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Involvement of RBP-J in biological functions of mouse Notch1 and its derivatives

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SUMMARY

Notch is involved in the cell fate determination of many cell lineages. The intracellular region (RAMIC) of Notch1 transactivates genes by interaction with a DNA binding protein RBP-J. We have compared the activities of mouse RAMIC and its derivatives in transactivation and differentiation suppression of myogenic precursor cells. RAMIC comprises two separate domains, IC for transactivation and RAM for RBP-J binding. Although the physical interaction of IC with RBP-J was much weaker than with RAM, transactivation activity of IC was shown to involve RBP-J by using an RBP-J null mutant cell line. IC showed differentiation suppression activity that was generally comparable to its transactivation activity. The RBP-J-VP16 fusion protein, which has strong transactivation activity, also suppressed myogenesis of C2C12. The RAM domain, which has no other activities than binding to RBP-J, synergistically stimulated transactivation activity of IC to the level of RAMIC. The RAM domain was proposed to compete with a putative co-repressor for binding to RBP-J because the RAM domain can also stimulate the activity of RBP-J-VP16. These results taken together, indicate that differentiation suppression of myogenic precursor cells by Notch signalling is due to transactivation of genes carrying RBP-J binding motifs.

Key words: RBP-J, RAM domain, transactivation, differentiation suppression, mouse, Notch

INTRODUCTION

The Notch receptor is involved in the cell fate determination of various lineages including nerve, muscle and germ cells in *Drosophila* (Muskavitch, 1994; Artavanis-Tsakonas et al., 1995). Although Delta and Serrate have been shown to be ligands for Notch (Rebay et al., 1991), most studies on the biological function of Notch have been done using the truncated intracellular form of Notch, which shows constitutively active phenotypes. Overexpression of the truncated Notch in the *Drosophila* embryo suppresses neuroblast segregation (Rebay et al., 1991; Lieber et al., 1993; Struhl et al., 1993). Expression of the intracellular region (RAMIC) of mouse Notch suppresses myogenesis from myogenic precursor cells and neurogenesis from neuronal precursor cells (Nye et al., 1994). Chromosomal translocations that cause expression of the truncated form of human Notch (TAN-1) are found in a subset of acute human T cell lymphoblastic lymphoma (Ellisen et al., 1991). More recently expression of human TAN-1 from a retroviral vector was shown to transform murine T cells with high frequency (Pear et al., 1996). In general Notch signalling brings cells into the proliferative phase and blocks cell differentiation.

Notch contains several functionally important motifs: a RAM domain, CDC10/ankyrin repeats, nuclear localization signals, OPA and PEST. The RAM domain, located immediately downstream of the transmembrane region, interacts with Suppressor of Hairless (Su(H)) or its mammalian homologue RBP-J (previously called RBP-Jκ) (Tamura et al., 1995; Minoguchi et al., 1997), a nuclear DNA binding protein recognizing a DNA sequence CGTGGGAA (Tun et al., 1994). The Notch RAMIC binds to RBP-J at the RAM domain. Interaction of RAMIC with RBP-J leads to transactivation of genes that have RBP-J-binding sequences in their promoter regions (Jarriault et al., 1995). Similar RBP-J binding and transactivation activities are found in all four mammalian Notch members (Kato et al., 1996). The CDC10/ankyrin repeats are also essential for the transactivation activity and suppression of differentiation (Kopan et al., 1994; Jarriault et al., 1995), but their precise function is still unknown. There are at least two nuclear localization signal sequences at both sides of the CDC10 repeats. The glutamine-rich OPA sequence is often found in homeobox genes but its function is totally unknown. The PEST sequence, which is suggested to be involved in recognition of proteolytic cleavage, is located at the C terminus of Notch, but its function in this receptor is totally unknown.

Since the DNA binding protein RBP-J and the cell surface receptor Notch directly interact, signaling through Notch is considered to be unique among other receptors. Binding of the transmembrane ligand Delta to Notch is proposed to induce pro-

teolytic cleavage of Notch, giving rise to RAMIC, which migrates to the nucleus and interacts with RBP-J (Jarriault et al., 1995; Tamura et al., 1995). Notch signalling regulates transcription of genes essential for differentiation of nerve and muscle cells. In *Drosophila* activation of Su(H) induces the expression of genes of the *Enhancer of split (E(spl))* complex (Lecourtois and Schweisguth, 1995; Furukawa et al., 1995), encoding basic Helix-Loop-Helix (bHLH) transcription factors that appear to negatively regulate the expression of another set of bHLH proteins encoded by the *achaete-scute* (*ac-sc*) proneural genes, leading to suppression of the neural phenotype. Essentially a similar sequence of events takes place in Notch signalling in vertebrate systems (de la Pompa et al., 1997; Wettstein et al., 1997). Although the *HES-1* gene, the mammalian homologue of the *Drosophila* hairy gene encoding a bHLH protein, contains the RBP-J binding sequence in its promoter and is transactivated by RAMIC in vitro (Takebayashi et al., 1994; Jarriault et al., 1995), expression of the *HES-1* gene was not significantly affected in *RBP-J*$^{-/-}$ mutants (de la Pompa et al., 1997). Thus key target genes regulated by mammalian Notch signalling are still unknown.

The RAM domain-deleted intracellular region of Notch, which is referred to as IC, has been shown to have differentiation suppression activity *in vitro* (Kopan et al., 1994). Recently Shawber et al. (1996) proposed that there may also be an RBP-J-independent signalling pathway of Notch IC because IC mediated differentiation suppression of myogenesis while it did not transactivate promoters carrying the RBP-J binding motif in HeLa cells. However, the genetic data in *Drosophila* show a close linkage between Notch and Su(H) (Fortini and Artavanis-Tsakonas, 1994). In addition, phenotypes of Notch$^{-/-}$ and RBP-J$^{-/-}$ appear to be similar (Swiatek et al., 1994; Conlon et al., 1995; Oka et al., 1995).

To clarify the signalling pathways we compared the activities of RAMIC and its mutants in RBP-J binding, transactivation and myogenic suppression. We found that both RAMIC and IC require RBP-J for their transactivation activity. Transactivation and myogenesis suppression activities of various constructs were in parallel. The results indicate that the negative regulation of muscle differentiation is under the control of genes carrying the RBP-J binding motif.

MATERIALS AND METHODS

Construction of plasmids

pEF-BOSneo-RAMIC was constructed from pCS2+MTmNotchIC (Kopan et al., 1994) and RAM23 (Tamura et al., 1995). The IC fragment and its 3′ truncated forms were derived from pCS2+MTm-NotchIC. Its mutant versions (ICM1 and ICM2) were synthesized by the polymerase chain reaction (PCR) as described (Kopan et al., 1994). RAM23 and RAMmM2-2 fragments synthesized by PCR were flanked by a c-myc-tag (Tamura et al., 1995). All of these fragments were cloned into pEF-BOSneo vector (Mizushima and Nagata, 1990). pEF-BOSneo-RBP-J or pEF-BOSneo-RBP(R218H) were constructed from CDM8-RBP-J or CDM8-RBP(R218H) (Chung et al., 1994) in the pEF-BOSneo vector. pGa981-6 contains the hexamerized 50-bp EBNA2 response element of the TP-1 promoter (ERE-TP1) in front of the minimal β-globin promoter driving the luciferase gene. *HES-1-luc* contains the −194 to +60 promoter fragment of the *HES-1* gene (Takebayashi et al., 1994) cloned upstream of the luciferase gene in the pGV-B-basic vector (TOYO-INKI Co Ltd, Tokyo). TK-MH 100 × 4 LUC (Kang et al., 1993) was kindly provided by K. Umesono. GAL4-ankyrin contains IC Ankyrin, amino acids 1848-2170 of mouse Notch1 (Tamura et al., 1995) fused to the GAL4 DNA binding domain (1-147) derived from pGBT9 (CLONTECH Lab. Inc.). pSG5Flag-RBP-VP16 was a gift from E. Manet (Walzer et al., 1995).

For the differentiation suppression assay, RAM23 and its RBP-J binding-defective mutant RAMmM2-2 (Tamura et al., 1995) were ligated to the *Bam*HI site within the hinge region of human IgG_1 and cloned into the pEFBOSneo vector. The pMKIT RBP-J gene10 was described previously (Tamura et al., 1995).

Transactivation assay

COS7 cells were transfected in 6-well plates by the lipofection method using the lipofectamine (GIBCO BRL), with 0.2 μg luciferase vector alone or in combination with pEF-BOSneo-RAMIC and various amounts of pEF-BOSneo-R218H or pEF-BOSneo-RAM23 construct; 100 ng *SV40-lacZ* construct was included in each transfection as an internal control. Three independent experiments were carried out. Bars in figures indicate s.d. The luciferase activity was measured 48 h after transfection in a luminometer LumatLB9501 (Berthold, Wildbach) and normalized according to the β-galactosidase activity. The induction of luciferase activity was calculated as ratio of the luciferase activity of the reporter plasmid containing the RBP-J binding site to that of a negative control reporter plasmid that has no RBP-J binding site, unless otherwise specified.

Establishment of RBP-J$^{-/-}$ cell line (OT-11)

Fibroblastic cell lines were established by culturing dissociated cells from embryos 9.5 days post-ferilization, generated by the cross of heterozygotes between RBP-J$^{-/-}$ (Oka et al., 1995) and H-2K^{b}-tsA58 (Jat et al., 1991), as described. The four lines (OT-11, OT-12, OT-13 and OT-21) obtained were examined for the genotype of the RBP-J gene by Southern blot hybridization. OT-11 and OT-12 were RBP-J$^{-/-}$ and OT-13 and OT-21 were RBP-J$^{+/+}$.

Differentiation suppression assay

C2C12 cells were transfected as described above. Following transfection cells were transferred to Dulbecco's Modified Eagle's Medium containing 3% horse serum, 100 U/ml penicillin and 2 mM L-glutamine. After 3.5 days the cells were fixed for 30 minutes in 2% paraformaldehyde, followed by washing with PBS(−), and permeabilized for 10 minutes in 100% ethanol at −20°C. Cell monolayers were then incubated with an anti-myoglobin polyclonal antibody (Nichirei) and with either anti-myc mAb (9E10) for Notch-related proteins, or K0043 (Sakai et al., 1995) for RBP-J-related proteins. Following washes the antibodies were detected with Texas Red-labeled anti-rabbit IgG antibodies (Organon Teknika N.V.-Cappel Products) and with either DTAF-labeled anti-mouse IgG antibodies (Chemicon International, Inc.) or FITC-labeled anti-rat antibodies (Organon Teknika N.V.-Cappel Products). RAM23 and RAM23mM2-2 coupled with human IgG were directly detected by FITC-labeled anti-human IgG antibodies (Organon Teknika N.V.-Cappel Products). DAPI was used for nuclear staining. Slides were mounted in glycerol and viewed with a Zeiss fluorescence microscope. The images were incorporated into the computer, overlayed with each other and signals were enhanced using Photoshop (Adobe Systems Inc.) for counting the number of nuclei. The proportion of areas that were positive for myoglobin staining was measured by a microcomputer imaging device (MCID) (Imaging Research Inc.) to monitor differentiation efficiency of non-transfectants.

RESULTS

Transactivation activity of RAMIC, IC and their mutants

Transactivation activities of RAMIC, IC and their mutants (Fig. 1) were compared using different read-out constructs.

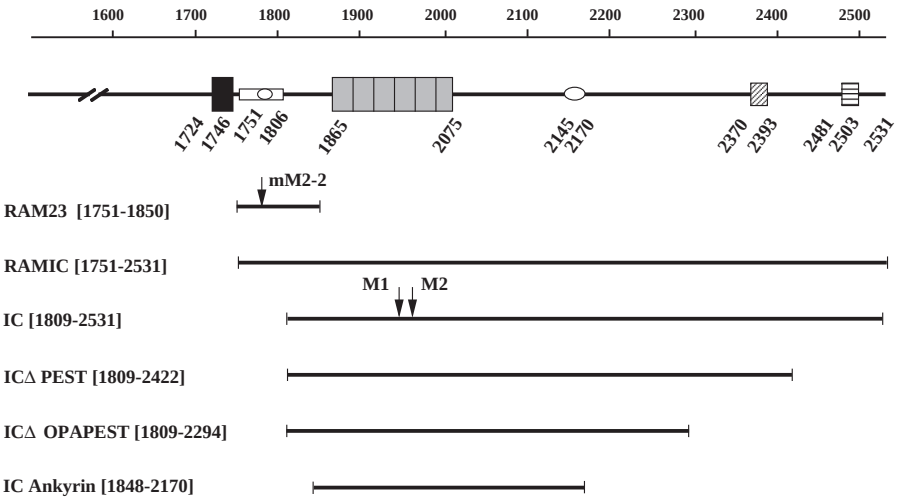

Fig. 1. Schematic representation of mouse Notch1 derivatives used in this study. The top lines show the amino acid positions of mouse Notch1 with structural motifs. Truncated forms of Notch1 are shown below by horizontal bars with amino acid residue numbers in brackets. Closed, open, dotted, diagonal-hatched and horizontal-hatched boxes indicate the transmembrane, RAM domain, CDC10/ankyrin repeats, OPA sequence and PEST sequence, respectively. Ellipses show nuclear localization signals. Mutation sites are indicated by vertical arrows.

RAMIC transactivated both *ERE-TP1* and *HES-1* promoters by cotransfection of COS7 cells (Jarriault et al., 1995; Kato et al., 1996). Endogenous RBP-J appears to be involved in transactivation by RAMIC because the RAMIC activities were blocked by competition with either RAM23 or a mutant of RBP-J (R218H) that cannot bind to DNA (Kato et al., 1996; our unpublished data). The RAM domain would compete with RAMIC for binding to endogenous RBP-J while the R218H mutant would compete with endogenous RBP-J for binding to RAMIC or unknown cofactors. IC, the Notch intracellular region devoid of the RAM domain, has been reported to have weak but significant transactivation activity of the *HES-1* promoter in HeLa cells (Jarriault et al., 1995). We have confirmed that IC has a weak but significant transactivation activity of both *ERE-TP-1* and *HES-1* promoters in COS cells as compared with RAMIC (Fig. 2A,B). In addition, the mutations in the CDC10/ankyrin repeats of IC (ICM1 and ICM2) that were shown to inactivate the biological function of IC (Kopan et al., 1994) abolished this transactivation activity (Fig. 2A,B). Furthermore, C-terminally truncated forms of IC, in which the PEST and OPA sequences are deleted, also showed transactivation activity that was similar to IC (Fig. 2C). However, a further C-terminal truncation product IC Ankyrin, which contains the ankyrin repeats and the nuclear localization signal, lost the transactivation activity. Furthermore, the transactivation of both *ERE-TP1* and *HES-1* promoters by IC showed dominant negative suppression by RBP-J (R218H), which cannot bind DNA (Fig. 3A,B). These results suggest that the transactivation activity of IC is mediated by direct or indirect interaction with RBP-J.

Direct evidence for requirement of RBP-J for the activity of IC

We then examined if the transactivation activity of IC is dependent on RBP-J. For this purpose we established a fibroblastic cell line (OT-11) from RBP-J$^{-/-}$ embryos. OT-11 cells were transfected with the IC construct and a reporter plasmid of *(CBF-1)$_6$-luc1* (Hsieh et al., 1996). As shown in Fig. 4, neither RAMIC nor IC alone showed any transactivation activity above a backgound level. In contrast, the addition of the RBP-J construct to either RAMIC or IC showed strong transactivation activity. However, the M1 or M2 mutant of IC did not show any transactivation activity even in the presence of RBP-J. Similar results were obtained with the other reporter constructs used in the present study, namely *ERE-TP-1-luc* and *HES-1-luc* (data not shown). These results convincingly indicate that the transactivation activity of IC as well as RAMIC is dependent on RBP-J.

Interaction between RBP-J and ankyrin repeats of IC

To examine the interaction between RBP-J and IC, a two-hybrid assay in a mammalian system was introduced. Two fusion protein constructs (one between the GAL4 DNA binding domain and the IC CDC10/ankyrin repeats, and the other between RBP-J and VP16) were cotransfected into COS7 cells with a reporter plasmid containing the basic TK promoter flanked by GAL4 DNA binding motifs (Fig. 5). Interaction between the ankyrin repeats and RBP-J would allow transcription of the reporter plasmid. GAL4-ankyrin did not show any transactivation activity in agreement with the the absence of activity in IC Ankyrin (Fig. 2C). As expected, however, RBP-J-VP16 together with GAL4-ankyrin augmented transcription in a dose-dependent manner (Fig. 5, lanes 4-6). On the other hand, the absence of the ankyrin repeats or the M1 mutation in the ankyrin repeats abolished augmentation of the transcription by RBP-J-VP16. These results indicate the functional interaction between RBP-J and the ankyrin repeats of IC.

The physical interaction between RBP-J and IC was examined by immunoprecipitation after expression of c-myc epitope-tagged IC and gene 10 epitope-tagged RBP-J in COS7 cells by transfection (Fig. 6). IC as well as RAMIC was coimmunoprecipitated with RBP-J by using anti-T7 monoclonal antibody (mAb) recognizing the gene 10 epitope, although the amount of IC co-immunoprecipitated with RBP-J was much lower than that of RAMIC because of the absence of the RAM domain in IC. The M1 mutant of IC was not coimmunoprecipitated with RBP-J. Control experiments indicate the amounts of RBP-J immunoprecipitated were almost identical. These results clearly indicate that the ankyrin repeats of IC interact with RBP-J, albeit less strongly than the RAM domain, in agreement with the two-hybrid assay described above.

Suppression of myogenesis by Notch and its derivatives

We then compared the relative differentiation suppressive activities of RAMIC, IC, RBP-J and their mutants. To quantitate differentiation suppression activity, two-color staining experiments that detect both transfected cells and their expression of myoglobin were carried out (Fig. 7). The differentiation frequency of cells expressing RAMIC, IC, RBP-J or their mutants in nuclei was measured. The differentiation frequency of non-transfectants in each dish was monitored and shown to be the same between compared sets.

IC, which had weaker transactivation activity than RAMIC, had a level of differentiation suppression activity comparable with its transactivation activity (Fig. 7A,B; Table 1). By contrast, transactivation-incompetent derivatives of Notch 1 such as ICM1, RAM23 and RAMmM2-2 all have negligible levels of differentiation suppression activity (Fig. 7C-E). The RBP-J-VP16 fusion protein, which was shown to have strong transactivation activity of the *TK* promoter fused with RBP-J binding motifs (Waltzer et al., 1995), suppressed myogenesis of C2C12 markedly (Fig. 7H). However, both RBP-J and RBP-J (R218H), which have no transactivation activity of the *ERE-TP-1* and *HES-1* promoters (Fig. 3), were negative for differentiation suppression activity (Fig. 7F,G). These results indicate that transactivation activities of mouse Notch1 and their derivatives generally parallel their differentiation suppression activities.

The functional cooperation between the RAM domain and IC

To our surprise, the transactivation activity of IC was synergistically augmented in a dose-dependent manner by the addition of RAM23, which has strong affinity to RBP-J but no transactivation activity by itself (Fig. 2A,B). The transactivation activity of IC in the presence of RAM23 reached a level similar to RAMIC. The synergistic effect with RAM23 was conserved even by the deletion of the PEST and OPA sequences (Fig. 2C). However, IC Ankyrin with the further deletion proximal to the nuclear localization signal greatly reduced the synergistic activity. The synergistic effect was also lost when loss-of-function mutations (ICM1 and ICM2) were introduced into the CDC10/ankyrin repeats of IC, suggesting that the ankyrin repeats are essential for the synergy with RAM23. Inversely, the synergistic effect was also abolished when RAM23 was replaced by its mutant (mM2-2), which cannot bind to RBP-J (Tamura et al., 1995) (Fig. 2D).

To understand the molecular mechanism underlying synergy

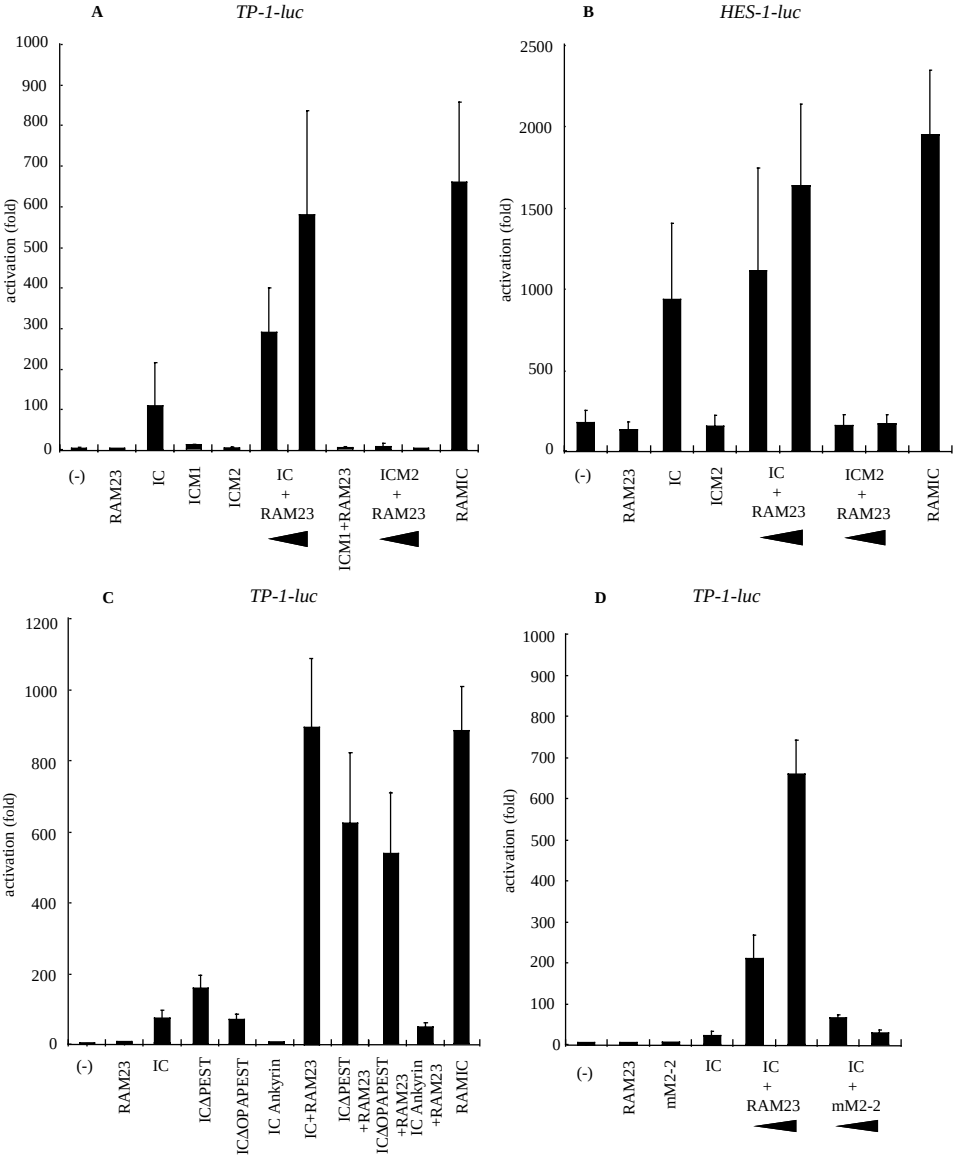

Fig. 2. Transactivation activity of IC and its synergy with RAM23. COS7 cells were transfected with a luciferase reporter plasmid alone (–) or together with various expression vectors (pEF-BOSneo-RAM23, pEF-BOSneo-mM2-2, pEF-BOSneo-IC, pEF-BOSneo-ICM1, pEF-BOSneo-ICM2, pEF-BOSneo-RAMIC) as indicated. 0.25 μg/3.5 cm dish of pEF-BOSneo-IC, pEF-BOSneo-ICM1 or pEF-BOSneo-ICM2 were introduced into COS7 cells together with a reporter plasmid *ERE-TP-1-luc* (A,C,D) or *HES-1-luc* (B) and different amounts (1.25 μg or 2.5 μg/3.5 cm dish) of pEF-BOSneo-RAM23 (A,B,D) or pEF-BOSneo-mM2-2 (D), as indicated by arrowheads. In C 1.25 μg of pEF-BOSneo-RAM23 were used. The ratio of the luciferase reporter plasmid/pEF-BOSneo-IC, -ICM1, or -ICM2/pEF-BOSneo-RAMIC was 1/1.25/1.25 in all co-transfection experiments. Transfection and assays were carried out as described in Materials and Methods.

Table 1. Relative suppressive effects on C2C12 cell differentiation by Notch and EBNA2 derivatives

Expt group	Transfected constructs	Differentiation % (nuclei counted)	Relative suppression (%)	Relative transactivation (%)	
				TP-1	HES-1
1	RAMIC	23 (333)	72.3	100	100
	IC	41 (242)	50.6	7.6	53.0
	IC-M1	83 (275)	0	0	0
2	RAM23	60 (479)	6	0	0
	RAM mM2-2	64 (823)	0	0	0
3	RBP-J-VP16	9 (225)	89.8	100	100
	RBP-J	83 (638)	5.7	0	0
	R218H	88 (235)	0	0	0

C2C12 cells were transfected with the plasmids indicated and differentiation into myogenic cells was monitored by double staining, as shown in Fig. 7. Differentiation frequency was calculated by counting numbers of transfected nuclei in myoglobin-expressing cells. Differentiation frequencies were comparable only among plasmids whose expression was measured by the same antibody because detection levels of transfected nuclei are variable with different antibodies. Differentiation frequencies of non-transfected cells were the same among the sets compared and almost identical to transfectants of mutants that had weakest suppression. Relative suppression was calculated by the ratio of their activity to the level of the non-transfected cells. Antibodies used for detection of transfectants were as follows: group 1, anti-c-myc mAb; group 2, anti-human IgG1 Ab; group 3, anti-RBP-J K0043mAb. Relative transactivation rate is the average value of our data.

between the RAM domain and IC, we first tested whether binding of the RAM domain to RBP-J would induce a conformational change of RBP-J, resulting in augmentation of interaction of IC with RBP-J. However, we did not see any change in the amount of IC coimmunoprecipitated with RBP-J in the presence or absence of RAM23 (data not shown). We then examined whether the synergistic effect of the RAM domain is dependent on IC or not. A fusion protein of RBP-J with the viral activation domain VP16 (RBP-J-VP16) has been shown to transactivate the promoter containing the RBP-J binding motif (Waltzer et al., 1995). RAM23 augmented the transactivation activity of RBP-J-VP16 in a dose-dependent manner (Fig. 8). The results indicate that the synergistic effect of RAM23 does not require IC. Interaction of RAM23 with RBP-J *per se* appears to stimulate the activity of the transactivation domain (VP16). Since RBP-J-VP16 is a fusion protein of the DNA binding protein (RBP-J) and the activation domain (VP16), augmentation of the transactivation activity of IC by RAM23 is unlikely to be due to augmented interaction between RBP-J and IC.

DISCUSSION

Correlation between transactivation and differentiation regulation

We have compared relative transactivation and myogenesis suppression activities among mouse Notch1, RBP-J and their variants (Table 1). We have shown that relative transactivation activities on two promoters (*ERE-TP-1* and *HES-1*) by mouse Notch 1 and its derivatives are in general agreement. Although recent studies indicate that the *HES-1* gene may not be the major target of Notch signalling (Shawber et al., 1996; de la Pompa et al., 1997), the *HES-1* promoter is useful for assessment of transactivation activities of Notch1 and its derivatives. A strong transactivator such as RAMIC suppressed myogenesis markedly. The correlation between the transactivation activity and differentiation suppression was also obvious for IC that has weaker transactivation and milder differentiation suppression activities than RAMIC. The replacement mutant (ICM1) of IC that has neither RBP-J binding nor transactivation activity is negative for differentiation suppression activity. Furthermore, RBP-J-VP16 that has the potent transactivation activity of promoters containing the RBP-J binding element suppressed myogenesis strongly. It is likely that differentiation suppression of myogenesis by Notch signalling is mediated by transcriptional acti-

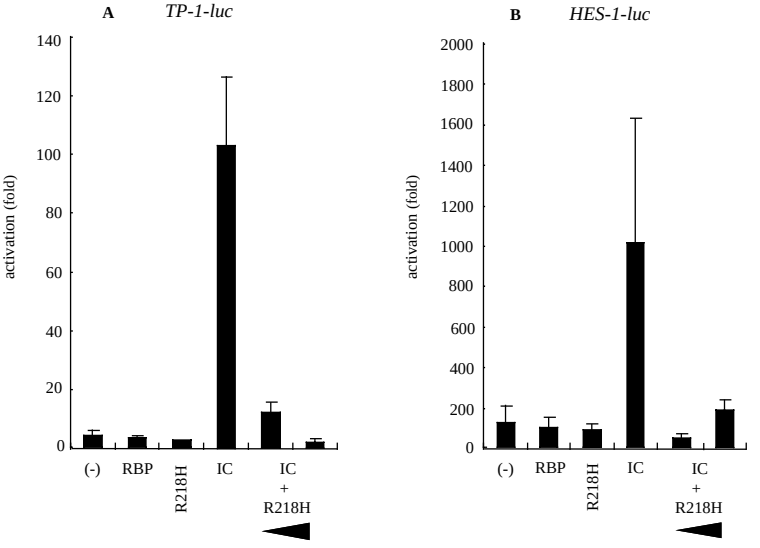

Fig. 3. The effects of RBP-J (R218H) on the transcriptional activity of IC. COS7 cells were transfected with a luciferase reporter plasmid alone (–) or together with various expression vectors. 0.2 μg/3.5 cm dish of pEF-BOSneo-IC were introduced into COS7 cells together with a reporter plasmid *ERE-TP-1-luc* (A) or *HES-1-luc* (B) and different amounts (0.4 μg or 2.0 μg/3.5 cm dish) of pEF-BOSneo-RBP-J or pEF-BOSneo-R218H, as indicated by arrowheads. The luciferase reporter plasmid/pEF-BOSneo-RAMIC-(IC) ratio was 1/1 in all co-transfection experiments. Transfection and assays were carried out as described in Materials and Methods. RBP, RBP-J. R218H, RBP-J(R218H).

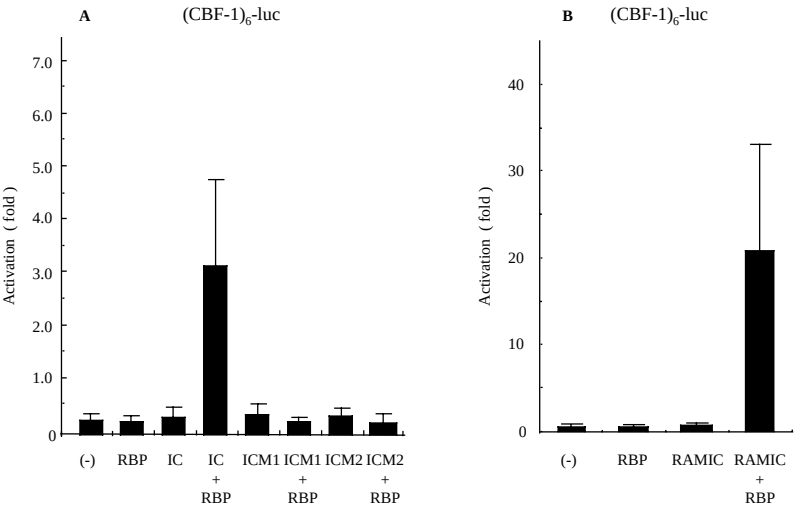

Fig. 4. Transactivation activities of IC, ICM1, ICM2 and RAMIC in OT-11 ($RBP^{-/-}$) cells. OT-11 cells were transfected using calcium phosphate coprecipitation method with a luciferase reporter plasmid alone (–) or together with various expression vectors (pEF-BOSneo-IC, pEF-BOSneo-ICM1, pEF-BOSneo-ICM2 and pEF-BOSneo-RAMIC), as indicated. 1.0 μg/3.5 cm dish of pEF-BOSneo-IC, pEF-BOSneo-ICM1, pEF-BOSneo-ICM2 (A) or pEF-BOSneo-RAMIC (B) were introduced into OT-11 cells together with a reporter plamid 6 × wt CBF1-luc (Hsieh et al. 1996) or 10 ng of pEF-BOSneo-RBP-J as indicated. 6 × mtCBF1-luc was used as a negative control reporter plasmid (Hsieh et al. 1996). RBP, RBP-J.

vation of genes that have the RBP-J binding element and negatively regulate myogenic genes.

Does IC utilize an RBP-J independent pathway for differentiation suppression?

The weak transcription activation activity of mouse IC was first reported in HeLa cells by Jarriault et al. (1995) and confirmed in COS7 cells in the present study. The ratios of the transactivation activities of IC to RAMIC were 7.6±0.56 and 53.0±8.6 for the *ERE TP-1* and *HES-1* promoters, respectively (Table 1). Recently Shawber et al. (1996) proposed that rat IC activates another signalling pathway which does not involve RBP-J because IC can suppress myogenesis of C2C12 cells in spite of the undetectable level of transactivation of the simian virus 40 promoter flanked by four copies of RBP-J binding motifs in HeLa cells. The difference in the transactivation activity of IC could be partly due to the sensitivity of the read-out system, because only 20-fold transactivation was seen by a construct similar to RAMIC as compared with vector alone in their system, whereas 160-fold and 600-fold transactivations were observed by Jarriault et al. (1995) and in the present study, respectively. In fact, we confirmed that IC can show about 20-fold and 11-fold transactivation of the β-globin basic promoter flanked by six copies of the RBP-J binding motif sequence identical to that used by Shawber et al. (1996) in COS7 cells (our unpublished data) and OT-11 cells (Fig. 4), respectively. However, we did confirm that the transactivation activity of IC is negligible (1.6% of RAMIC) in HeLa cells using the ERE TP-1 reporter (our unpublished data). The discrepancy could thus be due to cell lines used.

We have shown that the transactivation activity of IC involves RBP-J because it is synergized by RAM23 and suppressed dominant negatively by RBP-J (R218H) (Figs 2 and 3). Furthermore, the synergistic activity of RAM23 was abolished by the replacement mutation, which destroys RBP-J binding activity of RAM23. The transactivation activity of IC is completely dependent on RBP-J because IC and RAMIC have no activity in the RBP-J null cell line (OT-11) (Fig. 4). In addition, we have shown that the ankyrin repeats of IC interact weakly with RBP-J, in agreement with previous reports (Jarriault et al., 1995; Aster et al., 1997). Although IC and RAMIC have been shown to have the differentiation suppression activity, the relative differentiation suppression activity of IC and RAMIC has not been assessed (Kopan et al., 1994). We have shown that the levels of myogenesis suppression by IC and RAMIC are correlated generally with their transactivation activities. Taken together, IC most likely utilizes the transactivation pathway involving RBP-J for suppression of C2C12 cell myogenesis.

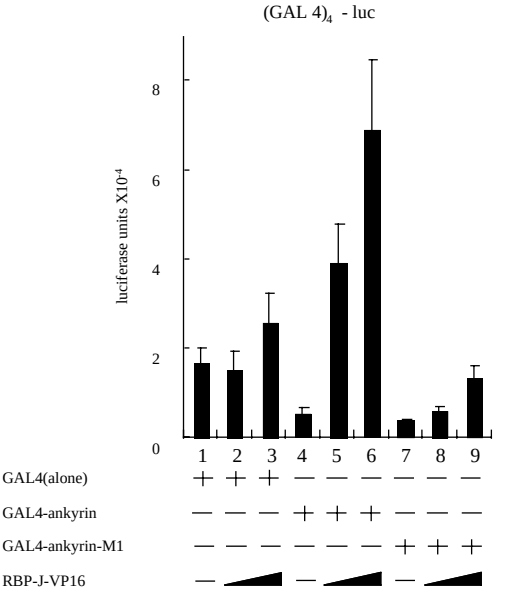

Fig. 5. Notch CDC10/ankyrin repeats interact with RBP-J in mammalian cells. COS7 cells were transfected with a luciferase reporter plasmid containing GAL4 binding sites (TK-MH 100 × 4 LUC) and indicated expression constructs. 0.4 μg of a reporter plasmid, 0.3 μg GAL4 (1-147) fusion constructs, and increasing amounts (0, 0.3 μg, 1.2 μg) of pSG5Flag-RBP-VP16 were used. Transfection and assays were carried out as described in Materials and Methods, except that the luciferase activity of the reporter plasmid containing the RBP-J binding site was shown.

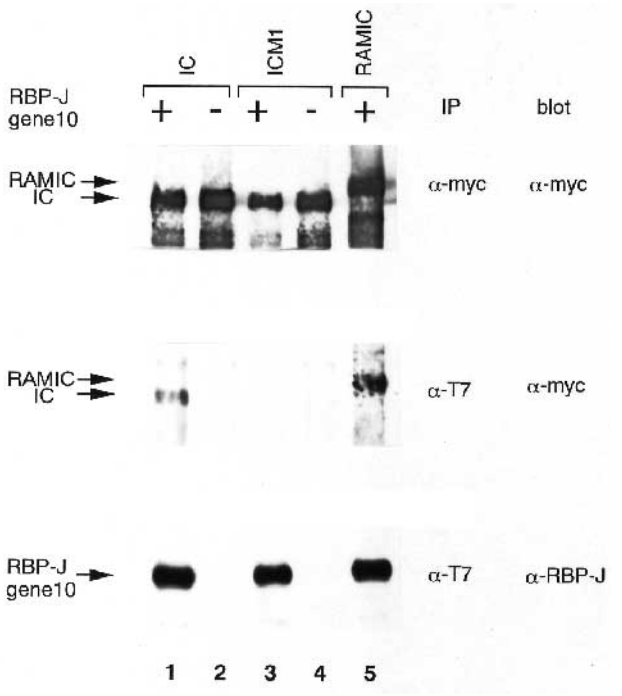

Fig. 6. Lysates from COS7 cells transfected with pEF-BOSneo-RAMIC (lane 5), pEF-BOSneo-IC (lanes 1 and 2), or pEF-BOSneo-ICM1 (lanes 3 and 4) with (lanes 1, 3, and 5) or without (lanes 2 and 4) T7 gene 10 epitope-tagged RBP-J (pMKIT RBP-J gene 10) were divided into two samples. One was subjected to immunoprecipitation (IP) with the T7 mAb for coimmunoprecipitation analysis and the other with the anti-myc mAb (α-myc) for confirmation of equivalent expression of the Notch variants. The immune complexes were purified with protein A-Sepharose beads and then separated by electrophoresis in a 8% sodium dodecyl sulfate-polyacrylamide gel and analysed by western blotting (blot). The gel of immunoprecipitates with the T7 antibody was cut horizontally into two. The upper part of the gel was used to monitor coprecipitation of the Notch variants with the anti-myc antibody, and the lower part was used to confirm precipitation of RBP-J with the anti-RBP-J monoclonal antibodies.

Separate roles of the RAM domain and the ankyrin repeats

RAMIC interacts with RBP-J at two regions, namely the RAM domain and the ankyrin repeats of IC. IC generated by deletion of the RAM domain from RAMIC retains the biological function of RAMIC, at least at a lower level. Furthermore, IC and RAM domains when mixed together exibit transcription activity similar to RAMIC. These results suggest that the RAM domain and the ankyrin repeats have different roles in the transactivation activity of RAMIC. The RAM domain does not appear to enhance interaction of IC with RBP-J. The synergistic activity of the RAM domain requires interaction with RBP-J but not the involvement of IC per se, because RAM23 augmented transactivation activity of RBP-J-VP16 (Fig. 5). We therefore propose that the RAM domain competes with a putative co-repressor for binding to RBP-J. The synergistic activity of RAM23 may be mediated by blocking repressor binding to RBP-J. This notion is supported by the fact that excess amounts of RAM23 are required to achieve the full activity. Furthermore, excess amounts of RAM23 augmented transactivation activity by IC in HeLa cells to a level comparable to RAMIC, even though the IC activity was negligible in the absence of RAM23 (unpublished data). The results can be interpreted to indicate that HeLa cells contain much more co-repressor(s) than COS7 and OT-11 cells.

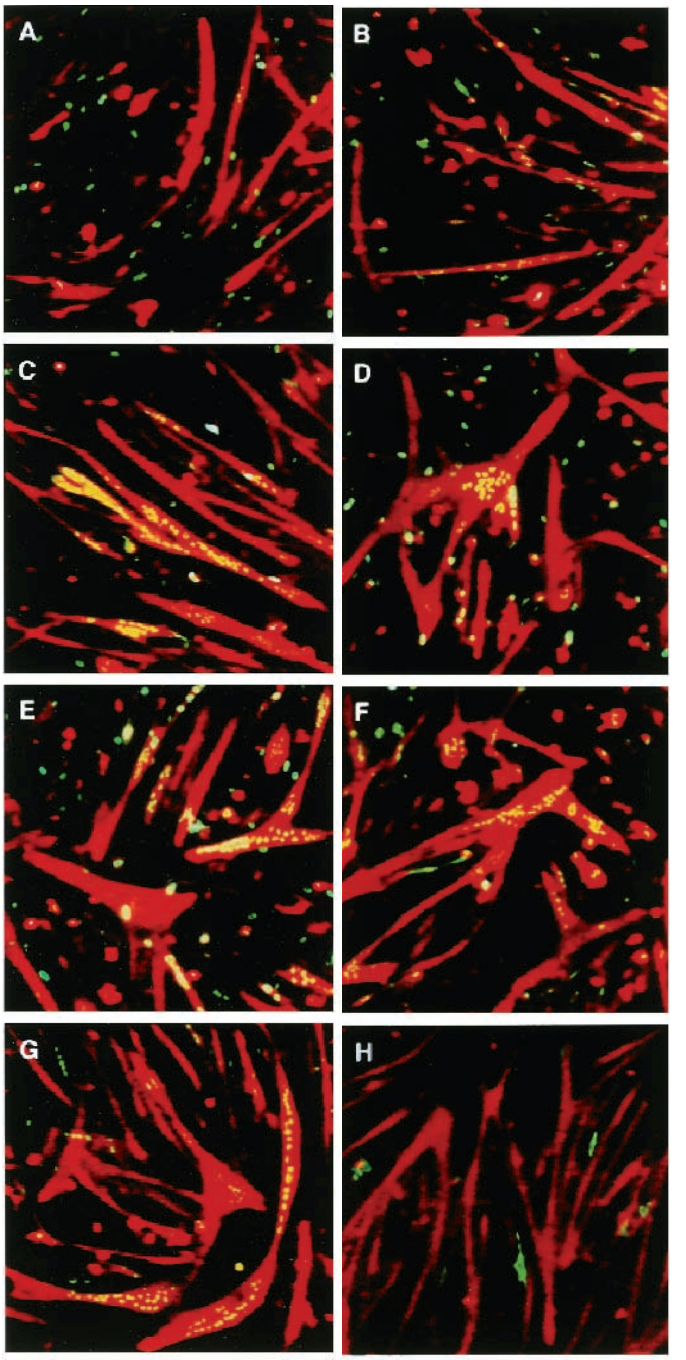

Fig. 7. Suppression of myogenic differentiation of C2C12 cells by Notch and RBP-J mutants. The Notch (A-E) and RBP-J (F-H) mutant proteins are stained green while the differentiated cells, as judged by their myoglobin expression, are stained red. Nuclei of transfected cells that underwent differentiation look yellow. Differentiation of C2C12 cells is suppressed strongly with RAMIC (A) and RBP-J-VP16 (H), and partially with IC (B). No or weak, if any, differentiation suppression was observed for IC-M1 (C), RAM23 (D), RAMm2-2 (E), RBP-J (F) and R218H (G).

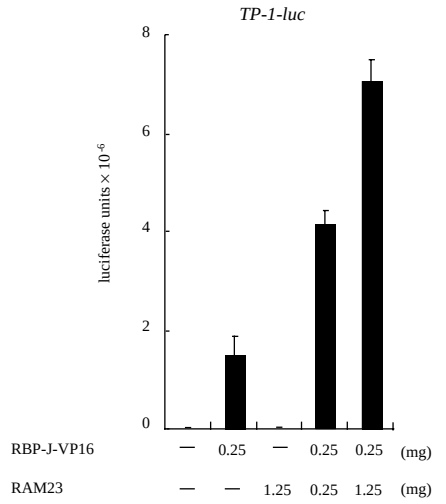

Fig. 8. RAM23 domain enhances transactivation activity of RBP-J-VP16. COS7 cells were transfected with a luciferase reporter plasmid *ERE-TP1-luc* (pGa981-6) and indicated expression constructs. 0.4 μg of a reporter plasmid and indicated amounts of pSG5Flag-RBP-VP16 and pEF-BOSneo-RAM23 were used. Transfection and assays were carried out as described in Materials and Methods, except that the luciferase activity of the reporter plasmid containing the RBP-J binding site was shown.

IC has transactivation activity by its weak interaction with RBP-J. The M2 mutation in RAMIC or IC, which abolishes interaction of the CDC10/ankyrin repeats with RBP-J, destroys the biological functions of RAMIC or IC, including transactivation (Jarriault et al., 1995) and differentiation suppression (Kopan et al., 1994). However, C-terminal truncation gradually reduces its activity. IC Ankyrin, which contains the ankyrin repeats and the nuclear localization signal, has negligible transactivation activity (Figs 2 and 5). The results suggest that IC may be further divided into two functional domains: RBP-J binding domain, i.e. the ankyrin repeats, and the transactivation domain, probably located in the region between residues 2170 and 2531.

A truncated form (ANK) of IC, comprising primarily the six ankyrin repeats, was first reported to have the biological function in cell fate determination in *C. elegans* (Roehl and Kimble, 1993). The ANK protein was primarily expressed in the cytoplasm as it has no nuclear localization signals. The rat ANK protein was also shown to have differentiation suppression activity of C2C12 cells (Shawber et al., 1996). Although the authors did not describe the cytoplasmic localization of the rat ANK protein, it is most likely to be cytoplasmic. Kopan et al. (1994) reported that IC with deletion of nuclear localization signals has weak differentiation suppression activity in C2 myoblasts. However, the *Xenopus* ANK protein was shown to be inactive in transactivation of *Xenopus ESR-1*, a RBP-J-regulated gene homologous to *Enhancer of split* (Wettstein et al., 1997). It is, therefore, likely that cytoplasmic ANK blocks differentiation by dominant negative interaction with some cofactors involved in myogenesis or other cell fate determination, but does not transactivate target genes regulated by RBP-J. Since overexpression of ANK in the cytoplasm may give rise to non-physiological interaction with other proteins, it remains to be seen whether differentiation suppression by ANK is physiological or not.

In summary, RAMIC interacts with RBP-J at the RAM domain and the ankyrin repeats. We propose that the RAM domain interaction with RBP-J derepresses the transactivation activity. The ankyrin repeat interaction with RBP-J is responsible for transactivation, probably through interaction with the basic transcription complex at the C-terminal region of IC.

CONCLUSIONS

Lines of evidence indicate that RBP-J is involved in transactivation of genes by Notch. Notch has been shown to suppress differentiation of progenitor cells of various lineages. Comparison of Notch1 mutants indicate that loss-of-function levels in the two activities i.e. RBP-J-dependent transactivation and differentiation suppression, are in parallel. Furthermore, the RBP-J-VP16 fusion construct can suppress differentiation of myogenic progenitor cells. We therefore conclude that genes regulated by RBP-J are responsible for myogenic differentiation suppression by Notch signalling. We have also shown that RAMIC can be separated into at least two functional domains, RAM and IC.

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