HERP, a Novel Heterodimer Partner of HES/E(spl) in Notch Signaling

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HERP1 and -2 are members of a new basic helix-loop-helix (bHLH) protein family closely related to HES/E(spl), the only previously known Notch effector. Like that of HES, HERP mRNA expression is directly up-regulated by Notch ligand binding without de novo protein synthesis. HES and HERP are individually expressed in certain cells, but they are also coexpressed within single cells after Notch stimulation. Here, we show that HERP has intrinsic transcriptional repression activity. Transcriptional repression by HES/E(spl) entails the recruitment of the corepressor TLE/Groucho via a conserved WRPW motif, whereas unexpectedly the corresponding—but modified—tetrapeptide motif in HERP confers marginal repression. Rather, HERP uses its bHLH domain to recruit the mSin3 complex containing histone deacetylase HDAC1 and an additional corepressor, N-CoR, to mediate repression. HES and HERP homodimers bind similar DNA sequences, but with distinct sequence preferences, and they repress transcription from specific DNA binding sites. Importantly, HES and HERP associate with each other in solution and form a stable HES-HERP heterodimer upon DNA binding. HES-HERP heterodimers have both a greater DNA binding activity and a stronger repression activity than do the respective homodimers. Thus, Notch signaling relies on cooperation between HES and HERP, two transcriptional repressors with distinctive repression mechanisms which, either as homo- or as heterodimers, regulate target gene expression.

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. Analyses of loss- and gain-of-function mutants of Notch in vertebrates and invertebrates have demonstrated that these repressive Notch functions are remarkably conserved throughout species (4, 14, 19).

Interaction of Notch with its ligands such as the Jagged and Delta families leads to cleavage of the Notch intracellular domain (NICD), which subsequently migrates into the nucleus. There, the NICD associates with a transcriptional factor, CBF1 [RBP-Jk/Su(H)/Lag-1], and the NICD-CBF1 complex up-regulates expression of primary target genes of Notch signaling (4, 14, 19). The recently discovered HERP family (for HES-related repressor protein) is downstream of Notch signaling (34, 37), and we elsewhere describe the HERP family as being an immediate and direct target of Notch signaling (23). The HERP family has thus joined the HES/E(spl) family of transcriptional repressors as primary targets of Notch signaling. We have now begun to elucidate the relationship between these repressor families.

HES/E(spl) is a basic helix-loop-helix (bHLH) protein with two unique, evolutionarily conserved features, a proline at a specific position within the DNA-binding basic domain, and a carboxyl-terminal tetrapeptide WRPW motif (Fig. 1) (15). The WRPW motif is both necessary and sufficient for the recruitment of the corepressor TLE or its *Drosophila melanogaster* orthologue Groucho and for transcriptional repression (16). Thus, HES acts as an effector of Notch signaling by repressing the expression of target genes that include tissue-specific transcriptional activators such as MASH1 and neurogenin (3, 9, 13, 22, 47).

The HERP family (also called Hesr [28], Hey [31], HRT [38], CHF [10], and gridlock [50]) has conserved domains similar to those in the HES/E(spl) family. In addition to the homologous bHLH domain, HERP and HES share the Orange domain (12) and the tetrapeptide motif at the carboxyl terminus (Fig. 1A). However, the invariant proline residue in the basic domain and the WRPW tetrapeptide of HES/E(spl) are replaced in HERPs by a glycine and by YRPW (or YQPW) (Fig. 1). Such features are also conserved in a *Drosophila* HERP orthologue (Fig. 1B and C) (28). These structural differences define the HERP family as related to, but distinct from, HES/E(spl). Our recent observations found that coculture of Notch-bearing cells with cells expressing Delta-like 1 or Jagged1 directly up-regulates HERP gene expression without de novo protein synthesis (23). Consistently, expression of HERP members is diminished in the presomitic mesoderm and nascent somite of Delta-like 1- and Notch1-null mutant mice (28, 30), and the transgenic mice expressing a constitutively active Notch show up-regulation of HeyL, another mem-

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FIG. 1. Alignment of HERP1, HERP2, and HES1 amino acid sequences. (A) Schematic diagram of mouse HES1, HERP1, and HERP2 amino acid sequences. The values are the percentages of protein sequence similarity in the bHLH domain, the Orange domain, and a region between the bHLH and Orange domains. Note that the HERP1 tetrapeptide is YQPW in mice and YRPW in humans. (B and C) Amino acid sequences of the basic domain (B) and the carboxyl terminus including the tetrapeptide motif (C) from mouse HES1, HERP1, and HERP2 and *Drosophila* HESr are aligned by using ClustalW. Identical amino acids are in black, and conserved residues are in gray. An arrowhead indicates the invariant amino acid residues in the basic domain of HERP1, HERP2, and *Drosophila* HESr (glycine) and HES1 (proline). Asterisks indicate the tetrapeptide motifs.

ber of the HERP family, in hair cuticles (32). The similarities in amino acid sequence between HERP and HES compellingly suggest the presence of intrinsic transcriptional repression domains in HERP. Consistent with this, overexpressed HERP can inhibit expression of transiently transfected reporter genes (10, 37). However, it is unknown whether this repression is mediated by direct and specific recruitment of HERP to the promoter region of target genes. Thus, although HERP is a primary target of Notch signaling, it remains to be determined whether HERP has intrinsic repression domains and actively represses transcription of Notch target genes as a Notch effector.

Here, we report that HERP indeed has intrinsic repression activities. Surprisingly, HES and HERP have distinct repression mechanisms: the repression activity of HERP resides in the bHLH domain rather than the tetrapeptide motif. Instead of TLE/Groucho, HERP engages the mSin3 complex, a major corepressor complex involved in transcriptional repression of a variety of genes. mSin3A is a large protein thought to act as a scaffold to form the mSin3 complex that contains at least seven subunits including histone deacetylase 1 (HDAC1) and HDAC2 (5, 27). The Sin3A complex can be associated with additional corepressors, N-CoR and SMRT, to facilitate transcriptional repression (5, 27). Consistently, HERP recruits

HDAC1 as well as N-CoR through the bHLH domain. Expression of HERP and that of HES are not always simultaneously up-regulated by Notch, as certain cells express only one of them (10, 28, 31, 38, 45). In cells where only HERP or HES is expressed, each binds DNA as a homodimer and represses gene expression. Strikingly, in cells coexpressing HES and HERP, the homodimers disappear while a HES-HERP heterodimer forms a distinct DNA-binding species with a DNA binding activity markedly higher than that of either homodimer. The HES-HERP heterodimer may be functionally important, since it generates more than an additive repression activity compared with the respective homodimers. Thus, Notch signaling elicits expression of two independent primary target genes, HES and HERP, and each works either individually or cooperatively to repress target gene expression through its specific DNA-binding site.

MATERIALS AND METHODS

Plasmids. The following constructs were generously provided: pCEP4 Flag-N-CoR by C. Glass (University of California, San Diego), pCS Myc-mSin3A by R. Eisenman (Fred Hutchinson Cancer Research Center), pBJ HDAC1-cFlag by S. Schreiber (Harvard University), UAS-tk-luc reporter gene carrying four repeats of Gal4 DNA-binding sites by R. Evans (Salk Institute), and pSV2-CMV-HES1 by R. Kageyama (Kyoto University). HDAC1 with a Flag tag was subcloned into pcDNA3 (Invitrogen). The hemagglutinin (HA) tag sequence was introduced at the amino terminus of human HERP1 by PCR [pcDNA3.1(-) HA-HERP1], and the FLAG tag was introduced at the amino terminus of mouse HERP1 by PCR [pcDNA3.1(-) Flag-HERP1]. A GAL fusion plasmid (pc3Gal) was generated by subcloning the Gal4 DNA-binding domain (amino acids 1 to 147) into the *HindIII-XhoI* site of pcDNA3 by PCR. Various deletion mutants of HERP1 and HES1 were subcloned into the *EcoRI-BamHI* site of pc3Gal by PCR (see Fig. 2C and D). For glutathione S-transferase (GST) fusion proteins, various fragments of HERP1 were subcloned into the *EcoRI-BamHI* site of pGEX2TK (Pharmacia) in frame by PCR (see Fig. 3G). For assays of the luciferase reporter gene, four repeats of the C-1 site were subcloned into the *KpnI-XhoI* site of pGL2 basic reporter (Promega) with a β -actin promoter.

Cell transfection and luciferase assay. Transfections of 70% confluent C3H10T1/2, HeLa, COS7, and C2C12 cells in 60-mm-diameter dishes were performed according to the 2-[bis(2-hydroxyethyl)amino]ethanesulfonic acid (BES)-buffered saline protocol as previously described (20). Two micrograms of reporter constructs was cotransfected with the indicated amounts of expression vectors for HERP or HES1. The total amount of plasmid DNA was adjusted to 9 μ g with the control plasmid DNA lacking the cDNA. Transactivation of reporter genes was evaluated by luciferase assay as described before (44). These assays were done in triplicate and repeated several times.

Immunoprecipitations and Western blots. For in vivo protein interaction studies, 70% confluent 293T cells in 10-cm-diameter dishes were transfected by the BES-buffered saline method as described above with 15 μ g of pCEP4 Flag-N-CoR plus 5 μ g of pcDNA3.1(-) HA-HERP1. For the mSin3A interaction studies, the cells were transfected with 5 μ g of pcDNA3.1(-) Flag-HERP1 plus 15 μ g of the myc-mSin3A expression vector. For HDAC1 interaction studies, the cells were transfected with 10 μ g of pcDNA3 HDAC1-cFlag plus 10 μ g of pcDNA3.1(-) HA-HERP1. For endogenous HDAC1 interaction studies, the cells were transfected with 20 μ g of either pcDNA3.1(-) Flag empty, pcDNA3.1(-) Flag-HERP1, or pcDNA3.1(-) Flag-HERP2. For HES1 interaction studies, the cells were transfected with 10 μ g of pcDNA3.1(-) Flag-HERP1 plus 10 μ g of pc3Gal-HES1. The cells were lysed in a lysis buffer (20 mM Tris-HCl [pH 8.0], 5 mM $MgCl_2$, 10% glycerol, 0.1% NP-40, 100 mM KCl) supplemented with freshly prepared protease inhibitors. The cell extracts were incubated with either anti-Flag M2 (Sigma) or control normal mouse immunoglobulin G (IgG) coupled to protein G agarose (Sigma) for 1 h at 4°C. After washing three times, bound proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by Western blot analysis with anti-myc antibody (9e10, ATCC CRL1729), anti-HA antibody (Y-11; Santa Cruz Biotech), anti-HDAC1 antibody (06-720; Upstate Biotechnology), or anti-GAL4 DNA-binding domain (GAL4-DBD) antibody (sc-577; Santa Cruz Biotech).

In vitro interaction assay. GST pull-down experiments were carried out as described previously (20). Various deletion mutants of mouse GST-HERP1

fusion proteins were prepared from *Escherichia coli* as described previously (20). In vitro-translated ^{35}S -labeled proteins were prepared using the TNT coupled transcription-translation system (Promega). Labeled proteins were incubated with equal amounts of GST fusion protein for 1 h at 4°C. Bound proteins were analyzed by autoradiography after SDS-PAGE.

Electrophoretic mobility shift assay. In vitro-translated proteins of HERP1 and HES1 were prepared using the TNT coupled transcription-translation system. Nuclear proteins were extracted as described before with minor modifications as follows (2). 293T cells were transfected with plasmids described in the figure legends. Three days after transfection, cells were harvested with ice-cold lysis buffer (20 mM HEPES [pH 7.6], 20% glycerol, 1.5 mM MgCl_2 , 0.2 mM EDTA, 0.1% Triton X, 10 mM KCl, 1 mM dithiothreitol, 1 μg of leupeptin per ml, 1 μg of pepstatin per ml, 1 μg of aprotinin per ml, 1 μM phenylmethylsulfonyl fluoride) and homogenized with 10 strokes of a Dounce homogenizer. The cells were centrifuged for 10 min at $900 \times g$ at 4°C. The pellet was resuspended in the same volume of lysis buffer augmented to 500 mM KCl and transferred to a microfuge tube. The lysate was incubated at 4°C for 1 h and then centrifuged for 15 min at $16,000 \times g$ at 4°C. Supernatants were collected and frozen at -80°C .

Gel mobility shift assays were carried out as described previously (24, 41, 45). The annealed oligonucleotides were labeled at both ends by filling in with Klenow enzyme in the presence of [α - ^{32}P]dCTP. Protein-DNA complexes were formed by incubation of proteins described above with 25 fmol of radiolabeled nucleotides in 40 μl of buffer (25 mM HEPES [pH 7.5], 100 mM KCl, 20% glycerol, 0.1% Nonidet P-40, 10 μM ZnSO_4 , 1 mM dithiothreitol). Poly(dI-dC) was included as a nonspecific competitor at 5 ng/ μl . After incubation with probes at room temperature for 20 min, DNA-protein complexes were resolved by electrophoresis on a 5% acrylamide gel. The dried gel was exposed to X-ray film at -80°C as well as being subjected to PhosphorImager analysis (Storm 840; Molecular Dynamics).

Antibody production. Anti-mHERP1-N antibody was affinity purified from rabbit antisera directed against a synthesized oligopeptide of the amino terminus of mHERP1, EETTSSEDLDETIDVGSENN (Bethyl Laboratories, Montgomery, Tex.). Anti-HES1 antibody was generously provided by T. Sudo (Toray Industries, Inc., Kamakura, Japan).

RESULTS

HERP is a transcriptional repressor. To determine whether HERP has an intrinsic transcriptional repression domain, we first fused two HERP members, HERP1 and HERP2, to a heterologous GAL4-DBD. Transient transfection of these expression vectors with the reporter gene (UAS-tk-luc) showed a strong, dose-dependent repression of reporter gene expression from GAL4 binding sites (Fig. 2A and B). HERP expression vectors without the GAL4-DBD did not inhibit transcription of the GAL-dependent reporter gene (data not shown). The finding that HERP shows repression activity only when tethered to a promoter indicates the presence of intrinsic repression domains in HERP.

HERP repression activity resides in the bHLH domain. Having confirmed the intrinsic repressor activity of HERP, we expected that repression activity would reside in the modified, but conserved, tetrapeptide motif in the carboxyl terminus of HERP (YQPW or YRPW), as it does in the WRPW motif of HES. After all, Runt domain proteins carry a variant (WRPY) of the tetrapeptide motif (WRPW) and yet are active in the recruitment of TLE/Groucho and transcriptional repression (16). Therefore, one might anticipate that the YQPW motif of HERP also would have a repression activity. Accordingly, a series of HERP1 deletion mutants were expressed in GAL fusion proteins and tested. Unexpectedly, deletion of YQPW had little effect on HERP's repression activity (Fig. 2C, ΔY , NBO), and the C-terminal region containing YQPW (CY) produced only weak repression. Similarly, the Orange domain, previously described as a putative repression domain (12), also

FIG. 2. (A and B) Both HERP1 (A) and HERP2 (B) are transcriptional repressors. C3H10T1/2 cells were transfected with UAS-tk-luc reporter constructs (2 μg) and the indicated GAL fusion expression vectors. The means of luciferase activities with standard deviations are shown. (C and D) The bHLH domain mediates the repressor activity of HERP1. C3H10T1/2 cells were transfected with UAS-tk-luc reporter constructs (2 μg) and the indicated GAL fusion expression vectors (1 μg). (C) Fold repression was calculated as a ratio of reduction in luciferase expression mediated by GAL-HERP1 fusion constructs relative to the empty GAL4-DBD. (D) Repressor activity is expressed as a percentage of fold repression with full-length HERP1 or HES1 set at 100% and GAL4-DBD alone set at 0%. Repressor activity is directly compared between HERP1 and HES1. All the GAL fusion constructs showed equal expression levels as judged by Western blot analysis (data not shown).

had little effect (O). To our surprise, the bHLH domain alone retained repression activity (B) fully comparable to that of wild-type HERP1. Similar results were obtained using segments of HERP2 (data not shown). Thus, the repression activity of HERP resides primarily in the bHLH domain, while the YQPW motif plays a relatively minor role, if any. This finding is in sharp distinction to the function of the bHLH domain of *hairy*, a *Drosophila* homologue of HES that has no intrinsic repression activity (17). These unexpected results

prompted us to directly compare repression domains of HERP and HES within a single experiment (Fig. 2D). In contrast to HERP, the WRPW-containing carboxyl-terminal region of HES1 (HES1-CW) has full repression activity, whereas its bHLH domain had no effect (HES1-B). Together, these results indicate that the closely related proteins HES1 and HERP1 repress transcription using very distinct mechanisms.

HERP associates with mSin3 corepressor complex. The relative dispensability of the tetrapeptide feature of HERP is in sharp contrast to the WRPW motif of HES that is required to recruit the TLE/Groucho corepressor. The distinct repression domains of HERP and HES imply that HERP1 is likely to engage different corepressors, and several were tested. Sin3 is a major corepressor that participates in a number of transcriptional repression activities in mammalian cells, *Drosophila*, and *Saccharomyces cerevisiae* (5, 27). When both mSin3A and HERP1 were simultaneously expressed in cells, we found a strong association between them (Fig. 3A, lane 1). The association was specific, as it was not observed when HERP1 was absent (lane 2) or when control mouse IgG was used for mock immunoprecipitation (lane 3). These data suggest that HERP specifically associates with the mSin3A complex in cells. To further support this conclusion, we next studied whether HERP also associates with the corepressor N-CoR, which is known to associate with the mSin3 complex. Consistently, N-CoR also was found specifically associated with HERP1 in the cells (Fig. 3B). Furthermore, we found a strong and specific interaction between HERP1 and HDAC1, a known subunit of the mSin3 complex (Fig. 3C). An additional study further verified the association of an endogenous HDAC1 with both HERP1 and HERP2 (Fig. 3D). These findings demonstrate that HERP recruits the mSin3 complex containing histone deacetylases, as well as another corepressor, N-CoR.

The bHLH domain of HERP directly associates with mSin3A and N-CoR. Given that HERP associates with the mSin3 complex in cells, we further asked whether HERP directly contacts mSin3A, N-CoR, and HDAC1 in vitro. Incubation of full-length GST-HERP1 or GST alone with in vitro-translated mSin3A protein clearly showed a strong specific interaction between HERP1 and mSin3A (Fig. 3E, lanes 2 and 7). A deletion analysis to determine the interaction domain of HERP1 revealed that the bHLH domain is responsible for this interaction (lanes 3, 4, and 9) and that neither the Orange domain nor the YQPW-containing C terminus is involved (lanes 5, 6, and 10). Interestingly, the same set of HERP1 regions that contains the bHLH domain also mediates the interaction with N-CoR (Fig. 3F, lanes 13, 14, and 19). These results (as summarized in Fig. 3G) indicate that the bHLH domain directly engages both mSinA and N-CoR corepressors. HERP1 does not interact with HDAC1 in these in vitro interaction assays (data not shown), indicating that the association of HERP1 with HDAC1 observed in vivo (Fig. 3C and D) is indirect and is mediated by either N-CoR, mSin3A, or other subunits of the mSin3 complex. Similar indirect HDAC associations with transcription factors have been reported previously (5, 27). Importantly, these findings are in full agreement with the results of our repression domain mapping studies (Fig. 2C and D) and identify the bHLH domain of HERP1 as a docking site for an HDAC-containing mSin3 corepressor complex to mediate transcriptional repression by HERP1.

HES and HERP bind both distinct and common DNA sequences. In certain cell types, HERP and HES are individually expressed, whereas in other cells they are coexpressed. Having established that HERP is a transcriptional repressor, we questioned why there are two Notch effectors coexpressed within some cell types. Given the difference in amino acid sequences of their DNA-binding basic domains (Fig. 1B), HES and HERP may bind distinct DNA sequences. To address this possibility, we studied DNA binding activities of HES and HERP using various bHLH-binding DNA sequences (class A, B, and C) (15, 24, 41) as probes in gel shift assays. Although HERP1 binds to all the tested probes (Fig. 4A, lanes 7 to 12), and HES1 also binds most probes (lanes 2 to 6) except the class A probe (lane 1), HES1 and HERP1 show distinct preferences for different DNA sequences. For instance, HES1 binds to class B and C-1 probes at equal efficiency (lanes 2 and 3), but only weakly to the other class C probes (lanes 4 to 6). Although HERP1 and HES1 bind similarly to the class B probe (lanes 2 and 8), the binding of HERP1 to C-1 and C-2 probes was much weaker than that of HES1 (compare lanes 3 and 4 and lanes 9 and 10). Unlike HES1, HERP1 bound the class A probe (lane 7), raising the possibility that HERP1 could directly compete with tissue-specific transcriptional activators for class A sequences. HERP2 showed DNA binding preferences nearly identical to that of HERP1 (data not shown). Thus, as summarized in Fig. 4A (bottom), while HES and HERP can bind to most of the same DNA sequences, they do so with clearly different preferences.

Formation of the HES-HERP heterodimer both in vitro and in vivo. The finding that HES and HERP can also bind certain DNA sequences with the same efficiency (e.g., Fig. 4A, lanes 3 and 8), together with their coexpression within single cells, raises the question of whether HES and HERP compete for the common DNA binding site or otherwise interact. When HES1 or HERP1 is incubated independently with the class B probe in an electrophoretic mobility shift assay, each shows a single specific band but with distinct mobility (Fig. 4B, lanes 2 to 4 and 5 to 7). Surprisingly, when the two are simultaneously incubated with the probe, a single new main band with intermediate mobility appears. Two important features are noted regarding this intermediate band. First, the intermediate band is generated at the expense of the respective homodimers of HES1 and HERP1, as the homodimer bands are no longer present (lanes 8 to 10). Second, the intermediate band has at least a two- to threefold-higher DNA binding activity than the sum of those of the two homodimers (compare, e.g. lanes 3, 6, and 9). Essentially identical data were obtained using the class C-1 and C-2 probes (lanes 12 to 20 and 22 to 30). These data suggest that the intermediate band represents a HES-HERP heterodimer and that the heterodimer is strongly preferred to the homodimers across a variety of different DNA docking sequences. As shown in lanes 41 to 50, the protein concentrations of HES1 and HERP1 remained the same when the two were coexpressed (compare lanes 42 to 47 and lanes 48 to 50). Thus, they represent higher binding activities of the heterodimer for the DNA rather than altered protein concentrations. No such heterodimer was observed with the class A probe (lanes 32 to 40) (which only HERP binds), further supporting the notions that the band with intermediate mobility represents a HES-HERP heterodimer and that stable het-

FIG. 3. HERP1 forms a complex with mSin3A, N-CoR, and HDAC. (A) HERP1 associates with mSin3A. Cells were transfected with the myc-mSin3A vector as well as with either pcDNA3.1(–)-Flag-HERP1 or pcDNA3.1(–) empty vector. The extracts from the transfected cells were incubated with an equal amount of anti-Flag M2 antibody or control normal mouse IgG. Bound proteins were separated by SDS-PAGE followed by Western blot analysis with anti-myc antibody. (B) HERP1 associates with the corepressor, N-CoR. 293T cells were transfected with pCEP4 Flag-N-CoR or pcDNA3.1(–) empty vector plus pcDNA3.1(–) HA-HERP1. The interaction was studied as described above using anti-HA antibody. (C) HERP1 associates with HDAC1. Cells were transfected with pcDNA3-HDAC1-cFlag or pcDNA3 empty vector plus pcDNA3.1(–) HA-HERP1. The

erodimer formation requires the two basic domains of HES and HERP both to contact the DNA. That the intermediate band indeed contains both HES1 and HERP1 was directly demonstrated using specific antibodies against these proteins (Fig. 4C, lanes 4 and 5; see below).

But is the heterodimer formed *in vivo*? Nuclear extracts from cells expressing only HES1 or only HERP1 did not have detectable specific DNA binding activities (Fig. 4C, lanes 6 and 8), suggesting low binding affinities of the homodimers. Although addition of HES1-specific antibody caused little change (lane 7), addition of HERP1-specific antibody revealed a supershifted band (lane 9). Most importantly, nuclear extract from cells expressing both HES1 and HERP1 proteins showed a marked increase in DNA binding activity (lane 10). Addition of either HES1- or HERP1-specific antibody (lanes 11 and 12), but not a control IgG (lane 13), supershifted the band, indicating that the band contains both HES1 and HERP1. Similar supershifted bands were also observed when *in vitro*-translated proteins were incubated with the antibodies (lanes 4 and 5). Furthermore, simultaneous addition of both antibodies caused an additional supershift (lane 17), suggesting that the supershifted bands engendered by the single antibodies (lanes 11 and 12 or 15 and 16) contain both HES1 and HERP1. We observed essentially identical results using HERP2 (data not shown). These data demonstrate that HES and HERP form a heterodimer both *in vitro* and *in vivo* and that the heterodimer has a striking DNA binding activity and is the exclusive entity that forms in cells expressing both proteins.

HERP associates with HES in solution. In order to form a DNA-bound heterodimer, HERP might associate with HES before DNA binding. We studied this possibility first *in vitro* by a GST pull-down approach. *In vitro*-translated HES1 was efficiently associated with GST-HERP1 but not with GST alone (Fig. 5A, lanes 1 and 3). A reciprocal study using *in vitro*-translated HERP1 and GST-HES1 confirmed a strong specific interaction between HES1 and HERP1 proteins. A similar observation has been made by others previously (30). Furthermore, we found that this association was also observed *in vivo*. Total cell extracts from the cells expressing tagged HERP1 and/or HES1 were subjected to immunoprecipitation followed by Western blot analysis. A specific *in vivo* interaction was observed between the two proteins (Fig. 5B, lanes 1 to 3). The interaction is very strong, as a majority of HES1 protein was found associated with HERP1 in the immunoprecipitate (compare lanes 1 and 4). These data confirmed the presence of a tight association between HES and HERP in solution.

interaction was studied as described above using anti-HA antibody. (D) Both Flag-HERP1 and -HERP2 associate with endogenous HDAC1. Cells were transfected with either pcDNA3.1(–)-Flag empty, pcDNA3.1(–)-Flag-HERP1, or pcDNA3.1(–)-Flag-HERP2. The interaction was studied as described above using anti-HDAC1 antibody. (E) HERP1 interacts with mSin3A via its bHLH domain. *In vitro*-translated ³⁵S-myc-mSin3A was incubated with equal amounts of the indicated GST-HERP1 fusion proteins for 1 h at 4°C. Bound proteins were analyzed by autoradiography after SDS-PAGE. (F) HERP1 interacts with N-CoR via its bHLH domain. Assays were performed as described above using ³⁵S-N-CoR. (G) Summary of the domain mapping studies. Ip, immunoprecipitation; Ab, antibody. Numbers to the right of the gels are molecular masses in kilodaltons.

FIG. 4. DNA binding properties of HES and HERP. (A) HES1 and HERP1 show distinct DNA binding preferences. Electrophoretic mobility shift assays were performed using various DNA probes. Autoradiographs after short and long exposures are shown. Top-strand oligonucleotide sequences used in this assay are indicated (bottom panel). Consensus binding elements are underlined. Sequences of classes A and B are derived from those described previously (24). The C-1, C-2, C-3, and C-4 oligonucleotide sequences are from the regulatory region of the known HES/E(spl) target genes; achaete (41), E(spl) genes (45), hASH (9), and HES1 itself (46), respectively. Radioactivities of each band were measured by a PhosphorImager with ImageQuant software to assess the relative binding activities shown in the bottom panel. The radiolabeling efficiency of the probes was comparable among the different probes, and excess amounts of the free probes were confirmed in all lanes in a parallel experiment with the same results (data not shown). (B) HES1 and HERP1 form a heterodimer. HES1 and HERP1 proteins were either individually translated (lanes 2 to 7, 12 to 17, 22 to 27, 32 to 37, and 42 to 47) or cotranslated (lanes 8 to 10, 18 to 20, 28 to 30, 38 to 40, and 48 to 50). In vitro-translated proteins prepared in parallel using [³⁵S]methionine are shown to verify equal protein product (lanes 42 to 50). (C) A gel shift assay was performed using either in vitro-translated proteins (lanes 1 to 5) or nuclear extract from the cells transfected with the expression vectors for HERP1 and HES1 (lanes 6 to 17). These proteins were incubated with the DNA probe C-1 at room temperature for 20 min, and the indicated antibodies (lanes 4, 5, 7, 9, 11 to 13, and 15 to 17) were added to the mixture, followed by additional incubation for 30 min on ice. DNA-protein complexes were resolved by electrophoresis on a 5% acrylamide gel. Note that nuclear extracts from the cells expressing both HES1 and HERP1 showed a dramatic increase of DNA binding activity. Symbols: thin arrow, nonspecific band; thin open arrow, supershifted homodimer band; thick open arrow, heterodimer band; thick solid arrow, supershifted heterodimer band. The amount of HES1 protein in the nuclear extract from the cotransfected cells (lanes 10 and 14) was half of that from singly transfected cells (lane 6), as confirmed by Western blot analysis, and the same was true for HERP1 (data not shown).

FIG. 5. HERP1 and HES1 associate in solution. (A) HERP1 directly associates with HES1 in the absence of DNA in vitro. In vitro protein-protein interaction assays were carried out as described for Fig. 3E. (B) HERP1 associates with HES1 in cells. 293T cells were transfected with pcDNA3.1(-)-Flag-HERP1 or pcDNA3.1(-) empty vector plus pc3Gal-HES1. The extracts from the transfected cells were incubated with an equal amount of anti-Flag M2 antibody or normal mouse IgG. Bound proteins were separated by SDS-PAGE followed by Western blot analysis with anti-GAL4-DBD antibody. Ip, immunoprecipitation; Ab, antibody. Numbers to the right of the gels are molecular masses in kilodaltons.

Whether this interaction is more stabilized upon binding appropriate DNA sequences by the heterodimer remains to be determined (Fig. 4B). Furthermore, whether such protein-protein interactions precede or are required for DNA binding of the heterodimer can only be answered by kinetic studies.

HES and HERP cooperate to repress gene transcription. Given that the HES-HERP heterodimer is the highly preferred DNA-binding species (Fig. 4B and C), we attempted to determine its functional relevance. To address this issue, we first studied repression activities of HES and HERP from the multimerized C-1 binding sites (Fig. 6). HES1 efficiently represses this promoter in a dose-dependent manner (lanes 2 and 3). HERP1 also inhibits gene expression (lanes 4 and 5). Neither HES1 nor HERP1 represses expression from a control reporter gene lacking the C-1 site (data not shown). The degree of repression by HERP1 was considerably less than that by HES1 (compare lanes 2 and 3 and lanes 4 and 5), which is consistent with the weaker DNA binding activity of HERP1 on this particular C-1 DNA sequence (Fig. 4A). Importantly, when both HES1 and HERP1 were coexpressed, more than additive repression was observed (compare lanes 2, 4, and 6 or lanes 3, 5, and 7). This repression is likely derived entirely from the HES1-HERP1 heterodimer, since it is the only DNA-binding species observed (Fig. 4C). Similar results were obtained using the C-2 probe as well and when we used other cell types (data not shown). These data indicate that the HES-

FIG. 6. HERP1 and HES1 synergistically repress transcription. C3H10T1/2 cells were transfected with a reporter plasmid containing multimerized C-1 sites (C1x4-βactin-luc), together with expression vectors for HES1 and HERP1. The data are means of duplicate data from three independent experiments with similar results.

HERP heterodimer functions as a transcriptional repressor when docked at its specific DNA-binding sites. Altogether, HES and HERP can individually repress target gene transcription as homodimers in cells expressing only one or the other protein, whereas the HES-HERP heterodimer is responsible for target gene repression in cells coexpressing both proteins.

DISCUSSION

HERP2 mRNA expression is directly up-regulated by Notch ligand binding without de novo protein synthesis, which renders HERP2 a direct target of Notch signaling (23). Our data show that HERP has intrinsic repression activity. Instead of the TLE/Groucho corepressor that is recruited to the tetrapeptide motif in HES, HERP recruits the mSin3 corepressor complex to its bHLH domain (Fig. 3). HES and HERP are individually expressed in different cells during development (10, 28, 31, 38, 45). In cells expressing either HES or HERP, the respective HES or HERP homodimers can repress gene transcription (Fig. 6). In cells coexpressing both HES and HERP, however, they form a heterodimer as the only DNA-binding species to repress target gene expression (Fig. 4C and 6). Thus, HERP, either as a homodimer or as a heterodimer with the known Notch effector HES, regulates gene expression from specific DNA binding sequences. These findings strongly support the idea that HERP represents a novel Notch effector.

HES and HERP: similar domains with different functions. The present study has revealed that the repression activity of HERP resides primarily in the bHLH domain rather than the YQPW motif, suggesting that the tetrapeptide motif may be dispensable for the HERP family. This is in sharp contrast to the well-established essential roles of the WRPW motif in the HES/E(spl) family (17, 18, 43, 49). However, two exceptions are reported: the WRPW domain of the HES/E(spl) family does not appear to be required for suppression of neurogenesis in zebrafish or for suppression of SCUTE activity in the sex determination pathway in *Drosophila* (12, 47). Thus, the requirement for the WRPW motif of HES/E(spl) is not absolute. It is an interesting possibility that the YQPW motif of HERP

might have a more significant role in repressing target gene expression in other contexts.

Another distinct feature of HERP is the presence of a glycine in its basic domain at a position that is invariably occupied by proline in the HES/E(spl) family. The strict conservation of glycine among all the HERP family members suggests an important role of the residue. Given that HERP had different DNA binding preferences than those of HES1, the proline-to-glycine substitution might contribute to defining different DNA binding preferences for the two proteins. In an analogous situation, however, a role for the proline in the DNA binding of HES1 is yet to be established, since a proline-to-asparagine mutation in an E(spl) protein largely diminished its DNA binding activity, whereas a proline-to-threonine mutation had little effect (48). Thus, further studies are needed to determine the contribution of the glycine in defining the DNA binding specificity of HERP.

Recruitment of an HDAC complex by HERP. HERP recruits HDAC1 as a subunit of the mSin3 complex. Interestingly, Chen et al. have shown for *Drosophila* that Groucho can interact with Rpd3, an orthologue of mammalian HDAC (8). This raises the possibility that mammalian TLE might also recruit HDAC and, therefore, that HES/E(spl) and HERP might share a partly common repression mechanism that includes chromatin remodeling by HDACs. However, HES and HERP engender their own unique protein-protein interactions. For instance, Groucho is involved in additional interactions with histones H3 and H1 (8, 42). In addition, the mSin3 complex (which is recruited to HERP) also has HDAC-independent repression activity (29), likely mediated by multiple subunits of the complex as well as other associating proteins such as N-CoR. It has recently been shown that HES1 retains its repression activity in cells lacking N-CoR (25). Thus, HES and HERP employ different repression mechanisms involving heterologous sets of proteins. The mSin3 complex is recruited to a number of DNA binding transcription factors including the nuclear hormone receptors (1, 21, 36), PLZF (11), MeCP2 (26, 39), Ski (40), p53 (35), and Mad (6). Although Mad is a bHLH-Zip transcriptional repressor, it uses its N-terminal amphipathic α -helical region to recruit the mSin3 corepressor complex rather than its bHLH domain (6). To our knowledge, the present study is the first to show that a bHLH domain of a transcription factor can serve as an interface to recruit the mSin3 complex and its component HDACs.

HERP, a new heterodimer partner for HES/E(spl). bHLH proteins form a dimer through their HLH domains that properly positions and orients the basic domains for specific DNA sequences, the E box (CANNTG) and its variants. A basic domain of each subunit of a dimer recognizes a half-site of the E box (7, 33). Thus, each of three dimers, HES-HES, HERP-HERP, and HES-HERP, should have its own DNA binding specificity. Consistent with this idea, the HERP homodimer showed a different DNA binding sequence profile than that of the HES homodimer (Fig. 4A). The finding that HES and HERP homodimers bind common DNA sequences but with distinct preferences suggests that they may regulate both common and different target genes.

Regulation by homodimers may occur primarily in cells that express either HES or HERP, since in the presence of both proteins homodimers disappear and a HES1-HERP1 het-

FIG. 7. Model for HES and HERP cooperation in Notch signaling. Upon Notch stimulation, HES and HERP expression might both be induced. In tissues where only HES or HERP is expressed, the respective homodimer binds promoters of target genes. The HES homodimers recruit TLE via their WRPW motif, whereas the HERP homodimers recruit the mSin3A-HDAC-N-CoR complex via their bHLH domain. In tissues where both HES and HERP are coexpressed, the HES-HERP heterodimers become the predominant complex that binds a specific DNA site newly defined by the basic domains of HES and HERP. Because of the higher DNA binding affinity of the heterodimers, a lower concentration of them may be sufficient to achieve repression. Repression by HES-HERP heterodimers may be reinforced by their ability to recruit a more diverse set of repressors. The model is based on the experimental data largely from HES1 and HERP1. Because of the significant similarity among members of each family, however, such a model may be apt for other members including those in humans, mouse, and *Drosophila*.

erodimer becomes the exclusive species for most binding sites tested. The heterodimer showed a moderate increase in DNA binding activity in vitro (Fig. 4B), whereas it showed a drastic increase in vivo (Fig. 4C). This discrepancy might reflect post-translational modification or additional cofactors present only in vivo. In any case, the higher DNA binding activity in vivo further supports the physiological relevance of the heterodimer. Heterodimer formation may be essential to bring a DNA binding activity above a threshold level for any physiologically meaningful repression, and thus, it may represent an on-and-off switch for Notch signaling rather than simply providing a linear increase of signal. Alternatively, it may represent an efficient mechanism to amplify the signal or to change target genes.

One critical issue regarding the heterodimer formation is whether Notch stimulation can simultaneously up-regulate expression of HES and HERP in a single cell type. We have indeed observed the coexpression of HES and HERP mRNAs after Notch stimulation in certain cell types (23). Interestingly, the degree of HERP mRNA induction is typically severalfold higher than that of HES. Thus, it is possible that HERP protein might be abundant enough to generate HERP homodimers after formation of the HES-HERP heterodimer, which could regulate a wider spectrum of genes including both the homodimer-specific and the heterodimer-specific target genes.

HERP as a novel Notch effector. Our data show that most of the HERP homodimer binding sites are also bound by HES, albeit with different efficiency (Fig. 4A). This suggests that the HERP homodimer, at least in part, may share common target genes with the HES homodimer, and these sites might include tissue-specific transcriptional activators such as neurogenin

and MASH1. Although target genes of HERP remain to be determined, the idea that HERP is a natural Notch effector is now strongly supported by the following observations: first, HERP expression is directly up-regulated by Notch signaling (23); second, HERP has intrinsic repressor activity (Fig. 2); third, HERP forms a heterodimer with the established Notch effector HES (Fig. 5); and fourth, the HES-HERP heterodimer binds the same group of target DNA sequences as does the HES homodimer, albeit with distinct preferences (Fig. 4B and C). The remarkable increase in DNA binding activities shown by the HES-HERP heterodimer in vivo, accompanied by the functional cooperation of the proteins (Fig. 6), raises the possibility that heterodimerization of effectors may be a more general strategy to amplify Notch signaling. Thus, in some tissues where only one or the other is known to be expressed, there might exist other Notch effectors yet undiscovered.

In summary, our data support a model (Fig. 7) in which the Notch effector HES and its novel partner HERP synergistically repress downstream target genes by preferentially forming a heterodimer. The heterodimers recruit more diverse repressive functions than can be mustered by homodimers of either partner.

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