Acid α-glucosidase (GAA) is a lysosomal enzyme that degrades glycogen. A deficiency of GAA is responsible for a recessively inherited myopathy and cardiomyopathy, glycogenosis type II. Previously, we identified an intronic repressor element in the GAA gene and demonstrated that Hes-1, a basic helix-loop-helix factor, binds to a C class E box within the element and functions as a transcriptional repressor in HepG2 cells. Hes-1 is a well studied downstream target gene in the Notch signaling pathway. In this study, over-expression and depletion of Notch-1 intracellular domain (NICD) strategies were used to investigate whether expression of the GAA gene is under the control of Notch-1/Hes-1 signaling. In cotransfection experiments, Hes-1, up-regulated by over-expressed NICD, enhanced the repressive effect of the DNA element with wild type Hes-1 binding sites but not with mutant Hes-1 binding sites. Conversely, depletion of Notch-1 with phosphorothioated antisense oligonucleotides, corresponding to the fourth ankyrin repeat within NICD, led to reduced Hes-1. Constitutively over-expressed Hes-1 and Notch-1 repressed GAA gene expression. Therefore, our data establish that the human GAA gene, encoding a lysosomal enzyme, is a downstream target of the Notch-1/Hes-1 signaling pathway.

Notch receptors, members of a transmembrane protein family, play essential roles in cell fate decision in several developmental processes from Drosophila to human (1–3). Four Notch receptors (Notch 1–4) and four ligands (Jagged-1, Jagged-2, and Delta-like 1 and 3) have been identified in mammals. The biochemical mechanisms of Notch signaling are conserved among the Notch receptors (4). Upon activation by Notch ligands, Notch intracellular domain (NICD) is cleaved, released from the whole receptor, and translocated into the nucleus. There it forms a complex with DNA-binding protein RBP-Jk (CSL/CFB1/Su(H)/Lag-1) and directly activates transcription of its downstream target genes (5–8).

Hes, the mammalian homologues of Drosophila hairy and Enhancer of Split proteins are basic helix-loop-helix proteins, which function as transcriptional factors (9). Among Hes family members, Hes-1, -2, -3, -5, -6, and -7 genes have been isolated in mammals (10–15). The Hes-1 gene has been established as a downstream target of Notch signaling (16–18). Like Notch receptors, Hes-1 is also essential to several developmental processes such as neurogenesis, myogenesis, hematopoiesis, and T cell development (19), and additional functions of this transcription factor continue to be reported (20, 21). As a transcriptional repressor, Hes-1 down-regulates its target genes, such as Mash-1 and CD4 genes, by binding to a C class E box (CANNTG) or an N box (CACNAG) (22–24). Transducin-like enhancer (TLE), a mammalian homolog of the Drosophila Groucho, interacts with Hes-1 as a co-repressor (25).

Acid α-glucosidase (GAA; acid maltase, EC 3.2.1.20) is a glycogen-degrading lysosomal enzyme, a deficiency of which causes glycogen storage disease type II (GSDII, also called Pompe’s disease or acid maltase deficiency), a recessively inherited muscle disease (26). GAA belongs to a large family of lysosomal enzymes. The regulation of this gene, as well as that of other lysosomal enzyme genes, is largely unknown. In previous studies (27) in human hepatoma cells (HepG2), we have demonstrated that Hes-1 functions as a repressor for transcription of the human GAA gene. Hes-1, binding to a C class E box (CAGGCC) within an intronic repressor element, down-regulates the human GAA gene expression.

To determine whether the human GAA gene is under the control of the Notch-1/Hes-1 signaling pathway, we have investigated the effect of Notch-1 and Hes-1 on expression of the human GAA gene in HepG2 cells. We have demonstrated the Notch-1/Hes-1 pathway in HepG2 cells and shown that the human GAA gene is a downstream target of Notch-1/Hes-1 signaling.

**EXPERIMENTAL PROCEDURES**

**Plasmid Constructs**—Expression plasmid constructs containing four copies of repressor element with wild type (4xwtHes-1/TK-CAT) or mutant Hes-1 binding E box (4xmutHes-1/TK-CAT) used for co-transfection experiments were described previously (27) and are shown in Fig. 1A. The expression construct for Hes-1, pcDNA3-wtHes-1, was a gift from Drs. Michael Caudy and Felix Loh (Cornell University Medical College, New York, NY). The expression construct for NICD, cytobOptiDNA3, was a gift from Prof. Spyros Artavanis-Tsakovas (Massachusetts General Hospital, Harvard Medical School, Charlestown, MA). All of the plasmids were prepared and purified for transfection with a Qiagen Maxi Kit (Qiagen, Valencia, CA).

**Cell Culture and Transfection**—HepG2 cells were grown in Dulbecco’s modified Eagle’s medium with 10% fetal calf serum and penicillin/streptomycin (Invitrogen). For co-transfection experiments, 4xwtHes-1/TK-CAT or 4xmutHes-1/TK-CAT (1.0 μg) were incubated with plasmid pcDNA3 or cytobOptiDNA3 (3.0 μg) together with 10 μl of LipofectAMINE. Expression plasmid pXGH5 for human growth hormone (0.5 μg) was included as an internal control of the transfection efficiency. Forty-eight hours later, the cells were harvested for CAT and hGH activity assays (CAT and hGH enzyme-linked immunosorbent assay kits, Roche Molecular Biochemicals). CAT activities were further standardized to hGH activities. For stable transfection, pcDNA3, cytobOptiDNA3, or pcDNA3-Hes-1 was transfected into HepG2 cells.
FIG. 1. The effect of NICD on the expression of the reporter gene and Hes-1. A, structures of reporter expression constructs for co-transfection experiments. B, HepG2 cells were transfected with plasmid pBLCAT2, 4xwtHes-1/TK-CAT, and 4xmutHes-1/TK-CAT, alone or in combination with pcDNA3 or cytOH1pcDNA3 at an amount ratio of 1:3. Plasmid pXGH5 was used to monitor transfection efficiency. CAT activities are normalized to hGH activities and expressed as a percent of expression relative to cells transfected with empty vector. The CAT activities from at least three independent transfection experiments. The bars represent the mean and standard deviation of CAT activities from at least three independent transfection experiments. *, p < 0.01 compared with 4xmutHes-1/TK-CAT alone or with pcDNA3. C, up-regulation of Hes-1 gene expression by over-expressed NICD. HepG2 cells were transiently transfected with cytOH1pcDNA3 and total RNA was extracted two h after transfection. Northern blot analysis was performed with Hes-1 cDNA as a probe. Ethidium bromide staining of 18 S RNA is shown for equal loading of sample wells. The experiment was repeated at least three times.

with LipofectAMINE. Individual colonies were obtained with G418 (0.6 mg/ml) selection for 2 weeks. The colonies were grown and expanded in Dulbecco’s modified Eagle’s medium with G418 (0.6 mg/ml) for further assays. Pools of pcDNA3-transfected HepG2 cells were obtained by G418 selection for 2 weeks and used as controls.

Phosphorothioated Oligonucleotide Treatment—Phosphorothioated antisense oligonucleotides of 30mer in length corresponding to fourth ankyrin repeat within NICD were used for depletion of Notch1 in HepG2 cells. The DNA sequence is 5’-GCCCGCATCATGATGTCGAC-GACGCCACTG-3’ (28). No homology of the sequence was found with a BLAST search of the GenBank™ data base. The corresponding phosphorothioated sense oligonucleotides were used as internal control. All of the oligonucleotides were synthesized and purified by Lofstrand Labs Limited (Gaithersburg, MD). Oligonucleotides (2.0 µM) were incubated with LipofectAMINE (5 µg/ml) (Invitrogen) and overlaid onto the cells as described (20, 29). The medium containing oligonucleotides-Lipo-fectAMINE complexes was changed daily for 1 week.

Northern Blot Analysis—HepG2 cells were transfected with pcDNA3 or cytOH1pcDNA3 and LipofectAMINE. Total RNA was extracted at intervals after transfection with TRIzol reagent (Invitrogen). Northern blot analysis was performed according to standard procedures. A Hes-1 probe (697 bp) was generated by PCR with pcDNA3-wtHes-1 as a template with the primers 5’-ACCATGTCACACGACCGGAT-AACC-3’ (forward) and 5’-GTGCGTGTTGAGACGGGATGACAG-3’ (reverse).

Western Blot Analysis—Cell lysates were prepared with radiomune precipitation lysis buffer (10 mM Tris-HCl (pH 7.5), 150 mM NaCl, 0.1% SDS, 1.0% Triton X-100, 1.0% sodium deoxycholate, aprotinin 2.0 µg/ml, leupeptin 2.0 µg/ml, pepstatin 1.0 µg/ml, N3-p-tosyl-l-lysine chloromethyl ketone 50 µg/ml, and phenylmethylsulfonyl fluoride 100 µg/ml). Western blot analysis was carried out as described previously (30, 31). NICD, Hes-1, and GAA were detected using anti-Notch1 antibody (Upstate Biotechnology, Inc., Lake Placid, NY), anti-Hes-1 anti-body (a gift of Dr. Barry J. Byrne, University of Florida, Gainesville, FL), respectively. Immunodetection of β-catenin (Santa Cruz Biotechnology, Santa Cruz, CA) was used for loading control.

GAA Enzyme Activity Assay—HepG2 cells were prepared with lysis buffer (0.9% NaCl, 0.25% Triton X-100). The GAA and β-hexosaminidase activities of transfected HepG2 cells were assayed by conversion of the substrates 4-methylumbelliferyl β-D-glucoside and 4-methylumbelliferyl N-acetyl-β-D-glucosamidine to the fluorescent product umbelliferone as described (32). Protein concentration of whole cell lysate was measured with the Bio-Rad protein assay kit (Bio-Rad Laboratories, Hercules, CA).
RESULTS

Notch-1/Hes-1 Pathway in HepG2 Cell Line—Hes-1 has been shown to be a downstream target of Notch-1 signaling in human cell lines (22). To determine whether Notch-1/Hes-1 signaling is functional in HepG2 cells, co-transfection experiments were carried out with reporter gene constructs containing Hes-1 binding sites and NICD expression plasmid. Expression of the reporter gene linked to the wild type Hes-1 sites was significantly down-regulated by NICD (Fig. 1B). In contrast, expression of the reporter gene linked to mutant Hes-1 sites was not affected (Fig. 1B), suggesting that intact Hes-1 sites are required for NICD effect.

To establish the relationship between Notch-1 and Hes-1 in HepG2 cells, NICD was transiently transfected into the cells, and Northern blot analysis was performed with Hes-1 cDNA as a probe at 2, 6, 12, 24, and 48 h post-transfection. A 2.3-fold up-regulation of the Hes-1 gene expression by NICD, compared
with empty vector, was observed at 2 h post-transfection (Fig. 1C). This up-regulation, however, was transient and became marginal at later time points (not shown).

The validity of the Notch-1/Hes-1 pathway in HepG2 cells was further confirmed by depletion of Notch-1 with phosphorothioated antisense oligonucleotides of 30mer corresponding to the fourth ankyrin repeat of NICD. Corresponding phosphorothioated sense oligonucleotide was used as a control. After treatment for 1 week the depletion of Notch-1 (62%) was accompanied by a decrease in Hes-1 gene expression, as shown by the 56% reduction of Hes-1 protein on Western analysis (Fig. 2). 

**Notch-1/Hes-1/GAA Pathway in HepG2 Cell Line**—To demonstrate the effect of Notch-1 on the endogenous GAA gene, HepG2 cells were transfected with Hes-1 expression plasmid and stably transfected colonies were analyzed. Over-expression of Hes-1 down-regulated GAA gene expression, resulting in a decrease in the GAA protein level as shown by Western analysis (Fig. 3A and B). Consistent with these results, up to 30% reduction in GAA enzyme activity was observed after Hes-1 over-expression (Fig. 3C). Another lysosomal enzyme, β-hexosaminidase, used as an internal control, was not affected by over-expressed Hes-1 (Fig. 3C). Thus, expression of the GAA gene is negatively regulated by Hes-1 in HepG2 cells.

The effect of NICD over-expression in HepG2 cells was similar to that of Hes-1-negative regulation of the GAA gene expression. HepG2 cells constitutively expressing ectopic NICD were obtained by transfection with cytobN1pcDNA3 and selection with G418. As shown in Fig. 4, over-expression of NICD confirmed by Western blot with anti-Notch1 antibody (Fig. 4A), resulted in a reduced amount of GAA protein (Fig. 4B) and reduced enzyme activity (Fig. 4C). The data strongly suggest that human GAA is regulated by Notch-1/Hes-1 pathway in HepG2 cells.

**DISCUSSION**

GAA is a typical housekeeping gene that is expressed in every tissue. It has been shown, however, that during development there are significant quantitative differences in the level of murine GAA expression in different tissues and even within a particular tissue (33), suggesting that the gene is transcriptionally regulated. In humans, both protein and enzyme activity are significantly higher in newborns compared with adults, again suggesting that the gene is transcriptionally regulated during development (34). In our previous studies (27, 35) we identified a cis-acting negative regulatory element in the first intron of the GAA gene and demonstrated that transcription factor Hes-1 binds to the element in a tissue-specific manner and functions as a repressor in HepG2 cells. The Hes-1 gene and its homologues are immediate downstream genes of the Notch signaling pathway in many cells. In this study, we provide evidence of the Notch-1/Hes-1 pathway in HepG2 cells, demonstrating that the human GAA gene is a downstream target of Notch-1/Hes-1 signaling. This pathway may contribute to the developmental control of GAA gene expression.

Several lines of evidence indicate that Hes-1 is under Notch-1 control in HepG2 cells; depletion of Notch-1 led to a decreased level of Hes-1, whereas over-expression of NICD led to up-regulation of endogenous Hes-1, which in turn down-regulated the reporter gene expression through binding to its recognition sequence. The up-regulation of endogenous Hes-1 transcription by over-expressed NICD in HepG2 cells, detectable at 2 h post-transfection, was transient, however; the effect became marginal afterward. Although several studies have demonstrated that Hes-1 is activated by constitutively over-expressed NICD (18, 36, 37), others have reported only marginal or no changes in Hes-1 gene expression under Notch-1 signaling (38, 39).

Transient activation of Hes-1 gene expression has also been observed under ligand-activated Notch-1 signaling and retinoic acid-induced differentiation (40–42). This transient activation of Hes-1 is most likely due to the ability of the protein to negatively auto-regulate itself because three stretches of recognition sequence (N boxes) are present within the promoter of Hes-1 gene (11, 43, 44). This autoregulation loop of Hes-1 gene expression may act as a regulator for the varied functions of Notch-1 signaling during development (45).

The link between GAA and Notch-1/Hes-1 pathway was established by demonstrating a repressive effect of both Notch-1 and Hes-1 on the levels of GAA protein and activity. The effect was not a dramatic one, which is hardly surprising. After all, only minor differences in the level of GAA activity separate the most severe rapidly progressive form of GSDII (Pompe’s disease) from milder late-onset variants of the disease.

Recently, another Hes-related repressor protein, HERP, has been shown to be a target of Notch-1 signaling, which functions as a heterodimer partner of Hes-1 (39, 45, 46). Although our data do not rule out the role of HERP, we believe that Hes-1 rather than HERP is mainly responsible for the transcriptional repression of the human GAA gene because the silencer in the GAA gene contains a recognition sequence a C class E box (CACGCG) preferred by Hes-1 (46). These new data thus show yet another aspect of the Notch-1/Hes-1 signaling pathway; however, this lysosomal enzyme may have still unrecognized physiological roles in development.

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Human Acid α-Glucosidase Gene Is a Target of Notch-1/Hes-1
