

## BRIEF REPORT

## Targeted Therapy for Inherited GPI Deficiency

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## SUMMARY

Disrupted binding of the transcription factor Sp1 to the mutated promoter region of the mannosyl transferase–encoding gene *PIGM* causes inherited glycosylphosphatidylinositol (GPI) deficiency characterized by splanchnic vein thrombosis and epilepsy. We show that this results in histone hypoacetylation at the promoter of *PIGM*. The histone deacetylase inhibitor butyrate increases *PIGM* transcription and surface GPI expression in vitro as well as in vivo through enhanced histone acetylation in an Sp1-dependent manner. More important, the drug caused complete cessation of intractable seizures in a child with inherited GPI deficiency.

**L**INKAGE TO GLYCOSYLPHOSPHATIDYLIINOSITOL (GPI) IS A MODE OF CELL-SURFACE expression used by proteins of diverse functions.<sup>1</sup> Acquired GPI deficiency, as seen in the clonal disorder paroxysmal nocturnal hemoglobinuria, is characterized by hemolytic anemia, thrombosis, and bone marrow failure.<sup>2,3</sup> In paroxysmal nocturnal hemoglobinuria, deficiency of GPI-linked proteins is caused by somatic mutations in the X-linked gene *PIGA*, resulting in a block in the addition of glucosamine to phosphatidylinositol.<sup>4,5</sup>

We recently described a new form of inherited GPI deficiency presenting in infancy, characterized by splanchnic vein thrombosis and seizures and inherited as an autosomal recessive trait.<sup>6</sup> As compared with paroxysmal nocturnal hemoglobinuria, inherited GPI deficiency does not result in clinically significant hemolysis and bone marrow failure. In the families described so far, partial yet severe GPI deficiency is caused by the blocked addition of the first mannose residue onto the GPI intermediate phosphatidylinositol–glucosamine, a step catalyzed by the  $\alpha$ 1,4-mannosyltransferase *PIGM*. The genetic defect in inherited GPI deficiency is a  $-270C\rightarrow G$  mutation in the core promoter of *PIGM*, which disrupts binding of the transcription factor Sp1 to its cognate motif, resulting in markedly reduced transcription.<sup>6</sup>

Sp1 influences transcription through heterotypic interactions with the basal transcriptional machinery and with other transcription factors and by recruiting histone acetyltransferases and histone deacetylases to the promoter.<sup>7</sup> Histone deacetylase inhibitors such as sodium butyrate enhance transcription and hold promise as therapeutic agents for a variety of diseases, including cancer.<sup>8</sup> For a small number of genes, the hyperacetylation effect of sodium butyrate requires the presence of Sp1-binding elements or a conserved sequence to which an as-yet-unknown transcription factor binds; these promoter elements are also referred to as butyrate response elements.<sup>9</sup> In Sp1-dependent genes containing butyrate response elements, sodium butyrate

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may modulate transcription through covalent modification of Sp1 as well as through histone hyperacetylation.<sup>7,9</sup>

We investigated whether the *PIGM* promoter contained butyrate response elements and whether modification of acetylation mediated by sodium butyrate could result in enhanced transcription of *PIGM*, even in the presence of the mutated Sp1-binding motif associated with inherited GPI deficiency.

## METHODS

### PATIENT AND STUDY DESIGN

The patient was seen at King's College Hospital and Hammersmith Hospital in London. The study was approved by the respective local research ethics committees, and oral informed consent was provided in accordance with the provisions of the Declaration of Helsinki.

### CELL LINES AND ANALYSES

The generation of Epstein–Barr virus (EBV)–transformed lymphoblastoid cell lines was described previously.<sup>10</sup> For details regarding flow cytometry,

real-time polymerase-chain-reaction analysis, reporter assays, and statistical analysis, see the Supplementary Appendix, available with the full text of this article at [www.nejm.org](http://www.nejm.org). Chromatin immunoprecipitation assays (Upstate Biotechnology) were performed according to the manufacturer's instructions except for the following modifications: immunoprecipitation was performed with anti-acetylated histone 4 (Upstate Biotechnology) or rabbit IgG (Santa Cruz). After DNA-protein cross-linking reversal and proteinase K digestion, DNA was isolated with the use of a MiniElute PCR purification kit (Qiagen). Primer sequences are available from the authors on request.

## RESULTS

### CLINICAL REPORT

In 1995, a 2-year-old girl (now 14 years of age) presented with hepatic vein thrombosis and the Budd–Chiari syndrome (Table 1 of the Supplementary Appendix). After one episode of hepatic decompensation owing to variceal bleeding at the age of 5 and two shunt procedures at the age of 7, portal hypertension eventually became well con-

#### Figure 1 (facing page). Effect of Butyrate In Vitro.

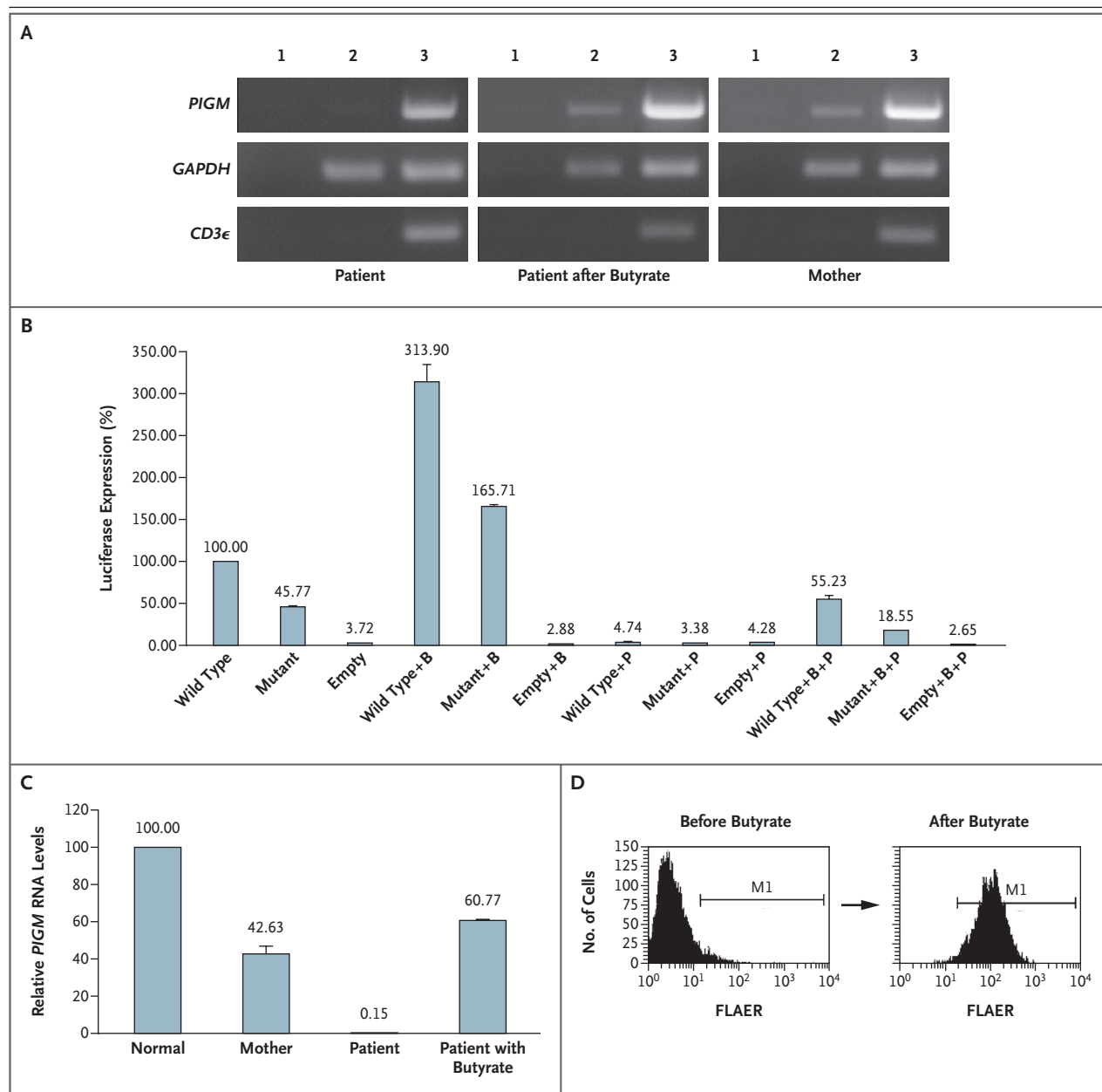
Panel A shows histone acetylation at the *PIGM* locus. Assessment of the acetylation status of histone 4 was performed with the use of chromatin immunoprecipitation assays. There was no evidence of acetylation at the *CD3ε* locus in lymphoblastoid cell lines from either the patient or her mother, and this finding did not change in the presence of sodium butyrate. Acetylation was detectable at the locus of *GAPDH* in both cell lines, and *PIGM* locus acetylation was present in the cell line of the mother but not of the patient. On exposure to sodium butyrate, acetylation was restored in the patient's cell line. Results of three independent experiments were similar and are represented as follows: lane 1, immunoprecipitation with IgG monoclonal antibody control; lane 2, immunoprecipitation with antiacetylated histone 4 monoclonal antibody; and lane 3, input nuclear extract. Panel B shows the effects of sodium butyrate (B) and plicamycin (P) on the reporter activity of the firefly luciferase reporter plasmid (pGL3) with wild-type and mutant *PIGM* promoter. The reporter assays were carried out in HeLa cells, and luciferase activity was measured 48 hours after transfection. The wild-type construct was considered to have 100% activity. Activity of the mutant construct was reduced to 45% (95% CI, 27 to 76). When incubated with 3 mM of sodium butyrate, the reporter protein activity of both wild-type and mutant constructs increased by a factor of 3.1 and 3.5, respectively (95% CI, 1.3 to 7.7 and 2.2 to 5.8, respectively). In the presence of plicamycin, luciferase activity was almost completely absent with both wild-type and mutant constructs, whereas activity of renilla luciferase reporter plasmid (pRL-CMV) was not affected. In the presence of butyrate and plicamycin, promoter activity was similarly reduced for both the wild-type construct (by 82%; 95% CI, 72 to 88) and mutant construct (by 88%; 95% CI, 87 to 89) below butyrate-induced and baseline levels; however, residual activities of 55% and 18%, respectively, were still observed. The columns labeled as empty refer to pGL3 without the *PIGM* promoter sequences. The means ( $\pm$ SE) of four independent experiments are shown. Panel C shows *PIGM* RNA levels, as determined by real-time polymerase chain reaction, in affected lymphoblastoid cell lines before and after sodium butyrate treatment. A normal cell line, homozygous for wild-type sequence, and one derived from the mother, heterozygous for the  $-270\text{C}\rightarrow\text{G}$  mutation, were used as controls. *PIGM* expression levels in the normal cell line were considered to be 100%. In comparison, *PIGM* RNA levels in the maternal cell line were 43% of normal (95% CI, 29 to 63), and they were less than 1% of normal in the patient's cell line before butyrate treatment. After 72 hours of incubation with 3 mM of sodium butyrate, *PIGM* RNA levels in the patient's cell line increased by a factor of 407 (95% CI, 257 to 644). The means ( $\pm$ SE) of four independent experiments are shown. Panel D shows the correction of the GPI-negative phenotype in the patient's cell line after exposure to sodium butyrate. GPI expression, as determined by fluorescent inactivated aerolysin (FLAER) staining and flow cytometry, was completely restored in the patient's cell line after incubation with 3 mM of sodium butyrate for 72 hours. M1 indicates positive events as determined by the isotopic control set at 1%.

trolled with conservative therapy (spironolactone, propranolol, and oral anticoagulants).

Absence seizures developed at the age of 4 years and were initially well controlled with sodium valproate; lamotrigine was added when the patient was 9 years old after an episode of status epilepticus, with good control of seizures. When she was first seen in the United Kingdom at the age of 9 years, tests for thrombophilia and the results of metabolic screening were normal; the diagnosis of inherited GPI deficiency was made by flow-cytometric analysis of GPI expression on blood cells.<sup>6</sup> During the next 5 years, the frequency of

seizures increased progressively despite increases in anticonvulsant therapy. Sodium valproate was stopped and levetiracetam started at the age of 12 years; a year later, topiramate was added. When the patient was seen in the United Kingdom again at the age of 14 years, she was having multiple absence seizures and approximately five tonic-clonic seizures per day; antiepileptic treatment consisted of lamotrigine, levetiracetam, topiramate, and clobazam (Table 1 of the Supplementary Appendix).

On examination, the patient was wheelchair-bound with global hypotonia, drooling, and extreme drowsiness. She was poorly responsive and



unable to feed herself, symptoms that may have reflected toxic effects of the antiepileptic therapy as well as the disease itself. Central nervous system imaging, including magnetic resonance imaging and magnetic resonance angiography and venography, showed no structural abnormalities or evidence of thrombosis. Findings on electroencephalography performed while the patient was having multiple absence episodes were grossly abnormal, with frequent multifocal and generalized epileptiform discharges and a massive photoconvulsive response, and were consistent with an electrographic diagnosis of nonconvulsive status epilepticus.

#### EFFECT OF SODIUM BUTYRATE IN VITRO

Since Sp1 could be important for histone acetylation,<sup>9,11</sup> we studied the effect of the  $-270C \rightarrow G$  mutation on the acetylation status of histone 4 in the promoter region of *PIGM*, using chromatin immunoprecipitation assays. As expected, there was no evidence of histone acetylation in the promoter of the T-cell-specific gene *CD3ε* in lymphoblastoid B-cell lines from either the patient or her mother (Fig. 1A). Consistent with the function of a housekeeping gene, histone 4 at the promoter of *GAPDH* was fully acetylated in both cell lines. Similarly, acetylation at *PIGM*, itself a housekeeping gene, was also readily detected in the maternal lymphoblastoid cell line. However, in the patient's cell line, there was no evidence of histone acetylation at the *PIGM* promoter, suggesting that the  $-270$  Sp1-binding motif is crucial for histone acetylation. Histone 4 acetylation was fully restored on exposure of the patient's lymphoblastoid cell line to sodium butyrate, which suggested the presence of promoter sequences that on inhibition of histone deacetylases could substitute for the disrupted  $-270$  motif in promoting histone hyperacetylation.

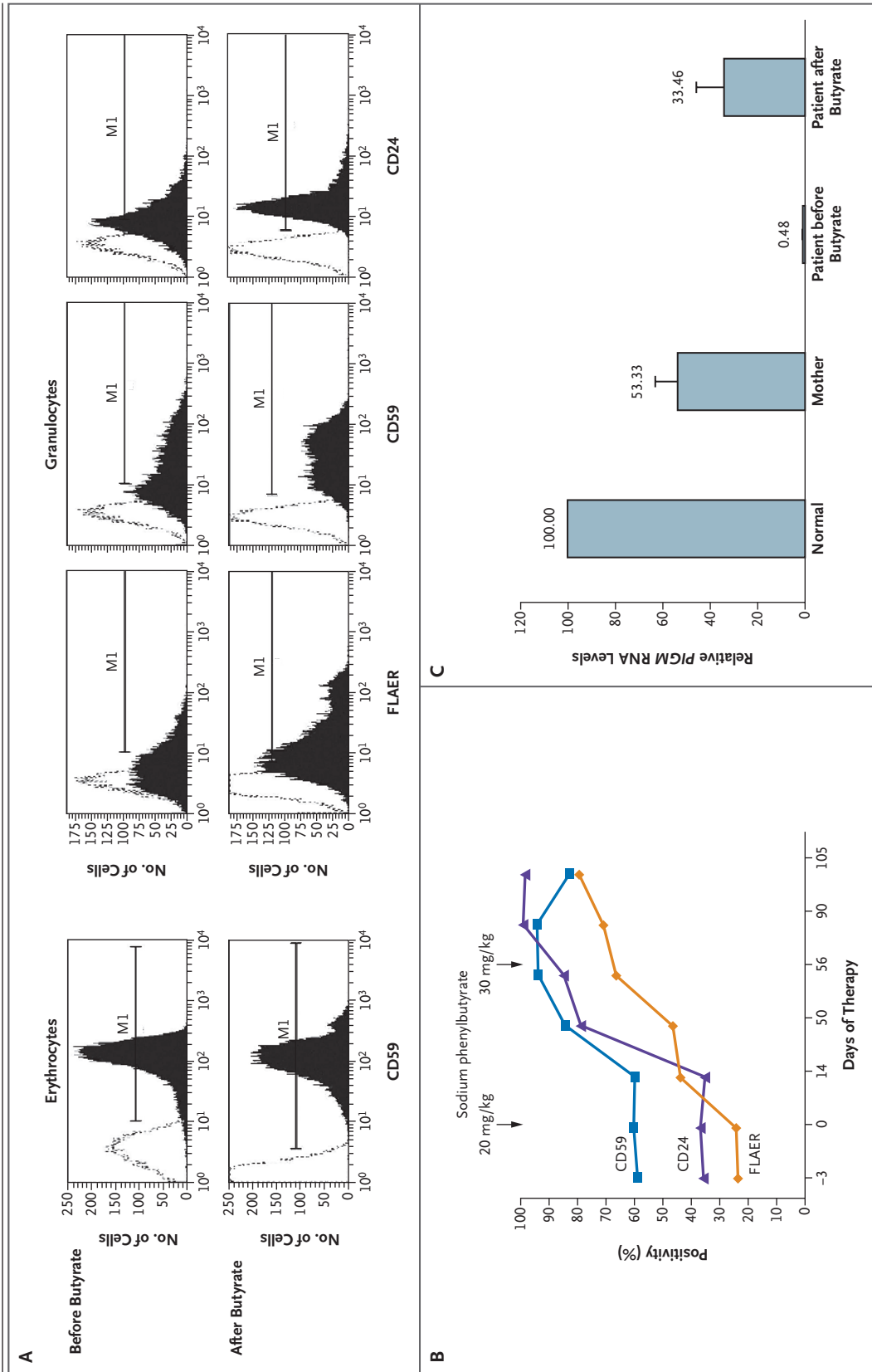
To test whether inhibition of histone deacetylases could affect *PIGM* transcription through Sp1, we tested the effect of sodium butyrate in luciferase reporter assays, using wild-type or mutant *PIGM* promoter constructs. As shown previously,<sup>6</sup> the  $-270C \rightarrow G$  mutation resulted in a 55% reduction in *PIGM* promoter activity (Fig. 1B). In the presence of sodium butyrate, transcriptional activity increased by a factor of approximately 3 with both constructs. In the presence of plicamycin (formerly called mithramycin), an agent that blocks Sp1 binding to DNA,<sup>12,13</sup> transcriptional activity was almost entirely abolished for both constructs,

#### Figure 2 (facing page). Effect of Butyrate In Vivo.

Panel A shows expression of GPI and GPI-linked proteins on the surface of erythrocytes and granulocytes before and 12 weeks after the initiation of butyrate therapy in the patient. There is a change in the pattern of expression of all antigens tested. In the erythrocytes, the small proportion of CD59-negative cells present before therapy almost completely disappeared after butyrate therapy. Staining of granulocytes with fluorescent inactivated aerolysin (FLAER) revealed a greater proportion of GPI-positive cells after treatment. This finding was mirrored in the expression of GPI-linked proteins, all of which showed a right shift with increased antigen expression after therapy. Events are negative if they are below the 1% mark of the isotypic control and positive if they are above it (designated as M1). Panel B shows an increase in expression of GPI and GPI-linked antigens over time during butyrate therapy. Sodium phenylbutyrate was started at a dose of 20 mg per kilogram of body weight and increased to 30 mg per kilogram on day 60. Throughout the therapy, there was a progressive increase in the number of cells staining positive for all three GPI-linked antigens tested (i.e., FLAER, CD59, and CD24) after 12 weeks, with a mean increase by a factor of 2.2 (95% CI, 1.0 to 4.8) from day 0 to day 90. Panel C shows *PIGM* RNA levels in the patient's peripheral-blood mononuclear cells before and after 12 weeks of butyrate therapy. *PIGM* RNA levels from a person without inherited GPI deficiency and from the patient's mother were used as controls. After 12 weeks of butyrate therapy, the patient's *PIGM* RNA levels increased by a factor of 70 (95% CI, 10 to 480). The means ( $\pm$ SE) of five independent experiments are shown.

suggesting that Sp1 binding is critical for efficient transcription of *PIGM*. Plicamycin similarly reduced but did not completely abolish sodium butyrate-enhanced transcriptional activity: 55% and 18% residual activity was observed for the wild-type and mutant constructs, respectively.

The increased transcriptional activity of the *PIGM* promoter in the presence of sodium butyrate was accompanied by an increase by a factor of 400 in *PIGM* messenger RNA (mRNA) levels in the patient's cell line (Fig. 1C). At the same time, expression of surface GPI, as assessed by fluorescent inactivated aerolysin (FLAER) staining, was completely restored (Fig. 1D). Taken together, these findings suggested that sodium butyrate-mediated transcriptional activation could take place even in the presence of the mutated Sp1 motif, causing inherited GPI deficiency. In addition to being histone hyperacetylation-dependent, this effect is also Sp1 binding-dependent and, to a lesser extent, Sp1-independent.



**EFFECT OF SODIUM BUTYRATE IN VIVO**

In view of the intractable seizures experienced by the patient and guided by the effectiveness of sodium butyrate in restoring *PIGM* transcription and GPI expression in vitro by the mutant as well as the wild-type promoter, we started clinical therapy with sodium phenylbutyrate at a dose of 20 mg per kilogram of body weight three times a day. At the same time, levetiracetam, clobazam, and lamotrigine were withdrawn and sodium valproate added.

The in vivo effect of sodium phenylbutyrate on *PIGM* RNA levels and on surface GPI was assessed prospectively in peripheral-blood cells. A progressive increase in the proportions of granulocytes staining positive for FLAER and two GPI-linked proteins was observed, as compared with levels before the administration of sodium phenylbutyrate. Assessment of the effect of this treatment on *PIGM* transcription showed that 3 months after therapy began, *PIGM* mRNA levels in primary mononuclear blood cells increased by a factor of nearly 70 from baseline (from 0.5 to 33.0%) (Fig. 2C). Thus, sodium phenylbutyrate increased *PIGM* transcription and cell-surface GPI expression in vivo as well as in vitro.

An equally dramatic clinical effect was observed (Table 1 of the Supplementary Appendix). The general condition of the patient improved, and she could perform activities that she had been unable to perform for 2 to 3 years — namely, to walk, interact, and feed herself. More important, within 2 weeks after the treatment modification began, she became entirely seizure-free and remained so 5 months later. No side effects were noted, even when the dose of sodium phenylbutyrate was increased to 30 mg per kilogram three times a day after 8 weeks.

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**DISCUSSION**


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We show here that binding of Sp1 to its core promoter motif is crucial for control of the acetylation status and transcriptional activity at the *PIGM* promoter, findings with important therapeutic implications for patients with inherited GPI deficiency. As shown in Figure 1A, the -270 motif is required for maintenance of histone acetylation in the *PIGM* promoter. Its disruption by the mutation responsible for inherited GPI deficiency results in significant reduction in acetylation, *PIGM* transcrip-

tion, and synthesis and cell-surface expression of GPI. These findings are consistent with the previously described function of Sp1 in regulating transcription through recruitment to the promoter of histone acetyltransferases,<sup>14,15</sup> histone deacetylases,<sup>7,15</sup> or both and its function through histone acetylation.

Despite disruption of Sp1 binding to the -270 motif, sodium butyrate increased histone acetylation and transcriptional activity of the mutant *PIGM* promoter, thereby restoring surface GPI expression in vivo as well as in vitro. Presumably, the other three predicted Sp1-binding motifs<sup>6</sup> were responsible for these effects. Our findings with the use of plasmid promoter constructs (Fig. 1B) also suggest that inhibition of histone deacetylases by sodium butyrate can lead to enhanced *PIGM* transcription through binding of Sp1 (and to a lesser degree of other transcription factors) to its cognate promoter motifs in its acetyl-modified form. This hypothesis is in line with previous reports showing that inhibition of histone deacetylases, as well as histone hyperacetylation, can lead to increased transcriptional activity through acetyl modification and increased affinity binding of Sp1.<sup>16,17</sup>

Regulation of acetylation and *PIGM* transcription by Sp1 might be important during embryonic and fetal development, when increased acetylation is required for coordinate expression of numerous genes. In this way, maintenance of an open chromatin configuration by histone acetylation would increase *PIGM* transcription and expression of GPI-linked proteins critical for neural development<sup>18,19</sup> to levels sufficient to prevent lethal neurodevelopmental defects, such as those seen in female mice with a deleted *Piga* gene<sup>20</sup> but inadequate to prevent severe epilepsy. We show that manipulation of acetylation in postnatal life with sodium phenylbutyrate can have a dramatic therapeutic effect on otherwise intractable seizures. The enhancing effect of sodium butyrate on *PIGM* mRNA and surface GPI expression on blood cells in vivo suggest that restoration of GPI biosynthesis in the central nervous system is responsible for the drug's clinical effects. However, since direct assessment of primary neural tissue was not possible, it cannot be ruled out that the neurologic improvement was caused by alternative actions of sodium phenylbutyrate on the central nervous system. Sodium valproate is an antiepileptic agent with properties of a histone deacetylase inhibi-

tor<sup>21</sup> and could have contributed to seizure control, along with sodium butyrate. Indeed, in our *in vitro* assays using concentrations of sodium valproate similar to the *in vivo* therapeutic range, we observed mild restoration of GPI expression in the patient's lymphoblastoid cell line but no synergistic or additive effect with sodium butyrate (data not shown). Presumably, these levels are not sufficient for control of epilepsy *in vivo*.

Whether treatment with sodium phenylbutyrate eliminates or reduces the risk of thrombosis in inherited GPI deficiency is not known. Thrombosis is the only clinical feature shared by inherited GPI deficiency and paroxysmal nocturnal hemoglobinuria; in the latter, the lack of CD59, an inhibitor of membrane-attack complex of complement, is thought to lead to hypercoagulable platelets and contribute to an increased risk of thrombosis.<sup>22</sup> In inherited GPI deficiency, 30 to 50% of platelets are CD59-deficient.<sup>6</sup> However, sodium phenylbutyrate had no effect on the level of CD59

expression on platelets (data not shown), despite increasing granulocyte CD59 expression.

In summary, we have shown that the mutation responsible for inherited GPI deficiency disrupts an Sp1-dependent butyrate response element and is associated with hypoacetylation at the promoter of *PIGM*. Modification of acetylation with sodium butyrate enhances transcription of *PIGM* and surface GPI expression *in vivo* as well as *in vitro* and is of great therapeutic value. Therefore, sodium butyrate may be an effective therapeutic option for other diseases caused by Sp1-dependent hypoacetylation.

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No potential conflict of interest relevant to this article was reported.

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