-1 signaling cascade. Our results demonstrate that Dl-1 can act as a ligand to activate the Notch-1 receptor and prevent cellular differentiation.

Previous results from our laboratory have shown that maturation of the Notch-1 receptor by a member of the furin family of convertases is required for cell surface expression (42). Here we confirm and extend these results by showing that inhibition of this proteolytic step prevents Notch signaling. In the presence of α1PDX the receptor is trapped within the cell (42) and therefore is not exposed to the ligand presented by QT6 cells. However, α1PDX is unlikely to affect ligand-dependent processing, since activation of HES-1-luc by ∆E was unaffected in these studies. Thus, the functional form of the mammalian Notch-1 receptor is the heterodimeric molecule composed of the two cleavage products, which are associated at the cell surface.

We also present evidence that mammalian counterparts of Drosophila components of the Notch pathway are indeed implicated in Dl-1-dependent Notch-1 signaling.

Genetic studies with Drosophila and C. elegans have highlighted a role for a metalloprotease, Kuzbanian/Sup-17, in at least a subset of Notch-dependent biological events (49, 52, 58, 68). Laser ablation experiments (68), as well as kuz genetic mosaic analysis during Drosophila neurogenesis, wing margin formation, vein width specification, and adult sensory bristle development (49, 52, 58) or nematode vulval development (68), have shown that Kuz acts cell autonomously in the Notch-expressing cell. In addition, gain-of-function phenotypes elicited by intracellular forms of either Notch or Lin-12 are similar in kuz/sup-17 mutant or kuz/sup-17 wild-type backgrounds, indicating that Kuz acts upstream of intracellular Notch signaling (49, 58, 68). Thus, Kuz may play a role in Notch maturation or activation. When using a truncated form of murine Kuz, which acts as a dominant-negative inhibitor when overexpressed in Drosophila (49), we observed a decrease in Dl-1-dependent transactivation of the HES-1 promoter. Moreover, this dominant-negative form of Kuz did not lower ∆E-induced HES-1-luc transactivation. ∆E is a membrane-tethered, active form of Notch-1 which lacks nearly all of the extracellular part of the receptor. We therefore conclude that Kuz activity is required to elicit transcriptional activation in HeLa-N1 cells in response to Dl-1-induced Notch-1 stimulation and that Kuz activity is required upstream of the postulated processing event which releases the intracellular region of Notch.

FIG. 7. Dl-1-expressing cells suppress C2 cell differentiation. The differentiation of C2 myoblasts was assessed by indirect immunofluorescence analysis of troponin T expression. (A) C2 cells; (B) C2 cells cocultivated with control QT6 cells; (C) C2 cells cocultivated with QT6-Dl1 cells. Magnification, ×40.

FIG. 8. Endogenous HES-1 expression is increased in C2 cells cocultured with Dl-1-expressing cells. Expression of the HES-1 gene was analyzed by Northern blotting with 2 μg of poly(A)+ RNA of C2 cells cocultured with QT6 (top panel, lane 1) or QT6-Dl1 (top panel, lane 2) cells for 17 h. mRNA sizes are indicated on the right. Normalization was done with the GADPH probe (bottom panel).
sistent with results obtained with invertebrates with intracellular Notch protein in a kuz mutant background (49, 58, 68), and furthermore, they confirm that Kuz acts upon the extracellular portion of mammalian Notch-1.

In Drosophila, Notch activation leads to an increase in E(spl) proteins through transcriptional activation of the genes, which represent downstream targets of the Notch cascade (6, 31, 40). The Su(H) protein has been suggested to be responsible for this transcriptional activation by directly binding to E(spl) gene regulatory regions (6, 17, 20, 40). We and others have shown that truncated active forms of Notch-1 stimulate transcription of a HES-1-derived promoter construct (29, 34). Reminiscent of E(spl) transactivation by Su(H), truncated Notch-dependent transactivation of the HES-1 promoter was mediated by the RBP-J protein through binding sites present in the HES-1 promoter (29). In agreement with these findings, we show in this study that Dl-1 failed to induce transactivation of a mutated HES-1 promoter construct, from which the two RBP-J binding sites have been deleted. Thus, RBP-J activity is indeed required for Dl-1-induced Notch-1 activation.

The results presented above also show that coculture of Dl-1- and Notch-1-expressing cells, besides inducing transactivation of a transfected HES-1 promoter in the Notch-1 expressing cells, also leads to an increase in the level of endogenous HES-1 mRNA, strongly suggesting that this increase is a result of RBP-J-mediated gene transactivation. These data indicate that the HES-1 gene is a bona fide target of Dl-1-dependent Notch-1 signaling in HeLa-N1 cells. HES-1 has been shown to inhibit cellular differentiation when overexpressed in MyoD-induced 10T1/2 cells (54) or murine retinal progenitors (27, 61), suggesting that it functions as a negative regulator of myogenesis and neurogenesis. Supporting this hypothesis, disruption of the HES-1 gene leads to up-regulation of proneural basic helix-loop-helix factors and premature neurogenesis in mouse embryos (26). These data strongly suggest that HES-1 and Notch-1 could function in the same pathway. However, Northern blot analysis revealed that the level of HES-1 mRNA was not changed in RBP-J and Notch-1 knockout embryos (13). Nevertheless, these results do not exclude the possibility that HES-1 expression could be regulated by Notch signaling in some particular developmental events, possibly depending on the cellular context. Development of the retina could be a good candidate, as overexpression of Delta-1 or activated forms of Notch-1 inhibits retinal progenitor differentiation (1, 5, 14, 24), which is accelerated in HES-1-null mutant mouse embryos (61).

We and others recently proposed a model in which ligand binding induces Notch processing. As an attempt to test this model, we tried to detect a processed intracellular form of Notch-1 following exposure to Dl-1. However, we were unable to detect such a molecule by Western blotting or to detect any nuclear antigenicity by immunofluorescence or gel shift experiments. One explanation could be that the actual number of activated Notch-1 receptors is too low to allow detection by these techniques, although it is sufficient to support transactivation. A recent report by Schroeter et al. also demonstrated that undetectable amounts of transactivated DeltaE are sufficient to transactivate the HES-1 promoter (55). Accordingly, when we performed immunoprecipitations on [35S]Met-labelled extracts of 293T cells transiently transfected with the DeltaE construct, we were unable to detect any intracellular processing (our unpublished data). In contrast, when the same experiment was performed with a C-terminally truncated DeltaE lacking the PEST domain, the processed form was clearly detectable (our unpublished data and reference 36). PEST sequences are found in proteins which undergo rapid turnover (51), and evidence exists that PEST regions can serve as signals for proteolytic degradation (see reference 50 for a review). Our observations suggest that the DeltaE-derived processed intracellular form has a very short half-life. Consistent with this hypothesis, the use of a specific proteasome inhibitor allowed detection of a DeltaE-derived processed form (our unpublished data). Thus, it is possible that the combination of a limited number of DeltaE-mobilized Notch-1 molecules with rapid turnover of the induced intracellular cleaved forms precludes detection of such a processed form. Alternatively, it may be that Dl-1-induced stimulation of the Notch-1 receptor does not lead to intracellular processing. Nevertheless, recent studies done with transgenic flies expressing a Notch-Gal4 chimera to allow detection of the intracellular cleaved fragment (39, 59), or overexpression of Notch-1 and Jagged-1 by transient cotransfection in mammalian cells (55), strongly suggest that ligand activation of the Notch receptor indeed results in processing of the intracellular part of the receptor.

We also showed that exposure of myoblastic C2 cells to Dl-1 results in the suppression of muscle differentiation and in increased levels of endogenous HES-1 mRNA in the C2 cells. These results suggest that Dl-1 activates similar Notch pathways in HeLa-N1 cells and in C2 cells. Since C2 cells endogenously expressed different Notch genes, it remains to be determined which Notch receptor is activated by Dl-1 in these cells and whether transactivation of HES-1 is required for suppression of muscle differentiation.

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REFERENCES


