Notch signaling is involved in many cell fate determination events in metazoans. Ligand binding results in proteolytic cleavage to release the signal-transducing Notch intracellular domain (NICD). The nuclear protein RBP-Jκ, when complexed with NICD, acts as a transcriptional activator which, in turn, induces a target gene of Notch such as the repressors HES/E(spl) and HERP2. Under physiological stimulation using co-culture with Notch ligand-expressing cells and target cells expressing Notch receptors, the HES1 gene and the HERP2 gene have been shown to be directly up-regulated by Notch ligand binding. However, expression of another member of the HERP family, HERP1, was not induced by ligand stimulation in any cells tested, leading to the suggestion that HERP1 may not be an immediate target gene of Notch or that Notch pathways can be cell type-specific. Because HERP1 appears to play a central role in the development of the aorta (Zhong, T. P., Rosenberg, M., Mohideen, M. A., Weinstein, B., and Fishman, M. C. (2000) Science 287, 1820–1824), we re-addressed the issue of its relationship with the Notch pathway by examining its expression in A10 smooth muscle cells derived from thoracic aorta. We show that in these specific cells HERP1 is also a direct target gene of Notch. NICD activates the HERP1 promoter in an RBP-Jκ-dependent manner, and induces expression of endogenous HERP1 mRNA as well as HERP1 protein in A10 cells. Co-culture with Notch ligand-bearing cells induces endogenous HERP1 mRNA expression in A10 cells, and these events occur even in the absence of de novo protein synthesis. In addition, RBP-Jκ proved essential for induction of HERP1 mRNA in Notch signaling because exogenous RBP-Jκ was sufficient to rescue HERP1 mRNA expression in RBP-Jκ-deficient cells. These findings provide the first solid evidence that HERP1 is a novel primary target gene of Notch and underscores the cell-specific complexity of the Notch regulatory pathway. Given that Notch signaling plays a crucial role in vascular development, Notch may derive its function via HERP family members.

The evolutionarily conserved Notch signaling pathway controls cell fate in metazoans through local cell-cell interactions.

Specific intercellular contacts activate this highly complex signaling cascade leading to down-regulation or inhibition of cell type-specific transcriptional activators. Cells are thus forced to take on a secondary fate or remain undifferentiated while awaiting later inductive signals (5–7). Interaction of Notch with its ligands such as the Delta and Jagged families leads to cleavage of the transmembrane Notch receptor, giving rise to the Notch intracellular domain (NICD) which subsequently migrates into the nucleus (8, 9). There the NICD associates with a transcriptional factor, RBP-Jκ (CSL/ICF1/Su(H)/LAG-1). In the absence of NICD, RBP-Jκ can act as a transcriptional repressor from its DNA-binding site (GTGGA) by associating with a co-repressor complex, and this association is disrupted in the presence of NICD (10, 11). The NICD-RBP-Jκ complex, which behaves as a transcriptional activator, up-regulates expression of primary target genes of Notch signaling such as HES and HERP2 in mammals and E(spl) in Drosophila (1–3, 5–7).

The recently discovered HERP family (for HES-related Repressor Protein, also referred to as Hesper/Hey/HR/CHF/gridlock) is structurally similar to the HES family (1, 4, 12–15). Both families share common domains including basic helix-loop-helix domain, orange domain (16), and tetrapeptide motif. Although the invariant proline residue in the basic domain and the WRPW tetrapeptide at the carboxyl terminus in HES/E(spl) are replaced in HERPs by a glycine and by YRPW (or YQPW), their overall structures are still similar to each other. The high sequence similarity among them led us to speculate that the HERP gene family might be new targets of Notch like HES and E(spl). In line with this, several observations suggest that the HERP family appears to be downstream of Notch (13, 17–20). However, this possibility requires more rigorous examination, because these conclusions rely primarily on experiments involving overexpression of the NICD transgene. In marked contrast to endogenous NICD, which is physiologically maintained at an extremely low concentration (8, 21), exogenously introduced NICD is expressed at easily detectable and supra-physiological levels (1, 22). Because NICD associates with a number of proteins through its multiple protein-interacting domains (23), such artificial unregulated overexpression of NICD by transient transfection could create misleading consequences. In fact, we have observed different results between NICD overexpression-induced and ligand-induced stimuli (1), clearly indicating the distinct nature of these stimuli.

By co-culturing Notch receptor-bearing target cells with Notch ligand-expressing cells, we have previously shown that the HERP2 (but not HERP1) mRNA was selectively up-regulated in several cell lines including C2C12 and C3H10T1/2 cells even in the presence of cycloheximide suppression of protein synthesis.

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The abbreviations used are: NICD, Notch intracellular domain; m.o.i., multiplicity of infection; RAM, RBP-Jκ-associated module.
synthesis (1). These experiments established HERP2 as a direct target of Notch. In contrast, however, we found no measurable induction of HERP1 mRNA in any of the ligand-stimulated cells that had shown marked induction of HERP2 mRNA. These observations suggested the presence of previously unappreciated differential regulatory mechanisms for the different HERP family members. Furthermore, whether HERP1 is a direct physiological target of Notch remains to be determined.

A number of observations indicate that both the Notch signaling pathway and HERP1 play roles in vascular development. Many Notch pathway components are expressed in the vascular system (24–27). In addition, mutations of genes encoding Notch ligands and receptors cause abnormalities in the vascular system (24, 27–30). HERP1 is abundantly expressed in the aorta (12). In zebrafish, mutation in the gridlock gene (zebrafish orthologue of HERP1) leads to selective disturbance of assembly of the aorta (4). Collectively, these findings suggest that Notch and HERP1 both play roles in vascular development. Although our previous experiments (1) failed to find a direct interaction of Notch signaling with HERP1 expression in some cell types, we have now re-examined their relationship in cells derived from aortic components.

Here we show in several different ways that HERP1 is a true target gene of Notch in aortic smooth muscle cells. NICD activates the HERP1 promoter in an RBP-Jκ-dependent manner and induces expression of endogenous HERP1 mRNA as well as HERP1 protein. Notch ligand stimulation induces endogenous HERP1 mRNA expression, which was observed even in the absence of de novo protein synthesis. HERP1 induction by Notch stimulation was not observed in RBP-Jκ-deficient cells, but exogenous expression of RBP-Jκ restores HERP1 mRNA expression in these cells. These findings provide the first solid evidence that HERP1 is a novel primary target of Notch that is directly up-regulated by a cellular signal transduction system (requiring RBP-Jκ) activated by physiological Notch stimulation in a smooth muscle cell.

MATERIALS AND METHODS

Isolation of Genomic DNA of Mouse herp1 and Construction of Luciferase Reporter Plasmids—The mouse genomic phage library from CCE embryonic stem cells (31) was screened with mouse herp1 cDNA (nucleotides 568–1135) (1). HERP1 genomic DNAs of different length (nucleotides –3520 to –8, –2017 to –8, –1042 to –8, and –528 to –8 relative to the start codon) containing the putting transcription regulatory regions were subcloned into the KpnI-XhoI site of pG2L basic vector (Promega) by PCR. The accession number of the clone (–3520 to –8) in GenBank™ is AY059384. To generate the mutated HERP1 reporter constructs (μ3, μ2, and μ1), the three consensus RBP-Jκ-binding sites in the HERP1 promoter (–2017 to –8), TGCTTC-CCACCTCC (–1579 to –1573), GCATTCCCCCCGC (–342 to –336), and ACAGGGGGAAAA (–139 to –133), were changed by PCR to TTCCAGACTTCTC, GCAGAACATCAGGC, and AGGCCGCCGAAAGA, respectively. The RBP-Jκ-binding sites are written in boldface, and the mutated residues are underlined. Luciferase reporter plasmids under the control of the HES1 (–467 to +46, HES1-luc) and the HESS (–800 to +32, HESS-luc) promoter were generously provided by Dr. Kageyama (Kyoto University) (32).

Generation of Recombinant Adenoviruses—Empty adenovirus (Adeno-empty) and adenoviruses expressing NICD and RBP-Jκ (Adeno-NICD and Adeno-RBP-Jκ, respectively) have been created as described elsewhere (1). High titer of these viruses (1.0 × 109 plaque-forming units/ml) have been routinely obtained.

Plasmids and Luciferase Assay—Parental pEF-BOS empty vector, the Notch-I-expressing vector, and its derivatives were kindly provided by Dr. G. Weinmaster (UCLA) (33). Plasmids encoding wild type RBP-Jκ and its mutants, R218H and R272S, were generously provided by Dr. T. Honjo (Kyoto University) (34). Luciferase assays were carried out as described elsewhere (35).

RESULTS

 Constitutively Active Notch Intracellular Domain Activates Transcription of HERP1—To determine whether HERP1 may be a downstream target gene of Notch, we first isolated a HERP1 genomic DNA clone and found therein several central core consensus sites for RBP-Jκ (Fig. 3). The gene spans 10.4 kb of genomic DNA and contains five exons as depicted in Fig. 1A. All the introns are located within the protein-coding regions, and the exon-intron boundaries meet GT-AG rules (Fig. 1A). Comparison of the predicted amino acid sequences of HERP1 and HES1 revealed remarkably conserved positions of introns in the two genes, suggesting that the genes have a common ancestral origin (arrowheads in Fig. 1B) (32), and further supporting the notion that HERP1 may also be a target of Notch. As noted previously, there is a Pro to Gly substitution in the basic region (shown as an arrow in Fig. 1B) and a WR to YQ substitution in the tetrapeptide motif (marked with asterisks in Fig. 1B). There was no typical TATA box upstream of the coding region, although a similar sequence, TATTTA, was present at –291 bp relative to the start codon. Most importantly, we found that upstream of the coding region of the HERP1 gene contains three classical docking sites and several incomplete sites for the NICD DNA-binding adaptor, RBP-Jκ. These are listed in Fig. 2C.

To examine the regulation of HERP1 gene transcription, we first compared the HERP1 promoter activity with that of HES1 and HESS5 which are thus far the only known effectors of Notch in mammals (36). The constitutively active form of Notch1 (NICD) similarly and strongly activated the promoters of HERP1, HES1, and HESS5 in C3H10T1/2 fibroblasts (Fig. 2A). This result strongly suggests that HERP1 as well as HES1 and HESS5 are targets of Notch. The transcriptional response of HERP1 further confirms that the –2017 to –8 upstream region contains a physiologically relevant promoter.

In order to study the functionality of the RBP-Jκ-binding sites, we first created several luciferase reporter gene constructs bearing different lengths of the HERP1 gene upstream regions. Cells were transfected with the respective luciferase reporter plasmids with or without expression vectors for NICD. Luciferase activities of all four reporter constructs were strongly activated by NICD, whereas a control β-actin promoter was hardly affected (Fig. 2B), suggesting that transacti-
vation by NICD depends on the presence of the HERP1 up-stream sequences. Longer fragments showed stronger induction by NICD. Each deleted DNA region contained some complete and/or incomplete RBP-Jκ sites (Fig. 2C). Although these findings do not identify any particular RBP-Jκ site as being required for NICD induced reporter gene activation, they suggest rather that multiple RBP-Jκ sites including the incomplete consensus sites may be involved in transactivation of HERP1 promoter by NICD.

To determine the contribution of specific RBP-Jκ-binding sites, we next individually mutated each of the three complete RBP-Jκ sites (ζ, η, and θ in Fig. 2C) in an HERP1-luciferase construct (−2017 to −8). Constructs with each mutant were transfected with these luciferase reporter plasmids in the presence or absence of NICD. When compared with wild type HERP1 promoter, transcription of μ1 was strongly impaired whereas that of μ2 was moderately reduced. In contrast, no apparent difference was observed between wild type and μ3. One possible explanation for the observed difference among these mutants is the different flanking sequences of RBP-Jκ-binding site that might contribute to DNA binding activities of RBP-Jκ. Because each mutation did not completely abolish transactivation by NICD, we next mutated all three RBP-Jκ-binding sites within an HERP1 promoter (named μ123). As shown in Fig. 2E, transactivation of the μ123 reporter by NICD was greatly diminished but was not completely abolished. The degree of reduction of μ123 was almost the same as that of μ1. Taken together, these results identify site ζ for RBP-Jκ as an important sequence for NICD-mediated transactivation, although other RBP-Jκ sites may also be involved to a lesser extent. Indeed, the μ123 construct retains four unmodified incomplete RBP-Jκ-binding sites whose functions, if any, remain to be determined.

The full-length Notch receptor is functionally silent unless it is cleaved by the process called “regulated intramembrane proteolysis” (Rip) (37). After the cleavage, the NICD is released from the membrane and functions as a transcriptional activator together with the nuclear protein RBP-Jκ (8). It has been reported that the subtransmembrane region, RAM (for RBP-Jκ-Associated Module), is essential for RBP-Jκ interaction and transactivation of target genes such as HES1 (33, 38–40). Accordingly, we next studied whether the same domain of Notch is necessary for transactivation of HERP1 by expressing Notch deletion mutants (Fig. 2F). The Notch1-ICD encodes the complete RAM domain, whereas Notch1-ICDΔRAM lacks most of the RAM but contains an intact ankynin repeat region involved in protein-protein interactions (23). As expected, Notch1-ICD strongly activated transcription of the HERP1 promoter, but both full-length Notch1 and Notch1-ICDΔRAM failed to transactivate. Thus, the RAM domain of Notch is required for transactivation of the HERP1 promoter, which is consonant with the critical role of RBP-Jκ-binding sites (Fig. 2, D and E). These data suggest that transactivation of HERP1 by Notch signaling is regulated, at least in part, by the same mechanism as other target genes such as HES1.

Expression of Both HERP1 and HERP2 Is Induced by Constitutively Active Notch—To investigate the physiological relevance of these reporter gene assays, we next studied whether endogenous HERP genes are similarly up-regulated by NICD. For this purpose, A10 cells derived from smooth muscle cells of adult rat thoracic aorta were used, because normal vasculature requires HERP1 expression during development and is among the tissues with the highest levels of HERP1 expression. When NICD was introduced and overexpressed following infection of A10 cells with recombinant adenovirus (Adeno-NICD), both HERP1 and HERP2 mRNAs were clearly induced (Fig. 3, lanes 5 and 6), whereas control infection with Adeno-empty had no effect (lanes 3 and 4). The Northern blot signals were quantitatively measured by PhosphorImager, and the summary of
**FIG. 2.** *HERP1* can be a downstream target gene of Notch. C3H10T1/2 cells were transfected with 2 μg of the indicated reporter construct plus 2 μg of parental pEF-BOS empty vector or one of indicated Notch-expressing vectors. Cells were harvested 3 days after transfection. The luciferase activity of each reporter construct with no pEF-BOS plasmid was set at 1.0. The bars show means of luciferase activity with standard deviations. A, the *HERP1* promoter like the *HES1* and *HES5* promoters is activated by the constitutively active form of the NICD. Note that all three constructs were similarly and strongly activated by NICD. B, longer fragments of *HERP1* promoter are more strongly activated by NICD. *HERP1* reporter constructs with increasing lengths of promoter regions were created as described under "Materials and Methods." The locations of the three complete consensus sequences for RBP-J binding are indicated with open boxes. A reporter construct with the β-actin promoter was used as negative control (pGL2-β-actin). Note that all *HERP1* reporter constructs were activated by NICD, but longer fragments showed stronger induction. C, putative RBP-J consensus binding sites found in the *HERP1* upstream region, −8 to −3520 nucleotides relative to the translation start codon. Matching consensus sequence is shown in bold. Three nucleotides of both the 3′- and 5′-flanking sequences are also shown. D, requirement of each full RBP-J-binding site for transactivation of *HERP1* gene. Each of three consensus sites for RBP-J was mutated within a *HERP1* promoter construct (−2017 to −8). These constructs were designated μ1, μ2, and μ3 as indicated in the figure. Note that all *HERP1* reporter constructs were activated by NICD, but longer fragments showed stronger induction. E, incomplete RBP-J-binding sites may also play a role in transactivation of the *HERP1* promoter by NICD. The three classical CBF1 sites in *HERP1* promoter (−2017 to −8) were mutated as described under "Materials and Methods" (μ123). A reporter construct with the β-actin promoter was used as negative control (pGL2-β-actin). Note that transactivation of *HERP1* promoter with three mutated RBP-J-binding sites was not completely abolished. F, RBP-J-associated module (RAM) of NICD is required for transactivation of *HERP1* promoter. Note that both NICD without RAM as well as full-length Notch1 failed to activate transcription of the *HERP1* promoter. TM, transmembrane domain; ANK, ankyrin repeats; ICD, intracellular domain.
the results was shown in Fig. 3B. Because mRNA expression is not always coupled with expression of the corresponding protein, we next evaluated whether HERP mRNA up-regulation is accompanied by expression of the cognate protein. A10 cells infected with the Adeno-NICD expression vector were subjected to double immunofluorescence with anti-Notch1 antibody plus either anti-HERP1 or -HERP2 antibody (Fig. 3C). Expression of endogenous HERP1 and HERP2 proteins was clearly induced in the Notch1-positive cells (open arrows, Fig. 3C). Cells with apparently less NICD expression also showed less HERP expression (closed arrows, Fig. 3C). We confirmed the specificity of these antibodies against HERP proteins by using the respective blocking peptides (data not shown). The specific localization of HERP proteins in the nucleus is consistent with its expected role as a transcription factor. These findings suggest that both HERP1 and HERP2 are downstream targets of Notch signaling in aortic smooth muscle cells.

**Induction of Both HERP1 and HERP2 mRNA Expression by Ligand Binding**—We remained concerned that the observed HERP induction by overexpressed NICD (Fig. 3) did not necessarily represent physiological regulation for HERP expression (1). Indeed endogenous NICD is expressed only at an extremely low level that usually escapes detection by immunofluorescence, whereas exogenously expressed NICD is expressed at a high level and is easily detectable (22) (see also Fig. 3C). Accordingly, we addressed this concern by using a co-culture approach that relies only on the interaction of endogenous Notch with its natural ligands. A10 cells were cocultured with cells expressing either of the two Notch ligands, Jagged1 and Dll1 (Fig. 4A). Co-culturing A10 cells with either Dll1-expressing QT6 cells or Jagged1-expressing L cells for 24 h strongly induced both HERP1 and HERP2 mRNA expression (Fig. 4A, lanes 3 and 4 and 7 and 8), whereas co-culturing with parental cells did not (lanes 1 and 2 and lanes 5 and 6). QT6 cells and L cells showed minimal amounts of HERP mRNA expression when cultured alone (lanes 10–13). These results (summarized in Fig. 4B) suggest that co-culture with the Notch ligand-expressing cells successfully stimulated Notch signaling and that both HERP1 and HERP2 are physiological targets of Notch in these aortic smooth muscle-derived cells.

HERP1 and HERP2 mRNA show a unique expression pattern during development in that they are expressed in a mutually exclusive manner in subcompartments of heart, brain, and craniofacial region (14, 15). In the heart, for instance, HERP1 mRNA is detected in the atria, whereas HERP2 mRNA is detected only in the ventricle. Both HERP1 and HERP2 have intrinsic transcriptional repression domains and act as transcriptional repressors from their specific DNA-binding sites (35). These findings have raised an interesting possibility that HERP1 and HERP2 may mutually repress transcription of each other and thus eliminate each other's expression in a given cell type. However, this idea is not supported by our observation that there is simultaneous expression of both HERP1 and HERP2 mRNA in the co-culture studies (Fig. 4A).

To reconcile these disparate sets of observations, we hypothesized that HERP1 and HERP2 expression may follow different time courses such that the mutual exclusivity is still possible. Based on this idea, we studied the expression level of HERPs mRNA at different time points following ligand stimulation (Fig. 4C). HERP1 mRNA induction by co-culture of A10 cells with Jagged1-expressing L cells peaked at 4–6 h (lanes 12 and 13) and declined after 9 h (lane 14). The expression level of HERP1 mRNA at 24 h is still higher than the control co-culture (compare lanes 8 and 16). In contrast, HERP2 mRNA was up-regulated only after 4–6 h (lanes 12 and 13) and peaked at 9 h (lane 14), as HERP1 mRNA expression was declining considerably. Interestingly, HERP1 expression declined as HERP2 expression increased. In contrast, HERP2 mRNA expression increased despite high level expression of HERP1. One potential explanation of these observations is that HERP2 may negatively regulate HERP1 expression, whereas HERP1 may not...
repress HERP2 expression in Jagged1-stimulated A10 cells. It should also be noted that HERP1 promoter activity may be negatively regulated by HERP1 protein (18), and therefore, the transient nature of HERP1 mRNA induction (Fig. 4D) might also be due to this negative auto-regulation.

**HERP1 Is an Immediate Target of Notch Signaling**—These findings demonstrate that the HERP1 and HERP2 genes are physiological targets of Notch in vascular smooth muscle cells. However, they do not establish whether their transcription is directly regulated by Notch or requires the expression and translation of other mRNAs. Therefore, we next studied whether HERP mRNA can be up-regulated by Notch signaling absent de novo protein synthesis. A10 cells were co-cultured with Jagged1-expressing cells in the presence of cycloheximide to block protein synthesis for various periods, and HERP mRNA expression was studied at each time point. The induction of both HERP1 and HERP2 mRNA expression was observed at 3 h (Fig. 5A, compare lanes 3 and 4 and lanes 9 and 10). At 24 h, expression of both HERP mRNA was diminished, presumably due to depletion of general cellular proteins including those involved in Notch signaling after the extended cyclo-

FIG. 4. Expression of both HERP1 and HERP2 mRNA is up-regulated by ligand stimulation in aortic smooth muscle cells. A, A10 cells were co-cultured with either parental L, Jagged1-expressing L, parental QT, or Dll1-expressing QT cells in growth medium. Twenty four hours later, total RNA was extracted, and Northern blot analysis was performed as described under “Materials and Methods.” Data are from duplicate co-cultures. Total RNA from each cell line was used as control (lanes 9–13). Note that expression of both HERP1 and HERP2 mRNA was clearly induced following stimulation by Notch ligands. Also note that the signal for glyceraldehyde-3-phosphate dehydrogenase (G3PDH) gene expression is weak in lanes 12 and 13 because QT6 cells are derived from quail. B, the radioactivity of each signal was measured. C, A10 cells were co-cultured with either parental L or Jagged1-expressing L cells. Total RNA was extracted at the indicated time points for Northern blot analysis. Data are shown in duplicate. Note that expression of HERP1 and HERP2 mRNAs was induced at different time points after ligand stimulation. D, the radioactivity of each signal from co-culture with Jagged1-expressing L cells (lanes 9–16) was measured.
hexamidine treatment. These findings establish that both HERP1 and HERP2 are primary and direct targets of ligand-stimulated Notch signaling in aortic smooth muscle cells.

Dysregulated Expression of Exogenous RBP-Jx Interferes with HERP1 mRNA Expression—Results presented earlier (Fig. 2) strongly suggest that RBP-Jx may be involved in the regulation of HERP1 mRNA expression. However, it remains to be determined whether the RBP-Jx protein is involved in the endogenous HERP1 mRNA expression and its induction by Notch signaling. To address this, we first utilized mutant RBP-Jx proteins (RBP-Jx R218H and RY227GS) that do not bind DNA and that have been considered as dominant negative mutants (34). We tested whether overexpression of these RBP-Jx mutants can abolish HERP1 transcription in reporter gene assay. As shown in Fig. 6A, transactivation of HERP1 promoter by NICD was reduced by expressing either the R218H or RY227GS mutant (Fig. 6A, compare lane 2 and lanes 4 or 5). Surprisingly, however, a similar or even higher degree of reduction was observed following expression of the wild type exogenous RBP-Jx protein (wt RBP-Jx) (Fig. 6A, lane 3). This is likely due to its uncontrolled expression that squelches the signaling molecules and disrupts their proper stoichiometry (see “Discussion”). Consistent with this finding are reports that overexpression of wild type RBP-Jx protein can result in transcriptional repression of different promoters even under conditions in which Notch signaling is stimulated (38, 41–43). We observed essentially the same result using an HES1-luc reporter gene (Fig. 6A, lanes 6–10).

To study further whether endogenous HERP1 mRNA expression is similarly regulated, we performed Northern blot analysis by transfecting cells with either wild type (Fig. 6B, lanes 3 and 4) or the putative dominant negative RBP-Jx clones (lanes 5 and 6), and we found the same effect of RBP-Jx on endogenous HERP1 mRNA expression by overexpressed NICD. Expression of HERP1 mRNA induced by NICD was similarly reduced by RBP-Jx and its R218H mutant. Although the mechanism underlying transcriptional repression by overexpressed wt RBP-Jx was not determined, the data are consistent with the idea that RBP-Jx was involved in the regulation of endogenous HERP1 mRNA expression and that proper stoichiometry of RBP-Jx may be critical for HERP1 expression.

RBP-Jx Is Necessary and Sufficient for HERP1 mRNA Expression by Notch—To address further the role of RBP-Jx in HERP1 mRNA expression, we next studied whether HERP1 mRNA expression can be induced in the absence of a RBP-Jx protein using RBP-Jx-deficient cells (OT11) derived from homozygous RBP-Jx null mice (38). We expressed constitutively active NICD in these cells using recombinant adenovirus vector. Importantly, HERP1 mRNA expression was not induced by the NICD introduced by Adeno-NICD infection in RBP-Jx-deficient OT11 cells (Fig. 7A, lanes 5 and 6), whereas wild type OT13 cells showed an overt HERP1 mRNA induction by the same stimulation (lanes 11 and 12). Infection with the Adeno-empty virus did not affect the expression level of HERP1 mRNA in either OT11 or OT13 cells (Fig. 7A, lanes 3, 4, 9, and 10). Expression of NICD protein following Adeno-vector infection was comparable in OT11 and OT13 cells, as determined by Western blot analysis (1). We observed a similar degree of NICD protein in the nucleus of both OT11 and OT13 cells by immunofluorescence (1). Thus, impairment of neither protein expression nor nuclear translocation is the reason for the failure of NICD to induce HERP1 mRNA expression in OT11 cells. The absence of HERP1 mRNA induction in OT11 cells could be due to phenotypic changes that are not directly associated with a lack of RBP-Jx. Therefore, we attempted to rescue the inability of OT11 cells to induce HERP1 mRNA expression by simply providing exogenous RBP-Jx protein. OT11 cells were infected at different m.o.i. values with recombinant adenovirus carrying RBP-Jx cDNA. Neither NICD nor RBP-Jx alone induced HERP1 mRNA expression in OT11 cells (Fig. 7C, lanes 1, 2, 9, and 10). Strikingly, when both NICD and RBP-Jx were co-expressed, robust HERP1 mRNA expression was clearly observed (lanes 3 and 4). Thus, the expression of RBP-Jx protein was sufficient to rescue HERP2 mRNA induction in OT11 cells. These results establish the involvement of RBP-Jx protein in Notch-targeted HERP1 mRNA expression.

DISCUSSION

The high degree of sequence similarity between HERP and HES suggested initially that the HERP gene family may be
question of whether HERP1 was regulated by Notch but in an unprecedented cell type-specific manner or whether it was regulated by a signaling pathway other than Notch. In the present work, we first demonstrated that HERP1 is indeed a *bona fide* direct target of Notch signaling by physiological ligand stimulation in A10 aortic vascular smooth muscle cells. We also demonstrated a requirement of RBP-Jk for induction of HERP1 mRNA. Because HERP1 mRNA was not induced by Notch ligand binding in any other cells we tested, it seems likely that the Notch pathway regulates HERP1 mRNA expression only in selected cell types. Thus our data provide new insights about the Notch signaling pathway by identifying HERP1 as a new primary target of Notch that acts in a cell type-specific manner.

**RBP-Jk-binding Sites**—Notch signaling is mediated by the nuclear protein RBP-Jk that binds to an essential core DNA sequence, GTGGGAA (11). In addition to the core sequence, the immediate 5′- and 3′-flanking sequences also have an impact on the binding affinity of RBP-Jk, and thus more stringent consensus elements incorporating flanking sequence have been proposed such as CGTGGGAA (44), and (C/T)GTG(G/A)GAA(A/C) (45). Among the putative RBP-Jk-binding sites on the HERP1 gene (Fig. 2C), only site 1 matches the stringent consensus sequences, which is consistent with the marked reduction of transcription we observed when it was mutated (Fig. 2D). The mutation of the second matching element, site 2, affected NICO-induced transcription moderately, whereas the mutation of site 3, whose flanking sequence does not match any of the reported sequences, did not attenuate transcription (Fig. 2D), thus supporting the crucial role of these flanking sequences.

Recently, it has been reported that promoter regions for a number of other genes contain slightly modified RBP-Jk-binding sites (41, 42, 46–51). For instance, the cyclin D1 promoter has a poorly conserved consensus sequence, GCTAGGAT, which still bind RBP-Jk assayed by electrophoretic mobility shift assay (50). These findings suggest that RBP-Jk-binding sites are not very strict and that variant RBP-Jk-binding sites can also function. Given the rather flexible sequence requirement and slight transactivation of mutated HERP1 gene (μ123) by NICO (Fig. 2E), the incomplete RBP-Jk-binding sites on the HERP1 promoter (other than 1, 2, and 3 in Fig. 2C) might also contribute to promoter activity. However, the binding activities of previously reported DNA sequences have only been confirmed by electrophoretic mobility shift assay, and the effect of these sites on transcription has only been determined following overexpression of NICO. Accordingly, we cannot yet conclude whether these modified RBP-Jk sites play a role in the Notch signaling pathway, nor can we conclude whether the incomplete sites on the HERP1 promoter are biologically functional. Further studies are needed to clarify whether the non-classical binding sites for RBP-Jk within HERP1 and other genes are essential in the Notch signaling pathway in vivo.

**Mechanisms Underlying Cell Type-specific Regulation of HERP1 mRNA by Notch**—Notch signaling utilizes multiple ligands, receptors, and effectors. To date, five Notch ligands (Jagged1 and -2, Dll1, -3, and -4) and four Notch receptors (Notch1–4) have been isolated, and as yet only HES1, HERP1, HERP2 have been proven to be immediate targets of Notch (Refs. 1–3 and this paper). Reporter gene analyses suggested that HES5 and HRT3 are also potential candidates as target genes of Notch (17, 18, 32). The existence of such multiple components at each step of Notch signaling raises the question, for example, of how different ligands might be linked to distinct receptors.

**Fig. 7.** A, forced expression of constitutively active NICD does not induce HERP1 mRNA expression in RBP-Jk-deficient OT11 cells. OT11 and OT13 cells were infected with Adeno-empty or Adeno-NICD at an m.o.i. of 20. Forty eight hours later, total RNA was extracted, and Northern blot analysis was performed as described under “Materials and Methods.” Data are from duplicate infections. Note the overt induction of HERP1 mRNA by Adeno-NICD only in OT13 cells but not in OT11 cells. B, rescue of HERP1 mRNA induction by exogenous RBP-Jk expression in RBP-Jk-deficient OT11 cells. OT11 and OT13 cells were infected with adenovirus expression vectors at the indicated m.o.i. Forty eight hours later, total RNA was extracted for Northern blot analysis. Data are from duplicate infections. Note that HERP1 mRNA is re-induced in OT11 cells only by co-infection with both Adeno-NICD and Adeno-RBP-Jk. B and D, the radioactivity of each signal was measured.
and effectors. Tissue distribution of these components does not necessarily indicate their specific relationships (14, 15, 54–57), and neither the specific links between ligands and receptors nor those between receptors and target genes have been rigorously delineated.

In the present study, HERP1 mRNA was induced by ligand stimulation specifically in A10 aortic smooth muscle cells (Figs. 4 and 5) but not in C2C12 muscle cells or in 10T1/2 fibroblasts (1). In contrast, HERP2 mRNA was induced in all these cell lines, and HES1 mRNA was hardly up-regulated at all by ligand stimulation (Ref. 1 and data not shown). All these cells expressed at least Notch1, -2, and -3 receptors as determined by reverse transcriptase-PCR (Ref. 1 and data not shown), and yet only A10 expressed HERP1 mRNA, suggesting that the selectivity of HERP1 induction does not depend on selective expression of these three receptors.

In marked contrast to selective induction of the endogenous HERP1 mRNA by ligand stimulation, previous reporter gene studies (17) showed that the HERP1 promoter (as well as HERP2 and HES1 promoters) was up-regulated indiscriminately by all four types of NICDs. Thus, transiently transfected reporter genes are much less stringently regulated than the endogenous genes. These data imply that cellular components of Notch signaling necessary to activate HERP1 may be present only in A10 cells and may be absent or inactive in cells other than A10. Recently, Notch5 and -6 have been isolated in zebrafish (GenBank™ accession numbers Y10353 for Notch5 and Y10354 for Notch6). Mammalian homologues of these receptors or other undiscovered Notch signaling components might participate in the cell type-specific expression of target genes such as HERP1. Clarifying relationship between ligands, receptors, and target genes is one of the key issues to explain tissue specificity in Notch signaling. Continued efforts toward discovery of new Notch components and their characterization should contribute to further understanding of mechanisms underlying cell type-specific regulation of Notch target gene expression.

Requirement of RBP-Jk in Notch Signaling—We showed that HERP1 mRNA induction by Notch stimulation was absolutely dependent on RBP-Jk using RBP-Jk-deficient OT11 cells (Fig. 7C). HERP1 mRNA was re-induced by NICD in OT11 cells by simply providing exogenous RBP-Jk. However, an excess of RBP-Jk protein expression attenuated HERP1 mRNA induction only in A10 cells but not in other vascular smooth muscle cells tested (data not shown). Therefore, it remains to be determined whether HERP expression is pivotal in molecular regulation of vascular smooth muscle cell gene expression.

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