Identification of Fructose 6-Phosphate- and Fructose 1-Phosphate-binding Residues in the Regulatory Protein of Glucokinase*

Maria Veiga-da-Cunha‡ and Emile Van Schaftingen
From the Laboratory of Physiological Chemistry, University of Louvain and Christian de Duve Institute of Cellular Pathology, B-1200 Brussels, Belgium

Glucokinase is inhibited in the liver by a regulatory protein (GKRP) whose effects are increased by Fru-6-P and suppressed by Fru-1-P. To identify the binding site of these phosphate esters, we took advantage of the homology of GKRP to the isomerase domain of GlmS (glucosamine-6-phosphate synthase) and created 12 different mutants of rat GKRP. Mutations of three residues predicted to bind to Fru-6-P resulted in proteins that were 5-fold (S110A) and 50-fold (S179A and K514A) less potent as inhibitors of glucokinase and had an at least 100-fold reduced affinity for the effectors. Mutation of another residue of the putative binding site (T109A) resulted in a 10-fold decrease in the inhibitory power and an inversion of the effect of sorbitol-6-P, a Fru-6-P analog. The replacement of Gly107, a residue close to the binding site, by cysteine (as in GlmS and Xenopus GKRP) resulted in a protein that had 20 times more affinity for Fru-6-P and 30 times less affinity for Fru-1-P. These results are consistent with GKRP having one single binding site for phosphate esters. They also show that a missense mutation of GKRP can lead to a gain of function.

Glucokinase is the main enzyme responsible for the phosphorylation of glucose in liver cells and in pancreatic beta cells and, as such, plays a major role in the control of blood glucose concentration (reviewed in Refs. 1–4). This role is best illustrated by the fact that mutations that make glucokinase less active or less stable cause maturity onset diabetes of the young type 2 (MODY2), a form of dominantly inherited diabetes (5, 6).

In hepatocytes, glucokinase activity is acutely regulated by a glucokinase regulatory protein (GKRP),† which binds to this enzyme and inhibits it competitively with respect to glucose (7–10). Physiologically, the interaction between glucokinase and GKRP is modulated by Fru-6-P, which binds to GKRP, and which residues interact with these effectors. Due to the ability of GKRP to inhibit glucokinase, it has been postulated that mutations in the gene encoding this protein may cause diabetes if such mutations increase the inhibition, e.g. by suppressing the “de-inhibitory” effect of Fru-1-P (1, 23–25). It is therefore of interest to determine whether there are one or two binding sites for the phosphate esters in GKRP and which residues interact with these effectors.

To guide such studies, two different observations can be exploited. The first one is that GKRP found in lower vertebrates, unlike its mammalian counterpart, is insensitive to Fru-6-P and Fru-1-P, inhibiting the enzyme even in the absence of these phosphate esters (26). The cDNA encoding Xenopus GKRP has been cloned and shown to encode a protein with 58% identity to rat liver GKRP (27). A comparison of the sequences of these two proteins may therefore allow identification of residues that potentially interact with Fru-6-P and Fru-1-P.

The second observation of interest is that GKRPs (whether the fructose phosphate-sensitive or -insensitive forms) are homologous to bacterial open reading frames of unknown function (Yfeu) as well as to a number of other proteins, including the isomerase domain of GlmS (glucosamine-6-phosphate synthase) (28). This enzyme converts Fru-6-P into GlcN-6-P or Glu-6-P depending on the presence or absence of glutamine (29). The crystal structure of the isomerase domain of GlmS is known (30), and the residues that interact with the substrate Fru-6-P have been identified. They lie at the interface of two subdomains with similar topology, known as SIS (sugar isomerase) domains (31).

The objective of this work was to identify the regions in GKRP that are responsible for its interaction with Fru-6-P and Fru-1-P. For this purpose, we mutated residues that potentially interact with Fru-6-P based on the homology of Yfeu to GKRP and GlmS or on the sequence comparison between Fru-6-P-sensitive and -insensitive GKRP.

EXPERIMENTAL PROCEDURES

Materials—The sources of materials were as previously reported (32, 33). Recombinant human islet glucokinase was expressed and purified as described (34).

Site-directed Mutagenesis of GKRP—Mutants of rat liver GKRP were prepared with a PCR-based technique using back-to-back primers (32)
Mutagenesis of the Regulatory Protein of Glucokinase

TABLE I

| Mutant | Primers | Diagnostic restriction site |
|--------|---------|----------------------------|
| G99D/G100S | 5'-tacctgtgtagctgtgaggc-3' | KpnI |
| 5'-gtctagctctcttagaactct-3' | |
| 5'-gaattcgcaggtcgtagat-3' | NheI |
| T109A | 5'-agctccctctgctttactcgg-3' | EcoRV |
| 5'-acgcaagcagcggtagcgcctg-3' | |
| 5'-agcccccagccgctcttgccg-3' | StyI |
| SI10A | 5'-gtagggtagctctctctgct-3' | |
| 5'-aacggttcgctctctctgct-3' | NheI |
| 5'-aagcccacagcgatgcctatgacgac-3' | EcoRI |
| T337A | 5'-gtgctgctctctctctgctg-3' | StyI |
| 5'-gtgctgctctctctctgctg-3' | |
| 5'-gtgctgctctctctctgctg-3' | NheI |
| 5'-aagcccacagcgatgcctatgacgac-3' | EcoRI |
| K514A | 5'-gtgctgctctctctctgctg-3' | |

and Pwo polymerase, an enzyme with proofreading activity. PCR was performed with the primers shown in Table I using the expression vector of rat liver GKRPs (pET-GKRPs) as a template. This plasmid, derived from pET3a (35), contains the entire coding sequence of wild-type rat liver GKRPs flanked at its 5'-end by an Ndel site (containing the initiator ATG) and at its 3'-end by a BamHI site. The reaction was carried out in the presence of 1.3% Me2SO and with an elongation time of 60 s at 72 °C to ensure the yield of the PCR product with the expected size (6.5 kb). After PCR amplification, the linear plasmids were purified on a preparative agarose gel and cloned in E. coli JM109. Plasmids that had incorporated the desired mutation could be distinguished from the wild-type plasmid by restriction analysis (Table I); they were further sequenced to rule out any PCR errors. The mutant plasmids were used to transform E. coli BL21(DE3) pLysS for expression of the recombinant proteins (35).

Expression and Purification of GKRPs—For the expression of both wild-type and mutant GKRPs, 40-ml precultures of the bacteria bearing the appropriate plasmid were grown overnight at 37 °C, and extracts (50 ml) were prepared (27). For the expression of recombinant GKRPs, polyethylene glycol 6000 was added to a final concentration of 22%. After gentle mixing for 20 min at 4 °C, the preparation was centrifuged at 4 °C for 10 min at 12,000 × g, and the clear supernatant was eliminated. The pellet was dissolved in 10 ml of buffer A (55 mM Hepes (pH 7.1), 1 mM dithiothreitol, and 10 μg/ml each of aprotinin and leupeptin). SDS-PAGE and chromatography on a 1.6 × 13-cm DEAE-Sepharose column equilibrated with the same buffer. The column was washed with 100 ml of buffer A, and the proteins retained were eluted with a 0–0.5 M NaCl gradient (in 2 × 100 ml of buffer A). The fractions collected (2.5 ml) were tested for their ability to inhibit recombinant human liver glucokinase in the presence of 5 mM glucose and the indicated concentrations of Fru-6-P, sorbitol-6-P, or Fru-1-P using a pyruvate kinase/lactate dehydrogenase-coupled assay (8). Recombinant human liver glucokinase was used instead of rat liver glucokinase because both proteins have virtually identical properties, including their affinity for GKRPs (33). The dissociation constants for the complexes formed between GKRPs and Fru-6-P or sorbitol-6-P were derived from plots (15) relating the effect of these phosphate esters on the activity of glucokinase in the presence of GKRPs. The dissociation constants for the GKRPs in the absence of any phosphate esters were calculated from saturation curves describing the effect of Fru-1-P on the activity of glucokinase in the presence of Fru-6-P and GKRPs, taking into account the dissociation constant of the GKRPs in the absence of phosphate esters (15).

Cloning, Expression, and Purification of Yfeu—The coding sequence of Hemophilus influenzae Yfeu was amplified by PCR using Pwo polymerase and a plasmid (GHIDB10) containing the appropriate region of the genome (kindly provided by The Institute of Genomic Research, Rockville, MD) as a template. The 5'-end primer (CATATGGGATG- CATTATTA) contained the ATG codon (boldface) flanked by a BamHI restriction site (underlined). The 3'-end primer (GGATCCCTTTA TAGAAGACCATTTCT) contained the stop codon (boldface) flanked by a Ndel restriction site (underlined). The ~0.93-kb amplification product was first ligated in the EcoRV restriction site of pBluescript, sequenced to confirm the nucleotide sequence, and cloned in the Ndel and BamHI restriction sites of the expression vector pET3a (pET-Yfeu). Recombinant Yfeu was expressed at 22 °C in E. coli BL21(DE3) pLysS cells harboring pET-Yfeu as described above for GKRPs. The presence of a soluble and abundant polypeptide with a molecular mass of ~51 kDa was detected on SDS-polyacrylamide gels only in the bacteria that harbored pET-Yfeu. SDS-PAGE was therefore used to identify Yfeu in the course of the purification. Yfeu was purified by polyethylene glycol 6000 precipitation (which was carried out at pH 6.7 to facilitate precipitation) and DEAE-Sepharose chromatography as described for GKRPs. Four fractions (10 ml) containing Yfeu were pooled, concentrated by 4-fold in an Amicon pressure cell equipped with a YM-10 membrane to obtain a yield of ~0.5 mg/ml. The dimerization of Yfeu was assayed by its ability to inhibit glucokinase in the presence of 5 mM glucose and the indicated concentrations of Fru-6-P, sorbitol-6-P, or Fru-1-P using a pyruvate kinase/lactate dehydrogenase-coupled assay (8). Recombinant human liver glucokinase was used instead of rat liver glucokinase because both proteins have virtually identical properties, including their affinity for GKRPs (33). The dissociation constants for the complexes formed between GKRPs and Fru-6-P or sorbitol-6-P were derived from plots (15) relating the effect of these phosphate esters on the activity of glucokinase in the presence of GKRPs. The dissociation constants for the GKRPs in the absence of any phosphate esters were calculated from saturation curves describing the effect of Fru-1-P on the activity of glucokinase in the presence of Fru-6-P and GKRPs, taking into account the dissociation constant of the GKRPs in the absence of phosphate esters (15).

Assays of GKRPs and Determination of Kinetic Constants—GKRPs was assayed by its ability to inhibit glucokinase in the presence of 5 mM glucose and the indicated concentrations of Fru-6-P, sorbitol-6-P, or Fru-1-P using a pyruvate kinase/lactate dehydrogenase-coupled assay (8). Recombinant human liver glucokinase was used instead of rat liver glucokinase because both proteins have virtually identical properties, including their affinity for GKRPs (33). The dissociation constants for the complexes formed between GKRPs and Fru-6-P or sorbitol-6-P were derived from plots (15) relating the effect of these phosphate esters on the activity of glucokinase in the presence of GKRPs. The dissociation constants for the GKRPs in the absence of any phosphate esters were calculated from saturation curves describing the effect of Fru-1-P on the activity of glucokinase in the presence of Fru-6-P and GKRPs, taking into account the dissociation constant of the GKRPs in the absence of phosphate esters (15).

Sequence Comparisons—Fig. 1 shows the alignment of rat, human, and Xenopus GKRPs with bacterial proteins of the Yfeu family. These bacterial proteins contain conserved motifs that align better either with the amino- or carboxyl-terminal half of GKRPs. For instance, the sequence GXGTSGSR (residues 75–81 in Yfeu) is conserved in the amino-terminal half, whereas the motif D/A/G/IEXCXCTT(Y/F) (residues 86–94 in Yfeu) is conserved in the carboxyl-terminal half. Similarly, the motif GPEXX(S/T)GS(S/T)TRKX (residues 191–202 in Yfeu) is conserved in the amino-terminal half of GKRPs, whereas the neighboring motif GXXNXM(V/L)DXXXNXKL (residues

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223–240) is conserved in the carboxyl-terminal half. This alternation in the conservation of the motifs is in agreement with the fact that the identity between the amino- and carboxyl-terminal halves of GKRPs is very low.

Psi-Blast searches (38) indicated that Yfeu is homologous to the isomerase domain of GlmS (28), which, like GKRP, binds Fru-6-P. The crystal structure of this enzyme (29, 30) reveals that it contains two SIS subdomains (31), each of which has an /\beta\_9251/\_9252/-structure consisting of a five-stranded parallel /\beta\_9252/-sheet with connecting /\alpha\_9251/-helices (schematized in Fig. 2; see also Fig. 2 of Ref. 30). The binding site for the Hex-6-P substrate lies at the interface of the two subdomains and is made up of six peptide segments: three (motifs A–C in Fig. 2) in SIS domain 1, one (motif D) in SIS domain 2, and one (motif E) in the carboxyl-terminal loop; because GlmS is a dimeric protein, the sixth peptide segment (a KHG motif) (30) is contributed by the other subunit (not shown in Fig. 2). These peptide segments are, as expected, extremely conserved in the GlmS proteins, and we have tried to align them with well conserved motifs in GKRPs and Yfeu (Fig. 1). For motifs A–C, these alignments are based on Psi-Blast searches with Yfeu, and for motifs A and E, on the MatchBox algorithm (39). Motif D is tentatively aligned with a well conserved region in Yfeu and in the carboxyl-terminal half of GKRP. No candidate was found for the sixth motif (KHG), in relation to the fact that GKRP is a monomeric protein.

### Choice of Mutations
These alignments allowed us to predict residues of rat GKRP that could potentially interact through their side chain with Fru-6-P and other ligands. Some of these (Thr\textsuperscript{109}, Ser\textsuperscript{110}, Ser\textsuperscript{179}, and Lys\textsuperscript{514}) were mutated to alanine. We mutated also to alanine other well conserved residues that were not expected to bind directly to Fru-6-P; these residues are either inside (Asp\textsuperscript{507}) or outside (Thr\textsuperscript{337}, Thr\textsuperscript{411}, and Lys\textsuperscript{499}) these motifs. Furthermore, the alignments allowed us to identify a few residues that are conserved in mammalian GKRPs as well as in Yfeu, but that are different in \textit{Xenopus} GKRP (which is not sensitive to phosphate esters). These were mutated to the
corresponding residues in the *Xenopus* protein (mutations G99D/G100S, G107C, and V180C).

**Effect of the Mutations in GKRP**—All mutant proteins, as well as wild-type rat GKRP, were produced in *E. coli* strain BL21(DE3) pLysS. Under the conditions used for expression, all mutant GKRPｓ were at least partially soluble. The amount of soluble recombinant GKRP present in the bacterial lysates averaged 10 mg/liter of culture, but some mutations increased (G107C, T109A, and S110A) or decreased (S179A, K499A, and D507A) this yield by 2–3-fold. The proteins were purified, and the following properties were investigated: 1) ability to inhibit glucokinase in the presence of a saturating concentration of Fru-6-P (Fig. 3), 2) sensitivity to Fru-6-P (Fig. 4) and to sorbitol-6-P in the presence of a fixed concentration of GKRP, and 3) sensitivity to Fru-1-P in the presence of a fixed concentration of GKRP and Fru-6-P (Fig. 5). *K*ₜₐₜ values for Fru-6-P and Fru-1-P were calculated according to Ref. 15; these values are independent of the GKRP concentration and, in the case of the *K*ₜ for Fru-1-P, also independent of the Fru-6-P concentration. A summary of these data is shown in Table II.

The three mutations made in motif A (G107C, T109A, and S110A), one of the two mutations made in motif B (S179A), and the two mutations made in motif E (K499A and D507A) dramatically affected the properties of GKRP. For the alanine substitutions, these effects consisted of a 5-fold (S110A and D507A), 10-fold (T109A), or 50-fold (S179A and K514A) decrease in the affinity for glucokinase (Fig. 3), combined with a complete (K514A) or partial (S110A, S179A, and D507A) loss of effect of Fru-6-P, a decrease in the apparent affinity for this phosphate ester (T109A, S110A, and S179A), and an increase in the effect of GKRP in the absence of Fru-6-P in the case of mutant S110A (Fig. 4 and Table II). The decrease in the apparent affinity for Fru-6-P observed with mutants S110A, S179A, and K514A was paralleled by similar changes in the affinity for sorbitol-6-P and Fru-1-P (Fig. 5 and Table II). Remarkably, mutation T109A caused an inversion of the effect of sorbitol-6-P, which no longer reinforced the inhibition exerted by GKRP, but quite to the contrary, suppressed it (Fig. 6). No such effect was observed with other mutants.

Mutation G107C (a "*Xenopus*" mutation) in motif A did not affect the affinity of GKRP for glucokinase, but caused a marked increase in the affinity for Fru-6-P and sorbitol-6-P and a marked decrease in the affinity for Fru-1-P (Figs. 4 and 5). Other mutations (G99D/G100S, V180C, T411A, and K499A) had little effect, with the exception of T337A (close to, but outside motif D), which most increased the inhibition exerted by GKRP in the absence of Fru-6-P while decreasing by 1.6–5-fold the affinity for the investigated phosphate esters (Fig. 4). V180C decreased the affinity for Fru-1-P by ~3-fold, whereas the double mutant G107C/V180C had properties similar to those of the single mutant G107C (Table II).

**Dimeric Structure of Yfeu**—Because the bacterial homolog of GKRP contains only one SIS domain and two such domains are needed to form a binding site, it was of interest to determine the subunit composition of this protein. Yfeu, the *H. influenzae* homolog of GKRP, was expressed in *E. coli* and purified by polyethylene glycol 6000 precipitation and DEAE-Sepharose chromatography to near homogeneity. The molecular size of the purified protein was determined by gel filtration on Sephacryl S-200. As shown in Fig. 7, the protein almost coeluted with glycerol-3-phosphate dehydrogenase, indicating a dimeric structure.
molecular mass of ~68 kDa and therefore a dimeric structure. This dimeric structure was confirmed by cross-linking experiments using glutaraldehyde, followed by reduction with sodium borohydride and analysis by SDS-PAGE (data not shown).

**DISCUSSION**

**GKRP as a Member of the SIS Protein Family**—Sequence comparisons indicate that GKRP belongs to the SIS protein family, as does GlmS, a protein for which the three-dimensional structure is known. Based on this distant homology, we have tried to identify residues in GKRP that bind Fru-6-P and other phosphate esters. Replacement of four residues predicted to interact with Fru-6-P through their side chains (T109A, S110A, S179A, and K514A) resulted in a marked decrease in the ability of the resultant proteins to inhibit glucokinase. For three of them (S110A, S179A, and K514A), this change was accompanied by a large decrease in the affinity for Fru-6-P, which may account for the decreased inhibition of glucokinase. By comparison, smaller effects, if any, were observed with other mutants (with the exception of mutant T337A; see below). These data therefore confirm that GKRP belongs to the SIS protein family and that the binding site for phosphate esters lies at the interface of the two SIS domains, as in GlmS.

Although the closest homolog of GKRP contains only one SIS domain per polypeptide chain, its dimeric structure (indicated by gel filtration and cross-linking experiments) suggests that it has two binding sites for its physiological ligand(s), each consisting of motifs A–C in one subunit and motifs D and E in the...
other one. Such architecture accounts for the fact that the five conserved motifs that are distributed between the two SIS subdomains of GKRP are present in one single SIS domain in the case of Yfeu.

**Mechanism for the Action and Regulation of GKRP**—Fru-6-P and Fru-1-P act in a competitive manner on mammalian GKRP (7, 15). Kinetic experiments using analogs of both compounds did not allow discrimination between models with one or two binding sites for these phosphate esters (15). The presence of one single binding site is now supported by the finding of a significant structural homology between GKRP and GlmS and by the fact that most mutations affect the affinity for Fru-6-P, sorbitol-6-P, and Fru-1-P in parallel. It is also consistent with the fact that sorbitol-6-P behaves like Fru-6-P with wild-type GKRP and most mutants, but like Fru-1-P in the case of mutant T109A.

It has been postulated that Fru-6-P and Fru-1-P bind to two different conformations of GKRP, only one of which is able to form a heterodimer with glucokinase. The position of the binding site for these phosphate esters at the interface between the two SIS subdomains suggests that the two conformations could

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**TABLE II**

Summary of the effects of the mutations in rat liver GKRP

| Rat GKRP | Equivalent residue in Xenopus GKRP | Equivalent residue in E. coli GlmS | Interaction with GlcN-6-P | Interaction with Fru-6-P | Interaction with Sor-1-P | Interaction with Fru-1-P | Maximal effect of Fru-6-P | Maximal effect of Sor-1-P | Maximal effect of Fru-1-P |
|----------|-----------------------------------|-----------------------------------|--------------------------|------------------------|-------------------------|-------------------------|--------------------------|--------------------------|--------------------------|
| Wild-type|                                   |                                   |                          |                        |                         |                         |                          |                          |                          |
| G99D/G100S| Asp<sup>99</sup>Ser<sup>100</sup> | Cys<sup>300</sup> (motif A)       | O-1P (m)                | 30                     | 15                      | 5                       | 1                       |                          |                          |
| T109A    | Thr<sup>109</sup>                  | Thr<sup>302</sup> (motif A)       | O-3 (s), O-4 (m)        | 300                    | 40                      | 14                      | 2.9                      | 3.6                      |                          |
| S110A    | Ser<sup>110</sup>                  | Ser<sup>303</sup> (motif A)       | O-4, O-1P, O-3P (s) O-4 (m) | 150                    | >2000                   | 1.6<sup>a</sup>          |                          |                          |                          |
| S179A    | Ser<sup>179</sup>                  | Ser<sup>347</sup> (motif B)       | O-1P (s)                | >1500                  | >2000                   | 5<sup>a</sup>            |                          |                          |                          |
| V180C    | Cys<sup>380</sup>                 | Gln<sup>348</sup> (motif B)       | O-2P (s)                | 5                      | 20                      | (Unchanged)            |                          |                          |                          |
| G107C/V180C| Thr<sup>336</sup>                 |                                    |                          | 20                     | 1                       | (Unchanged)            |                          |                          |                          |
| T377A    | Thr<sup>336</sup>                 |                                    |                          | 30                     | 100                     | 4                       | 8                       | 3.3                      |                          |
| T411A    | Thr<sup>411</sup>                 |                                    |                          | 30                     | 20                      | 11                      | 3.5                      | 0.5                      |                          |
| K499A    | Lys<sup>499</sup>                 |                                    |                          | 30                     | 20                      | (Unchanged)            |                          |                          |                          |
| K514A    | Lys<sup>514</sup>                 |                                    |                          | 150                    | 27                      | 4                       | 4                       | 1.3                      |                          |
| D507A    | Asp<sup>506</sup>                 | Asp<sup>596</sup> (motif E)       | O-2P, O-3P (m)          | >1500                  | >2000                   | 1.7<sup>a</sup>          |                          |                          |                          |
| K514A    | Lys<sup>514</sup>                 |                                    |                          |                        |                         |                         |                          |                          |                          |

<sup>a</sup>At 2 mM Fru-6-P.
<sup>b</sup>At 1 mM sorbitol-6-P.
<sup>c</sup>At 1 mM Fru-1-P.

**FIG. 6. Inversion of the effect of sorbitol-6-P on mutant T109A.** Glucokinase (GK) activity was measured in the presence of 10 μg/ml wild-type (WT) or T109A GKRP, 5 mM glucose, 0 or 200 μM Fru-6-P (F6P), and the indicated concentrations of sorbitol-6-P.

**FIG. 7. Gel filtration of Yfeu and marker proteins on Sephacryl S-200.** Fractions of 1.1 ml were collected. PGII, phosphoglucose isomerase; Gly-3-P-DH, glycerol-3-phosphate dehydrogenase; Cyt. c, cytochrome c.
differ from each other by the relative disposition of the two SIS subdomains. This hypothesis is consistent with the effect of mutation T337A, which increased the effect of GKRP in the absence of Fru-6-P, presumably by favoring the conformation of GKRP able to bind to glucokinase. Thr\textsuperscript{337} is predicted to be in the ligand of Fru-6-P, and the mutation T337A, which increased the effect of GKRP in the subdomains. This hypothesis is consistent with the effect of GKRP could therefore result in a compensatory increase in the amount of glucokinase without significant perturbation or even with a slight improvement of glucose tolerance. Accordingly, adenovirus-mediated overexpression of GKRP was recently shown to improve glucose tolerance in a mouse model of type II diabetes (47). The mutants that we have produced may be helpful to understand the effect of GKRP on glucokinase expression, most particularly to determine whether the interaction between glucokinase and GKRP is essential for this effect.

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Maria Veiga-da-Cunha and Emile Van Schaftingen

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