"Identification of protein-ribulosamine-5-phosphatase as human low-molecular-mass protein tyrosine phosphatase-A."

Fortpied, Juliette ; Gemayel, Rita ; Vertommen, Didier ; Van Schaftingen, Emile

Abstract
Ribulosamines, which are substrates for the deglycating enzyme fructosamine-3-kinase-related protein, are presumably formed intracellularly through glycation of proteins with ribose 5-phosphate followed by dephosphorylation of resulting RN5Ps (ribulosamine 5-phosphates) by a putative RN5Pase (ribulosamine-5-phosphatase). Ribose 5-phosphate is known to be a potent glycating agent and we show in the present study that it reacts approximately 10 and 80-fold more rapidly with protein than ribose and glucose respectively. We also show that tissue extracts and, most particularly, erythrocyte extracts contain a protein-RN5Pase. We have purified this enzyme from human erythrocytes to near homogeneity and shown it to correspond to LMWPTP-A [low-molecular-mass ('weight') protein tyrosine phosphatase-A]. Human recombinant LMWPTP-A displayed an RN5Pase activity that was higher than its tyrosine phosphatase activity, indicating that this phosphatase may participate in protein deglycation, a new f...

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Identification of protein-ribulosamine-5-phosphatase as human low-molecular-mass protein tyrosine phosphatase-A

Juliette FORTPIED, Rita GEMAYEL, Didier VERTOMMEN and Emile VAN SCHAFTINGEN

INTRODUCTION

Sugars with a free carbonyl group spontaneously react with the amino groups of proteins or low-molecular-mass compounds to form Schiff bases, which slowly rearrange into Amadori products (ketoamines). This reaction, designated glycation, is known to take place in vivo with glucose, giving rise to fructosamines. It must also take place with other monosaccharides or sugar phosphates, in particular glucose 6-phosphate and ribose 5-phosphate, which, although usually present at lower concentrations than glucose, are intrinsically more reactive than this hexose [1–4]. It should be noted, however, that the higher reactivity of ribose 5-phosphate has only been demonstrated by measuring the formation of advanced glycation end-products and that this difference does not necessarily reflect a difference in the rate of early glycation.

Protein glycation is apparently detrimental and it is therefore not surprising that cells have enzymes that repair proteins by removing the accessible glycation adducts. The first deglycation enzyme that has been described is FN3K (fructosamine-3-kinase) which by phosphorylating fructosamines on their third carbon causes their destabilization and shedding from proteins [5,6]. The physiological occurrence of enzymatic deglycation was demonstrated by showing that FN3K deficiency leads to an increase in intracellular protein glycation [7,8]. FN3K takes care also of the glycation adds formed from glucose 6-phosphate, after these have been dephosphorylated by an FN6Pase (fructosamine-6-phosphatase) recently identified as MDP-1 (magnesium-dependent phosphatase-1) [9].

FN3K is only found in higher vertebrates, such as mammals and birds. However, many species, including mammals [10], birds, fish [11], plants [12] and bacteria (R. Gemayel, unpublished work), contain an FN3K homologue [designated FN3K-RP (FN3K-related protein)] which does not act on fructosamines, but only on ketoamines with a hydroxy group in the D-configuration on C-3, most particularly ribulosamines. We previously postulated that ribulosamines are produced by enzymatic dephosphorylation of RN5Ps (ribulosamine 5-phosphates), which form spontaneously from ribose 5-phosphate. The phosphatase involved in this process is, however, unknown. In the present study, we have quantified the rate of early glycation by ribose 5-phosphate and identified in human erythrocytes a phosphatase, distinct from FN6Pase/MDP-1, that uses protein-RN5Ps as its best substrate.

EXPERIMENTAL

Materials

Reagents, of analytical grade whenever possible, were obtained from Sigma, Acros Organics or Merck. DEAE-Sepharose, SP-Sepharose (sulfopropyl-Sepharose), Q-Sepharose, Sephacryl S-200 and Superdex-200 10/300 GL column were purchased from Amersham Biosciences; Biogel P2 and AG 50W-X4 (100–200 mesh) were from Bio-Rad. Dowex 1-X8-200 was purchased from Acros Organics and Vivaspin-2 centrifugal concentrators from Vivascience. Radiolabelled compounds were purchased from Amersham Biosciences or American Radiolabeled Chemicals, or synthesized as described in [9]. [1-13C]Ribose 5-phosphate was prepared by phosphorylation of [1,14C]ribose with 25 m-units of human recombinant ribokinase; the product was purified as described previously for [1-14C]glucose 6-phosphate [9]. FN3K-RP and MDP-1 were prepared as previously described [9,13].

Preparation of RN5Ps

Lysozyme glycated with [32P]ribose 5-phosphate was prepared as previously described for lysozyme glycated with [1-14C]glucose 6-phosphate [9], but the incubation time was reduced to 4 h and...
Tris was used instead of Hepes. For the preparation of lysozyme glycated with unlabelled ribose 5-phosphate, hen’s-egg lysozyme (60 mg/ml) was incubated for 16 h at 50 °C with 20 mM ribose 5-phosphate in a medium containing 25 mM Tris/HC1 (pH 7.1), 1 mM EGTA and 0.5 mM AIF3. The samples were purified on Dowex 1-X8 and Biogel P2 [9] and the concentration of lysozyme was determined [13a].

Ribuloselysine 5-phosphate was prepared by incubating a solution (3 ml) containing 200 mM N-α-Boc-lysine (where Boc is t-butoxycarbonyl) and 100 mM ribose 5-phosphate for 4 h at 50 °C. The Boc group was removed by overnight incubation in 1 M HCl at 22 °C. The sample was diluted in 12 ml of water and loaded on to an AG 50W-X4 column (H+ form, 3 cm3) equilibrated with water. The column was washed with 12 ml of water and 36 ml of 0.5 M NaCl. Fractions of 3 ml were collected, and those containing ribuloselysine 5-phosphate (assayed by the P, released by alkaline phosphatase, in the elution fractions) were concentrated under vacuum and desalted on Biogel P2 equilibrated with water.

Preparation of tissue extracts and purification of RNSPase (ribulosamine-5-phosphatase)

Tissues were collected from anaesthetized male Wistar rats. They were quick-frozen between Wollenberner clamps cooled in liquid nitrogen and kept at −70 °C until used. The frozen tissues (2 mg) were homogenized in a Potter–Elvehjem device in buffer containing 25 mM Tris/HC1 (pH 7.1), 1 mM DTT (dithiothreitol), 1 µg/ml leupeptin, 1 µg/ml antipain and 10 mM KCl (buffer A). Rat packed erythrocytes were washed twice with a solution containing 150 mM NaCl and 0.5 mM EDTA and once with 150 mM NaCl. One volume of washed erythrocytes was diluted in 10 vol. of buffer A. The homogenates and haemolysates were centrifuged for 20 min at 11 000 g and the resulting supernatants were used for enzyme assay. Protein concentration in tissue extracts was determined as described in [14] using bovine γ-globulin as a standard; Hb concentration in erythrocyte extracts was determined by the method of Drabkin and Austie [15].

For the purification of human RNSPase, 80 ml of packed erythrocytes was washed three times with 150 mM NaCl and diluted in 400 ml of a buffer containing 10 mM Tris/HC1 (pH 8), 1 mM DTT, 1 µg/ml leupeptin and 1 µg/ml antipain. The haemolysate was centrifuged for 20 min at 11 000 g. The supernatant (300 ml) was diluted twice in buffer B [20 mM Tris/HC1 (pH 8), 2 mM DTT, 1 µg/ml leupeptin and 1 µg/ml antipain] and applied to a DEAE-Sepharose column (200 cm3) equilibrated with the same buffer. The column was washed with 400 ml of buffer B and protein was eluted with a 0–0.25 M NaCl gradient in 1000 ml of buffer B. The most active fractions were pooled (55 ml), brought to 200 ml with buffer C (20 mM Mes, pH 6, 2 mM DTT, 1 µg/ml leupeptin and 1 µg/ml antipain), and applied to an SP-Sepharose column (20 cm3) equilibrated with buffer C. The column was washed with 50 ml of equilibration buffer and protein was eluted with a 0–0.75 M NaCl gradient in 200 ml of buffer C. Fractions of 3 ml were collected. Three peaks of RNSPase were found, each in the flow-through and washing fractions (peak I), and active fractions were pooled (55 ml), brought to 200 ml with 400 ml of buffer B and protein was eluted with a 0–0.5 M NaCl gradient. Fractions of 2.5 ml were collected. The most active ones (10 ml) were concentrated to 0.5 ml (with Vivasin-2) and gel-filtered on a Superdex-200 10/300 GL column equilibrated with buffer E [25 mM Tris/HC1 (pH 7.1), 1 mM DTT, 1 µg/ml leupeptin, 1 µg/ml antipain and 100 mM NaCl]. Peaks II (18 ml) and III (21 ml) of the SP-Sepharose column were also concentrated and gel-filtered on Superdex-200 equilibrated with buffer E. Fractions of 0.5 ml were collected. The bands co-eluting with the RNSPase activity in each Superdex-200 chromatography were cut out from an SDS/14% (w/v) polyacrylamide gel and digested with trypsin or chymotrypsin. Peptides were analysed by nanoelectrospray ionization tandem MS as previously described [16].

Expression and purification of human LMWPTPs (low-molecular-mass ‘weight’) protein tyrosine phosphatases

The open reading frame of human LMWPTPs A and B (accession numbers AAP35800.1 and AAC52065.1), two isoforms generated by alternative splicing [17], were PCR-amplified using Pwo polymerase, human liver cDNA as template, a 5’ primer containing the initiator codon (GCATATGGCGGAAACAGCTCAACAGT) in an Ndel site (in boldface) and a 3’ primer containing the putative stop codon (CGGATCTTCAGTGGCCCTTCTCCAAGAAC) flanked by a BamHI site (in boldface). The 480 bp PCR product was subcloned into pBlueScript. Restrictions with BstUI, HaeIII and AvaII were performed to discriminate between the two isoforms, which were checked by sequencing. Ndel–BamHI fragments of each form were removed from pBlueScript and inserted into pET-3a. The resulting plasmids were used to transform Escherichia coli BL21(DE3) pLYs [18]. The expression and preparation of bacterial extracts were performed as described previously [12]. The extract (12.5 ml) was diluted 3-fold with buffer F [25 mM Tris/HC1 (pH 7.1), 5 mM DTT, 1 µg/ml leupeptin and 1 µg/ml antipain] and applied to a DEAE-Sepharose column (12 cm3) equilibrated with the same buffer. This column was washed with 25 ml of buffer F and the flow-through and washing fractions of this column were pooled, concentrated and gel-filtered on Sephacryl S-200 (70 cm3) equilibrated with buffer F. The fractions containing LMWPTP, as determined by SDS/PAGE analysis, were supplemented with 10% (w/v) glycerol and stored at −70 °C. Protein concentration was estimated by measuring A280 (absorbance) assuming molar absorption coefficients of 18 260 and 15 130 M−1·cm−1 for LMWPTP-A and LMWPTP-B respectively. Starting from 250 ml of culture, we obtained for both isoforms ~10 mg of pure protein.

Assay of phosphatase activities

FN6Pase and PNPPase activities were assayed as previously described [9]. All other enzymatic assays were performed at 30 °C in a medium containing 25 mM Tris/HC1 and 1 mM DTT and the indicated concentration of substrate. Ribuloselysine-5-phosphatase activity was measured through the formation of P, [19]. Activities on 32P-componds were determined in 50 µl of a solution containing 1 µM of phosphogroups (i.e. ~7000 c.p.m.); this corresponded to a protein concentration of 0.5 mM (lysozyme), 15 µM (myelin basic protein) and 50 µM (Hb). The reactions were stopped by the addition of 1 mg of BSA (as a carrier) and 100 µl of ice-cold 10% (w/v) trichloroacetic acid. The samples were centrifuged for 10 min at 10000 g and the radioactivity in the supernatant was counted. Unless otherwise stated, RNSPase activity was measured using lysozyme glycated with [32P]ribose 5-phosphate as a substrate (tested at 1 µM of phosphogroups).
Phosphorylation by FN3K-RP of the dephosphorylation products of LMWPTP

Lysozyme glycated with ribose 5-phosphate (5 mg/ml) was incubated for 10 min at 37 °C in 20 µl of a solution containing 25 mM Tris/HCl (pH 7.1), 1 mM DTT, and 20 µg/ml LMWPTP-A. The samples were then incubated in 50 µl of the same medium containing 1 mM MgCl₂, 1 mM EGTA, 100 µM γ-[32P]ATP, and 60 µg/ml spinach leaf ribulosamine-3-kinase [12] or 280 µg/ml of human erythrocyte FN3K-RP [13]. After the indicated times, aliquots (12 µl) were spotted on P81 papers, washed and counted for radioactivity as described previously [5].

RESULTS

Glycation of proteins with ribose 5-phosphate

To get a better appraisal of the relative reactivity of ribose 5-phosphate and other sugars in early glycation, we measured the incorporation of [14C]ribose 5-phosphate, ribose and glucose 6-phosphate into lysozyme and albumin. As shown in Figure 1, ribose 5-phosphate reacted with lysozyme and albumin at rates that were 10- and 6-fold higher than ribose, and 15- and 24-fold higher than glucose 6-phosphate respectively. As glucose 6-phosphate is itself 3–8-fold [9] more powerful than glucose as a glycating agent, we calculated that ribose 5-phosphate is approx. 80-fold more reactive than glucose towards proteins, being therefore a very significant glycating agent (see the Discussion section).

Figure 2 Tissue distribution of RN5Pase

The enzymatic activity was determined on crude extracts (1–3 mg of protein/ml in the assay), using lysozyme glycated with [32P]ribose 5-phosphate as a substrate (tested at 1 µM of phosphogroup). Conditions used for these assays were such that the activity was proportional with time and with extract concentration and that less than 20% of the protein-RN5Ps were dephosphorylated. The results are the means ± S.E.M. for independent determinations performed on samples from three different animals. They are expressed as the activity per mg of protein or Hb (in the case of erythrocytes).

Tissue distribution and purification of RN5Pase

As RN5Ps are not substrates for FN3K-RP (see below), we searched for an enzyme able to dephosphorylate RN5Ps in tissues and erythrocyte extracts. For this purpose, we used a radioactively labelled substrate obtained by incubating lysozyme with [13P]ribose 5-phosphate. As shown in Figure 2, this substrate (tested at a phosphogroup concentration of 1 µM) was dephosphorylated in all rat tissue extracts that were tested. Interestingly, the highest activity was observed with extracts derived from erythrocytes, a cell type in which both FN3K-RP and FN3K are particularly active [11]. In all cases, the enzymatic activity was > 90% inhibited by 50 mM Hepes (a property of LMWPTP, see below).

The purification of RN5Pase was started by fractionation of a human erythrocyte lysate on DEAE-Sepharose at pH 8. RN5Pase was eluted from this column as a single peak, which was separated from the peak of FN6Pase/MDP-1 but coincided with the peak of PNPPase activity (Figure 3A). Unlike FN6Pase/MDP-1, RN5Pase was not dependent on the presence of Mg²⁺ (results not shown) but was inhibited by Hepes (IC₅₀ = 1.2 mM). Upon further purification on SP-Sepharose at pH 6 (Figure 3B), approx. 60% of the RN5Pase activity was found in the flow-through and washing fractions (‘peak I’), and the rest was eluted as two distinct peaks with ~200 (peak II) and ~300 (peak III) mM NaCl. Peaks II and III were further purified by gel filtration on Superdex-200. In both cases, the enzyme co-eluted with an 18 kDa polypeptide, as determined by SDS/PAGE analysis (Figures 4B, 4C, 4E and 4F). This 18 kDa polypeptide was the only band present in some fractions obtained at this stage reveled the presence of several bands co-eluting with the enzymatic activity (Figures 4A and 4D), including an 18 kDa band that was also identified as LMWPTP-A (Figure 4G). The other bands found in these fractions were

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digested with trypsin and MS analysis led to their identification as (i) phosphohistidine phosphatase (NP-054891), (ii) thioredoxin (NP-003320) and (iii) SH3 domain (Src homology 3 domain)-binding glutamic acid-rich protein (NP-112576).

Expression and substrate specificity of LMWPTPs

Human LMWPTP-A and LMWPTP-B, which differs from LMWPTP-A by an internal sequence due to an alternative splicing event [17,21], were overexpressed in E. coli and purified to near homogeneity by chromatography on DEAE-Sepharose and gel filtration on Sephacryl S-200 (results not shown). The activities of both recombinant proteins and of FN6Pase/MDP-1 were tested on 32P-labelled RN5Ps bound to hen’s-egg lysozyme or bovine Hb, on Fn6Ps (fructosamine 6-phosphates) bound to hen’s-egg lysozyme and on phosphophytose bound to myelin basic protein. All substrates were used at the same, very low concentration of phosphogroups (1 µM). As illustrated in Table 1 and Figure 5, RN5Ps (whether bound to lysozyme or to Hb) were the best substrates for both forms of LMWPTP, followed by protein-phosphohistidine and Fn6Ps, whereas a different substrate preference (FN6P-RN5P-phosphorylase) was observed with FN6Pase/MDP-1. Despite its high activity on protein-bound RN5Ps, LMWPTP-A was unable to dephosphorylate completely protein-RN5Ps: RN5Ps bound to lysozyme and Hb were dephosphorylated to the extent of 75% (Figure 5) and 55% (results not shown) respectively. This suggests that some of the RN5P residues are inaccessible to the phosphatase. Neither of the two forms of LMWPTP enhanced the decomposition rate of RN3Ps (ribulosamine 3-phosphates) bound to lysozyme, indicating that they did not act as RN3Pase (ribulosamine 3-phosphatase) (results not shown).

Interestingly, LMWPTP-A acted much better than LMWPTP-B on all four protein substrates (Table 1) as well as on free ribuloselysine 5-phosphate (activities at 1 mM: 1.6 ± 0.14 versus 0.08 ± 0.01 µmol · min⁻¹ · mg⁻¹, n = 3), whereas a similar activity was found when PNPP (p-nitrophenyl phosphate) was used as a substrate (activities at 1 mM: 4.7 ± 0.35 versus 4.9 ± 0.49 µmol · min⁻¹ · mg⁻¹, n = 3).

Conversion of RN5Ps into substrates for FN3K-RP

Protein-bound RN5Ps were phosphorylated neither by human FN3K-RP nor by its spinach leaf homologue, as indicated by the lack of incorporation of radioactive from [32P]ATP into protein (Figure 6). As ribulosamine 3,5-bisphosphates are probably more labile than RN3Ps, we also measured the formation of [32P]RN5P by both rhoamin-3-kinases. As expected, pre-incubation of lysozyme-bound RN5Ps with LMWPTP-A led to the formation of a substrate for both plant and human ribulosamine-3-kinases (Figure 6).

DISCUSSION

Previous work has shown that ribose 5-phosphate is a potent glycat agent [4]. However, the technique used to measure the glycation rate – the formation of UV-absorbing material – does not allow one to make a precise comparison of the reactivity of different sugars, because the formation of UV-absorbing material involves reactions downstream of the formation of Amadori products and therefore largely depends on the reactivity of the Amadori products themselves. The present study shows that ribose 5-phosphate reacts with proteins approx. 10-fold more rapidly than ribose and 80-fold more rapidly than glucose. This higher reactivity was obtained with freshly prepared, purified radiolabelled compounds, and identical results were obtained with [14C]ribose 5-phosphate and [32P]ribose 5-phosphate (results not shown). This indicates that there was no interference of contaminating, highly reactive radioactive impurities, which could potentially lead to an overestimation of the glycation rate [22].

Since the intracellular ribose concentration appears to be <10 µM (G. Delpierre and E. Van Schaftingen, unpublished work) as compared with 6–45 µM for ribose 5-phosphate [23,24], the much higher reactivity of ribose 5-phosphate compared with ribose indicates that ribulosamines probably arise mainly from ribose 5-phosphate. At a (physiological) concentration of 25 µM, ribose 5-phosphate has a glycat power amounting to approx. 40% of that of 5 mM glucose, which indicates that it is indeed a significant glycat agent. It may therefore make sense to have a system allowing the deglycation of RN5Ps.

FN3K-RP and its plant homologue are devoid of RN5P-3-kinase activity. A phosphatase is therefore needed to convert RN5Ps into ribulosamines. We show in the present study that such a phosphatase is present in mammalian tissues and erythrocytes. The human erythrocyte enzyme was purified and identified as LMWPTP-A. This identification was based on MS analysis of purified RN5Pase, on the finding that, like LMWPTP, it is not dependent on Mg²⁺ but is inhibited by Hepes [25], and on the demonstration that recombinant LMWPTP-A acts as a RN5Pase. Erythrocyte LMWPTP-A separated into three peaks upon chromatography on SP-Sepharose. This is possibly the result of genetic heterogeneity (a common polymorphism replaces Gln¹⁰⁰ with arginine [26]), post-translational modification, oligomerization or association with other proteins.
Protein-ribulosamine-5-phosphatase

Figure 4  Further purification of the three peaks of RN5Pase on Superdex-200 and co-purification with an 18 kDa polypeptide

The three peaks resulting from the SP-Sepharose chromatography were further purified on Q-Sepharose (peak I only; results not shown) and Superdex-200. Protein concentration ([H17033] and RN5Pase activity ([H17039] were measured ([A, B, C]), and the fractions were analysed by SDS/PAGE ([D, E, F]). The arrows (and numbers) show the bands that were cut out from the gel, digested with chymotrypsin (or trypsin) and analysed by tandem MS. The sequence of human LMWPTP-A and the variable region corresponding to LMWPTP-B are shown; the identified peptides are underlined ([G]).

Table 1  Substrate specificity of human LMWPTPs and MDP-1

The activities were determined with pure recombinant enzymes. Values are means ± S.E.M. (n = 3) or individual values.

<table>
<thead>
<tr>
<th>Substrate</th>
<th>LMWPTP-A (nmol · min⁻¹ · mg⁻¹)</th>
<th>LMWPTP-B (nmol · min⁻¹ · mg⁻¹)</th>
<th>MDP-1/FN6Pase (nmol · min⁻¹ · mg⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lysozyme glycated with [32P]ribose 5-phosphate</td>
<td>5.95 ± 0.005</td>
<td>0.28 ± 0.007</td>
<td>1.46 ± 0.156</td>
</tr>
<tr>
<td>Lysozyme glycated with [32P]glucose 6-phosphate</td>
<td>0.26 ± 0.121</td>
<td>0.04 ± 0.001</td>
<td>10.6 ± 0.298</td>
</tr>
<tr>
<td>Myelin basic protein-[32P]phosphotyrosine</td>
<td>0.93 ± 0.038</td>
<td>0.14 ± 0.014</td>
<td>0.31 ± 0.049</td>
</tr>
<tr>
<td>Hb glycated with [32P]ribose 5-phosphate</td>
<td>2.63 ± 3.05</td>
<td>0.66 ± 0.86</td>
<td>1.16 ± 1.29</td>
</tr>
</tbody>
</table>

* At 1 μM of phosphogroups.

LMWPTP was initially identified as a phosphatase acting on low-molecular-mass substrates, most particularly FMN and PNPP but very poorly on sugar phosphates [27]. Later work showed it to act also as a protein tyrosine phosphatase, but not as a protein-serine/threonine-phosphatase. This, together with the presence of a sequence motif reminiscent of tyrosine phosphatases, led to this enzyme being renamed ‘low-molecular-mass protein tyrosine phosphatase’. We now show that this enzyme acts particularly well as a protein-RN5Pase. Interestingly, this substrate is structurally similar to one of the best low-molecular-mass substrates, FMN, which contains a ribitol 5-phosphate portion. The crystal structure of LMWPTP shows it to have a deep and narrow catalytic pocket [25,28,29]. This is presumably why this enzyme is unable to remove phosphate groups bound to the first atom of an amino acid side chain (serine and threonine phosphates), but that it is highly active on tyrosine phosphates and RN5Ps, where the phosphate is bound to the 5th and 10th atoms of the amino acid side chain respectively. The low activity of LMWPTP on fructosamine 6-phosphates is probably due to the fact that the latter are essentially under their furanose forms [30], which are...
phosphorylation levels were measured after different times. Results are means ± S.E.M. (n = 3).

probably too bulky to enter the catalytic site. By contrast, RN5Ps are unable to cyclize. The lack of action of LMWPTP on RN3P is presumably also related to the narrowness of the catalytic site, which is probably unable to accommodate a secondary phosphate group when the neighbouring carbons bear a hydroxy group. The lack of RN3Pase activity is functionally important: its presence in cell signalling. This work was supported by grants from the European Foundation for the Study of Diabetes, the Juvenile Diabetes Foundation, the Interuniversity Attraction Pole Programme – Belgium Science Policy (Network P6/05) and the Actions de Recherches Concertées of the French Community of Belgium. R.G. is a Fellow of the Fonds pour l’Encouragement à la Recherche dans l’Industrie et dans l’Agriculture. D. V. was supported by the Interuniversity Attraction Pole Program – Belgian Science Policy (P6/28).

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