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Biochemical and Biophysical Research Communications 302 (2003) 127–132
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Activation of phospholipase D1 by ADP-ribosylated RhoA

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Received 23 January 2003

Abstract

Clostridium botulinum exoenzyme C3 exclusively ADP-ribosylates RhoA, B, and C to inactivate them, resulting in disaggregation of the actin filaments in intact cells. The ADP-ribose resides at Asn-41 in the effector binding region, leading to the notion that ADP-ribosylation inactivates Rho by blocking coupling of Rho to its downstream effectors. In a recombinant system, however, ADP-ribosylated Rho bound to effector proteins such as phospholipase D-1 (PLD1), Rho-kinase (ROK), and rhotekin. The ADP-ribose rather mediated binding of Rho-GDP to PLD1. ADP-ribosylation of Rho-GDP followed by GTP-γ-S loading resulted in binding but not in PLD activation. On the other hand, ADP-ribosylation of Rho previously activated by binding to GTP-γ-S resulted in full PLD activation. This finding indicates that ADP-ribosylation seems to prevent GTP-induced change to the active conformation of switch I, the prerequisite of Rho–PLD interaction. In contrast to recombinant systems, ADP-ribosylation in intact cells results in functional inactivation of Rho, indicating other mechanisms of inactivation than blocking effector coupling.

Keywords: ADP-ribosylation; C3; PLD1; RhoA-GTPase; Rho-kinase

Rho-GTPases are major regulatory proteins of the mammalian cell being in the regulation of cell morphology, gene expression, tumor progression, cell cycle progression, and apoptosis. GTP-bound (active) Rho-GTPases bind to their effector proteins, initiating downstream signaling. Rho effector proteins comprise Ser/Thr-kinases, lipid kinases, lipases, and scaffold proteins [1–3]. The common mechanism of the activation of the protein kinases (e.g., ROK, PKN) and scaffold proteins (e.g., rhophilin, Dia) is the disruption of an autoinhibitory intramolecular interaction caused by the binding of active Rho [1]. Among the effector proteins is also the phosphatidylcholine-hydrolyzing phospholipase D (PLD), that, however, does not contain an autoinhibitory domain [4]. The immediate PLD reaction product, phosphatidic acid, has been implicated in the regulation of early and late cellular responses, i.e., calcium mobilization, secretion, endocytosis, activation of NAPDH oxidase, translocation of GLUT4 transporter, the organization of the actin cytoskeleton, and cellular growth and differentiation [4,5]. From the two mammalian PLD enzymes cloned and characterized, only PLD1 is strongly activated in vitro by members of the Rho subfamily [5,6]. PLD1 regulation by Rho-GTPases is indirectly mediated through the Rho-kinase and the phosphatidylinositol 4-phosphate 5-kinase, but also a direct interaction has been reported [6]. Critical amino acid residues for the direct interaction of RhoA with PLD1 reside in the effector region (switch I) of RhoA [7].

Clostridium botulinum C3 is the prototype of the family of C3-like transferases which ADP-ribosylate the Rho subtypes A, B, and C at asparagine-41 [8,9]. ADP-ribosylation of Rho in intact cells results not only in disaggregation of actin filaments and all actin-dependent processes, but also in inhibition of all Rho-dependent cell functions such as cell-cycle progression and gene transcription [9–11]. The cellular effects of C3 can be explained only by an inactivation of cellular Rho by ADP-ribosylation. Because the bulky and strongly negatively charged ADP-ribose moiety resides in the

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doi:10.1016/S0006-291X(03)00112-8

effector region (switch I), it was thought that ADP-ribosylation blocks effector coupling thereby preventing downstream signaling [12,13].

Here we present evidence that ADP-ribosylation does not block the binding of RhoA to the effector proteins PLD1, ROK, and rhotekin, but that ADP-ribosylation rather hampers GDP-GTP transition of the RhoA switch I to prevent the formation of the active GTP conformation.

Materials and methods

Materials. 1-Palmitoyl-2-[^{3}H]palmitoyl-glycerophosphocholine ([^{3}H]PtdCho, 37.5 Ci/mmol) was purchased from NEN, Germany. Unlabeled PtdCho and TNM-FH insect medium were obtained from Sigma, and PtdIns(4,5)P_2 and GTP-γ-S were obtained from Roche, Germany. The antibody against RhoA was purchased from Santa Cruz.

Recombinant proteins. Human RhoA and PLD1 were expressed in *Sf*9 insect cells using baculovirus expression system (PharMingen). DNA encoding human PLD1 subcloned into pAcGHLT was a gift from Drs. A. Morris and M. Frohmann [14]. *Sf*9 cells (1×10^6 cells/ml) were cultured at 25 °C in TNM-FH insect medium containing 10% fetal calf serum, 100 U/ml penicillin G, and 100 mg/ml streptomycin. Infection with pAcGHLT containing RhoA or PLD1 baculovirus transfer vectors (MOI of 5–10) was performed at 25 °C for 48 h. Thereafter, the cells were lysed by sonication in buffer A (50 mM NaCl, 10 mM $MgCl_2$, 1 mM dithiothreitol, 10 μM phenylmethylsulfonyl fluoride, 1% (v/v) Triton X-100 (RhoA only), and 10 mM Tris–HCl, pH 7.5) and centrifuged at 20,000*g* for 1 h. GST-RhoA or GST-PLD1 from the supernatant was precipitated with glutathione Sepharose beads at 4 °C for 30 min followed by intensive washing with buffer A to remove unbound proteins. RhoA and PLD1 were released from the parent GST-fusion proteins by incubation with thrombin (Pharmingen, 10 U) at 4 °C overnight in buffer (150 mM NaCl, 5 mM $MgCl_2$, 2.5 mM $CaCl_2$, 1 mM dithiothreitol, and 50 mM Tris–HCl, pH 8.0). The beads were removed by centrifugation and thrombin was precipitated using *p*-aminobenzamidine beads. The homogeneity of the recombinant RhoA and PLD1 was proven by SDS–PAGE.

ADP-ribosylation reaction. Isoprenylated RhoA was purified as described above. ADP-ribosylation reaction was carried out in the presence of 50 μg/ml RhoA, 0.2 μg/ml exoenzyme C3, and 100 μM NAD in reaction buffer (50 mM Tris, pH 7.4, 150 mM NaCl, 5 mM $MgCl_2$, 1 mM dithiothreitol, and 1 mM PMSF) at 37 °C for 30 min.

Nucleotide exchange reaction. ADP-ribosylated and unmodified RhoA were incubated in the presence of 200 μM GDP or GTP[S] on ice for 1 h.

Pull down with GST-PLD1. Freshly prepared GST-PLD1 (approximately 15 μg) immobilized to glutathione Sepharose was incubated with recombinant RhoA from *Sf*9 cells (2 μg) at 4 °C for 30 min. Thereafter, the beads were washed three times with buffer A to remove unbound proteins and beads were eluted with glutathione (10 mM glutathione, 5 mM $MgCl_2$, and 50 mM Tris–HCl, pH 8.0). The supernatant was mixed with Laemmli buffer and incubated at 95 °C for 10 min. Samples were subjected to SDS–PAGE on 15% gels and analyzed by immunoblot for RhoA.

Pull down with MBP-RBD(ROK). Freshly prepared MBP-RBD(ROK) immobilized to agarose was incubated with recombinant RhoA from *Sf*9 cells (2 μg) at 4 °C for 30 min. Thereafter, the beads were washed three times with buffer (50 mM Tris–HCl, pH 7.4, 150 mM NaCl, 5 mM $MgCl_2$, 1 mM dithiothreitol, and 1 mM PMSF) to remove unbound proteins and beads were eluted with 10 mM maltose. The supernatant was mixed with Laemmli buffer and incubated at 95 °C for 10 min. Samples were subjected to SDS–PAGE on 15% gels and analyzed by immunoblotting for RhoA.

Pull down with GST-C21. Pull down experiments were performed as described by Reid and coworkers [15]. Two micrograms of recombinant RhoA modified and nucleotide loaded as indicated was added to 500 μl buffer (50 mM NaCl, 20 mM Tris–HCl, pH 7.4, 3 mM $MgCl_2$, 10 mM dithiothreitol, and 100 μM PMSF). Twenty microliters of beads slurry of the Rho-binding domain GST-C21 from rhotekin bearing approximately 30 μg of fusion protein, was added to each sample and rotated at 4 °C for 30 min. The beads were collected by centrifugation at 10,000 rpm and washed twice with lysis buffer. To each sample 20 μl Laemmli buffer was added to the beads and 15% SDS–PAGE was performed. RhoA was detected by Western blot using specific antibody.

Immunoblot analysis. Proteins were separated on 15% acrylamide gels and transferred onto nitro-cellulose for 2 h at 250 mA. The membranes were blocked for 1 h with 5% (w/v) non-fat dried milk. Blots were incubated overnight with anti-RhoA mab (Santa Cruz) in PBS containing 0.05% Tween and then for 45 min with a horseradish peroxidase-conjugated secondary antibody. The proteins were visualized by enhanced chemiluminescence (ECL).

PLD1 activity assay. To measure recombinant PLD1 activity, [^{3}H]PtdCho was mixed with PtdIns(4,5)P_2 in a molar ratio of 8:1, dried, and resuspended in 50 mM Hepes, pH 7.5, 3 mM EGTA, 80 mM KCl, and 1 mM dithiothreitol, followed by sonication on ice. Recombinant PLD1 activity (20–30 μg protein) was determined with [^{3}H]PtdCho/PtdIns(4,5)P_2 (200 μM/25 μM) as substrate vesicles in the presence or absence of the indicated concentrations of recombinant RhoA for 60 min at 37 °C. Termination of the reaction and isolation of labeled phospholipids and the specific PLD product, [^{3}H]phosphatidylethanol ([^{3}H]PtdEtOH), were performed as described before [16]. Data shown are means ± SEM from four independent experiments.

Results

Rho-dependent activation of PLD1

To directly test the notion that ADP-ribosylation blocks RhoA coupling to its effectors, stimulation of phospholipase D1 activity by RhoA was measured in a recombinant system. PLD1 activity was determined by

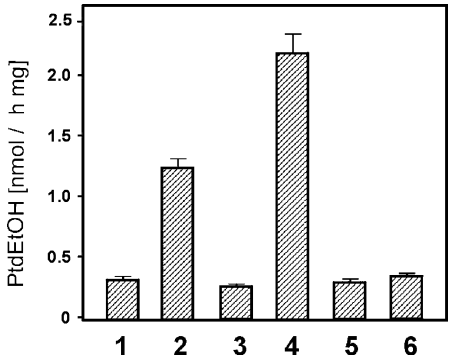

Fig. 1. Effect of ADP-ribosylation of RhoA on PLD1 activity. Recombinant PLD1 activity was measured as described under Materials and methods with [^{3}H]PtdCho/PtdIns(4,5)P_2 substrate vesicles in the presence of: 1. GTP-γ-S alone; 2. RhoA plus GTP-γ-S (but not pre-bound); 3. RhoA-GDP; 4. RhoA-GTP-γ-S pre-bound; 5. ADP-ribosylated RhoA-GDP; and 6. ADP-ribosylated RhoA-GTP-γ-S. Modification of RhoA was carried out before nucleotide loading. All data are means ± SEM of four experiments.

measuring the formation of the stable product [^{3}H] PtdEtOH. As shown in Fig. 1, unmodified RhoA exclusively enhanced PLD1 activity in the presence of GTP-γ-S whereby Rho pre-bound to GTP-γ-S was most efficacious. ADP-ribosylated RhoA bound to GTP-γ-S did not stimulate PLD1 nor in its GDP-bound form. ADP-ribosylation at Asn-41 does not alter GTP-binding and release [17,18], but it is not known whether binding of GTP to ADP-ribosylated RhoA does in fact induce switching of the effector region into the active GTP conformation. To test this notion, the sequences of GTP-loading and ADP-ribosylation were performed in reversed order, i.e., unmodified RhoA was first loaded with GTP-γ-S followed by ADP-ribosylation. This sequence of reaction is feasible, because GTP-γ-S binding does not block ADP-ribosylation [19]. Applying this sequence of reaction, ADP-ribosylated RhoA bound to GTP-γ-S was capable of stimulating PLD1 activity (Fig. 2). The amount of stimulation was comparable to that of unmodified RhoA-GTP-γ-S. ADP-ribosylated RhoA first modified followed by GTP-γ-S loading as well as ADP-ribosylated GDP-bound RhoA remained inactive (Fig. 2). Thus, active GTP-bound Rho subsequently ADP-ribosylated is capable of stimulating PLD1. In this case, the ADP-ribose moiety seems to be inert.

Effector coupling of ADP-ribosylated RhoA

The property of ADP-ribosylated RhoA to activate PLD1 implicates that ADP-ribosylated RhoA functionally binds to the effector protein PLD1 excluding non-specific interaction with basic protein domains. Binding of RhoA to PLD1 was studied by co-precipitation of RhoA with immobilized PLD1. To this end

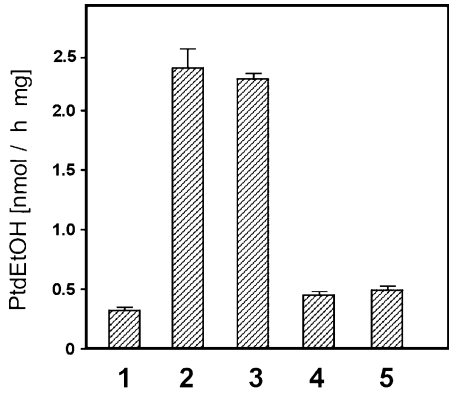

Fig. 2. Effect of the sequence of GTP-γ-S loading and ADP-ribosylation of RhoA on PLD1 activation. Recombinant PLD1 activity was measured as described under Materials and methods with [^{3}H]PtdCho/PtdIns(4,5)P_2 substrate vesicles in the presence of: 1. GTP-γ-S alone; 2. RhoA-GTP-γ-S; 3. ADP-ribosylated RhoA (GTP-γ-S loading followed by incubation with C3 and NAD); 4. ADP-ribosylated RhoA (incubation with C3 and NAD followed by GTP-γ-S loading); and 5. ADP-ribosylated RhoA-GDP. All data are means ± SEM of four experiments.

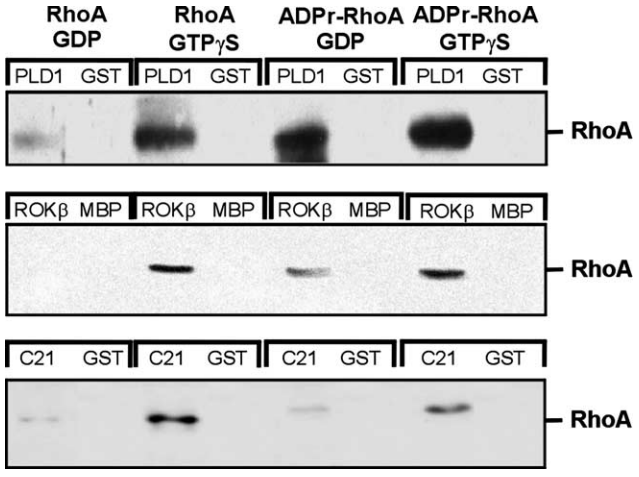

Fig. 3. Binding of ADP-ribosylated RhoA to GST-PLD1, MBP-RBD (ROK), and GST-C21 (Rhotekin). RhoA from *Sf*9 cells was first ADP-ribosylated followed by loading with GDP or GTP-γ-S as described under "Materials and methods." GST-PLD1, GST-C21, MBP-ROK, MBP alone, and GST alone as control all immobilized to beads were incubated with RhoA samples as indicated. Modification of RhoA was carried out before nucleotide loading. Precipitated proteins were separated by SDS–PAGE and probed for RhoA content by immunoblot analysis.

isoprenylated RhoA was ADP-ribosylated by transferase C3 or treated with C3 in the absence of NAD^+, followed by nucleotide exchange with GDP and GTP-γ-S, respectively. PLD1 (as fusion protein with GST) immobilized to Sepharose was added and bound RhoA was pulled down. Unmodified RhoA bound only to PLD1 when loaded with GTP-γ-S but not when loaded with GDP (Fig. 3). However, ADP-ribosylated RhoA bound to PLD1 in the GDP- as well as in the GTP-γ-S bound form (Fig. 3). Denaturation of GTP-γ-S-loaded RhoA (non-modified and ADP-ribosylated) by heat completely abolished binding to PLD1 (data not shown), excluding non-specific interaction. Comparable data were obtained for the co-precipitation of RhoA with effector proteins of other effector classes [20]: RhoA was pulled down using the Rho-binding domains of rhotekin (C21) and of rhokinase (ROK) both immobilized to beads. ADP-ribosylated Rho as well as unmodified Rho specifically bound to C21 and rhokinase (ROK) exclusively in the GTP-γ-S form. Only, a faint binding of ADP-ribosylated Rho-GDP to ROK was also observed (Fig. 3).

Discussion

ADP-ribosylated RhoA does bind to PLD1 in a nucleotide-independent manner. Whereas the typical sequence of reaction, i.e., first ADP-ribosylation, then nucleotide exchange, results in binding but not in stimulation of PLD1, the reverse order of reaction makes ADP-ribosylated RhoA functionally active to stimulate

PLD1. A partial activation of membranous PLD1 by ADP-ribosylated RhoA has also been observed by Exton and coworkers [21]. Thus, if RhoA possesses the active GTP conformation, ADP-ribosylation is an inert modification that does not alter signaling properties of RhoA. Because ADP-ribosylation does not block GTP-loading but activation, it has to be concluded that ADP-ribosylation seems to inhibit the GTP-induced conformational change of switch I. This notion is conceivable because the acceptor residue of ADP-ribosylation is located at the end of switch I, a hinge-like region. In the GDP-form the B2 β-strand covers residues from Tyr-42 to Val-48. After GTP binding, the β-strand starts from Phe-39 [22,23]. Because Phe-39 fulfills the most dramatic displacement after the switching of the effector region, it is conceivable that the ADP-ribose moiety may interfere with these conformational changes (Fig. 4). This view is supported by comparable structural findings on the mono-glucosylation of H-Ras by lethal toxin from *Clostridium sordellii* [24]: The glucose at Thr-35 is reported to strongly stabilize switch I in the inactive "GDP" conformation. The structural consequences of the ADP-ribose on the GTP/GDP switching of the effector region, however, remain to be evaluated using 3D structural analysis.

Direct activation of PLD1 by RhoA has been shown to strictly depend on the residues Tyr-34, Thr-37, and Phe-39 of RhoA that all reside in switch I [7]. Mutation of Asn-41 or the adjacent residue Glu-40 has no effect and exchange of Tyr-42 has only a minor effect on PLD stimulation [7]. Furthermore, Asn-41-acceptor for the ADP-ribose is surface exposed in both the GTP- and GDP-conformations [22,23]. Therefore, it is conceivable that a mobile modification of a residue non-essential in effector interaction does not directly hamper Rho-dependent PLD activation. The function of the ADP-ribose moiety is not to block effector coupling, but rather to prevent attaining the active, i.e. signaling conformation of switch I. However, ADP-ribosylated RhoA-GTP is only transiently active because GAP (GTPase activating protein) activity to produce GDP-bound RhoA is not inhibited by ADP-ribosylation [17,18].

The non-functional binding of inactive ADP-ribosylated RhoA to PLD1 and phosphatidylinositol 4-P 5-kinase [28] appears to be less clear, since binding of RhoA to its effector proteins is thought to be based on the active conformation of switch I and II attained only after binding to GTP [1,25]. It is conceivable that the negatively charged ADP-ribose moiety does bind to any positively charged surface region of PLD1 or is mediated by electrostatic interactions allowing non-specific binding that is not sufficient for PLD1 stimulation. Thus, in the case of PLD1 there is detectable effector binding that, in fact, does not correlate with effector activation.

In contrast to PLD1, Rho-kinase (ROK), rhotekin, and PKN belong to a Rho effector class that is regulated by an autoinhibitory intramolecular interaction. Our findings that ADP-ribosylated Rho binds to rhotekin and ROK are in line with our earlier observation that ADP-ribosylated Rho-GTP-γ-S binds to protein kinase N (PKN) [18]. Based on the structure of the Rho-PKN complex, Hakoshima and coworkers suggest that—from the sterical point of view—the bulky ADP-ribose moiety at asparagine-41 cannot block the binding of Rho to PKN [26]. In contrast to PLD1, GTP-loading of ADP-

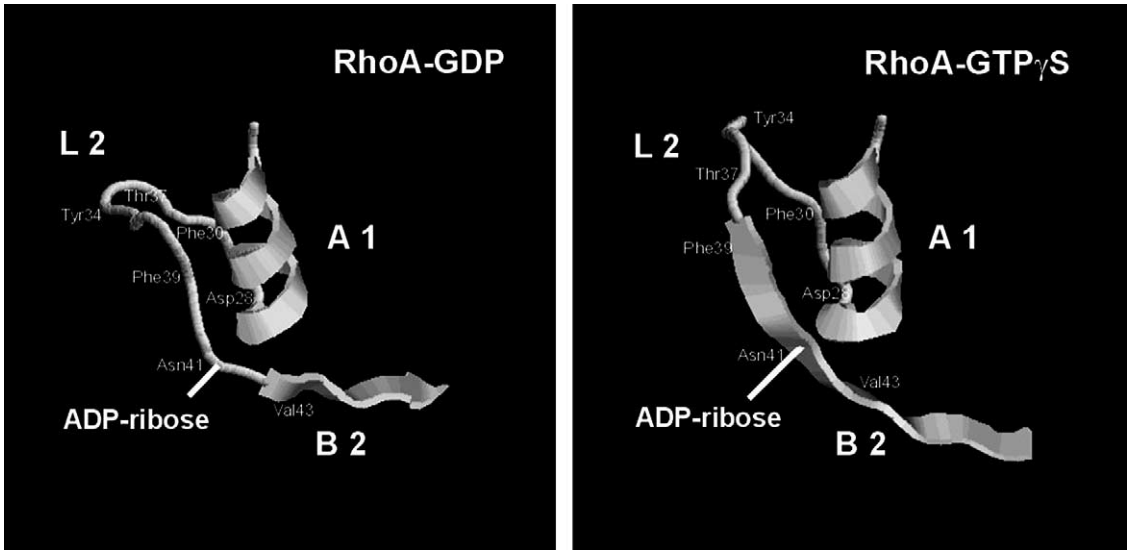

Fig. 4. GDP/GTP-γ-S switching of the RhoA effector binding region (aa Asp-28 to Val-43). The C-terminal part of RhoA switch I region (Phe-39 to Tyr-42) changes its conformation within GDP/GTP-γ-S exchange. ADP-ribosylation at Asn-41 is suggested to hamper the transition to the active state of the effector region. RhoA-GDP, Protein Data Bank code 1FTN [23]; RhoA-GTP-γ-S, Protein Data Bank code 1A2B [22]. Figures were generated using RasMol software.

ribosylated Rho was shown to result in the activation of ROK [Genth and Just, unpublished]. In the case of ROK, effector binding of ADP-ribosylated Rho correlates with activation. This activation appears to be possible, even if the sequence of reaction is to prevent the active conformation of switch I. From these findings, it is to be concluded that rather switch II is involved in ROK activation, whereby the ADP-ribose may stabilize binding but is not functional.

In conclusion, ADP-ribosylation has been suggested to alter Rho-dependent signaling by a blockade of effector coupling. From our findings on PLD1, this model is to be refined: ADP-ribosylated Rho binds to several effector proteins such as PLD1, ROK, rhotekin, PKN [18], and phosphatidylinositol 4-P 5-kinase [28]. The activation of the effector PLD1 strictly depends on the active conformation of the Rho effector loop [7]. GDP/GTP switching of the loop is suggested to be hampered by the ADP-ribose at Asn-41. In the case of ROK, GTP-binding of ADP-ribosylated Rho resulted in functionally active Rho; in the case of PLD1, GTP-binding of ADP-ribosylated Rho resulted in functionally inactive Rho incapable of PLD activation.

Therefore, in terms of the GTPase model of Rho, no general explanation on the consequences of ADP-ribosylation can be expected on the effector level. This notion underlines our former suggestions that the inactivation of Rho by ADP-ribosylation may be based on the reduced activity of the guanine nucleotide exchange factor (GEF) on ADP-ribosylated Rho [27] and the impaired release of ADP-ribosylated Rho from guanine nucleotide dissociation inhibitor (GDI-1) [Genth and Just, unpublished].

Acknowledgments

This work was supported by Hannover Medical School HiLF grant to H.G. We thank Ilona Klose for the expert technical assistance.

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