



HAL
open science

Evolution of vertebrate glucokinase regulatory protein from a bacterial N-acetylmuramate 6-phosphate etherase

Maria Veiga-Da-Cunha, Tatiana Sokolova, Fred R. Opperdoes, Emile van Schaftingen

► To cite this version:

Maria Veiga-Da-Cunha, Tatiana Sokolova, Fred R. Opperdoes, Emile van Schaftingen. Evolution of vertebrate glucokinase regulatory protein from a bacterial N-acetylmuramate 6-phosphate etherase. *Biochemical Journal*, 2009, 423 (3), pp.323-332. 10.1042/BJ20090986 . hal-00479216

HAL Id: hal-00479216

<https://hal.science/hal-00479216>

Submitted on 30 Apr 2010

HAL is a multi-disciplinary open access archive for the deposit and dissemination of scientific research documents, whether they are published or not. The documents may come from teaching and research institutions in France or abroad, or from public or private research centers.

L'archive ouverte pluridisciplinaire **HAL**, est destinée au dépôt et à la diffusion de documents scientifiques de niveau recherche, publiés ou non, émanant des établissements d'enseignement et de recherche français ou étrangers, des laboratoires publics ou privés.

Evolution of vertebrate glucokinase regulatory protein from a bacterial N-acetylmuramate 6-phosphate etherase

Maria VEIGA-DA-CUNHA*^{1, 2}, Tatiana SOKOLOVA*¹, Fred OPPERDOES†, and Emile VAN SCHAFTINGEN*²

¹ Both authors contributed equally to the work

From the *laboratory of Physiological Chemistry and †Research Unit for Tropical Diseases, de Duve Institute, Université Catholique de Louvain, Avenue Hippocrate 75, B-1200 Brussels, Belgium

Running head: Etherase activity of GKRP

²Address correspondence to: Emile Van Schaftingen or Maria Veiga-da-Cunha, Groupe de Recherches Métaboliques, de Duve Institute, 75, av. Hippocrate, B-1200 Brussels, BELGIUM.

Tel. 32 (0)2 764 75 65.

Fax: 32 (0)2 764 75 98;

E-mail: emile.vanschaftingen@uclouvain.be or maria.veiga@uclouvain.be

Keywords: muramyl-peptide, N-acetylmuramate, fructose metabolism, GKRP, fructose 6-phosphate, N-acetylmurate 6-phosphate

Abbreviations used: GK, glucokinase; hiGK, human islet glucokinase; xGK, xenopus glucokinase; GKRP, glucokinase regulatory protein; rGKRP, rat glucokinase regulatory protein; xGKRP, xenopus glucokinase regulatory protein; MurNAc6P, N-acetylmuramate 6-phosphate; GlcNAc6P, N-acetylglucosamine 6-phosphate; Glc6P, glucose 6-phosphate; Man6P, mannose 6-phosphate; Sol6P, sorbitol 6-phosphate; Fr6P, fructose 6-phosphate; Fr1P, fructose 1-phosphate; R5P, ribose 5-phosphate; Rol5P, ribitol 5-phosphate.

SYNOPSIS

Mammalian glucokinase regulatory protein (GKRP), a fructose 6-phosphate and fructose 1-phosphate sensitive inhibitor of glucokinase (GK), appears to have resulted from the duplication of a gene similar to bacterial N-acetylmuramate 6-phosphate etherase (MurQ). In the present work, we show that several genomes of primitive eukaryotes encode a GKRP-like protein with two MurQ repeats. Recombinant *Haemophilus influenzae* MurQ and the GKRP homologue of the amoebaflagellate *Naegleria gruberi* both behaved as excellent N-acetylmuramate 6-phosphate etherases, with K_{cat} values (83 and 20 sec^{-1}) at least as high as that reported for *Escherichia coli* MurQ. By contrast, rat and xenopus GKRP displayed much lower etherase activities ($K_{\text{cat}} = 0.08$ and 0.05 sec^{-1} , respectively). The etherase activity of rat GKRP was inhibited by ligands (fructose 6-phosphate, fructose 1-phosphate, sorbitol 6-phosphate) known to regulate its interaction with GK and by mutations affecting the binding of these phosphate esters. This indicated that these phosphate esters all bind to a single regulatory site, which evolved from the original catalytic site. Sorbitol 6-phosphate and other phosphate esters also inhibited the etherase activity of xenopus GKRP, but did not affect its ability to inhibit GK. Thus, unlike what was previously thought, xenopus GKRP has a binding site for phosphate esters, but this site is uncoupled from the GK binding site. Taken together, these data indicate that duplication of the *murQ* gene led to a eukaryotic type etherase, which subsequently evolved to GKRP by acquiring a new binding specificity whilst losing most of its etherase activity.

INTRODUCTION

Studies on the effect of fructose to stimulate the phosphorylation of glucose by glucokinase (GK) in rat hepatocytes led to the identification of glucokinase regulatory protein (GKRP) [1, 2]. This ≈ 68 kDa protein binds and inhibits GK, and its effect is reinforced by fructose 6-phosphate and antagonized by fructose 1-phosphate [3]. Fructose 1-phosphate, which is formed from fructose by ketohexokinase, acts at concentrations in the micromolar range and accounts for the stimulatory effect that low concentrations of fructose have on the phosphorylation of glucose. Similar regulatory properties are observed with human GKRP [4]. GKRP is also involved in the control of the subcellular localization of GK, which is present in the nucleus at low concentrations of glucose, but moves to the cytosol when the concentration of glucose is elevated or if low concentrations of fructose are present [5, 6]. Mice deficient in GKRP have reduced GK activity and this enzyme is exclusively cytosolic in their hepatocytes, suggesting that GKRP serves also to maintain in the nucleus a functional reserve of GK that is quickly available in the presence of glucose [7, 8]. Recently, two polymorphisms in the GKRP gene were shown to be linked with the blood glucose and triglyceride levels in various human populations [9, 10, 11, 12], emphasizing the importance of GKRP in the control of carbohydrate metabolism, but also suggesting a link between this regulatory molecule and lipid metabolism.

Detailed characterization of rat GKRP showed that several sugar or polyol-phosphates, most particularly sorbitol 6-phosphate, mimic the effect of fructose 6-phosphate, whereas others mimic the effect of fructose 1-phosphate [13]. Several residues that are involved in the binding of these phosphate esters were identified by site-directed mutagenesis [14, 15]. Intriguingly, xenopus GKRP, which shares about 60 % sequence identity with its mammalian homologues, inhibits GK in a fructose 6-phosphate and fructose 1-phosphate insensitive manner [16].

Sequence comparisons indicate that GKRP belongs to the sugar isomerase (SIS) family of proteins [17]. Its closest homologues are bacterial proteins (MurQ) involved in the recycling of the peptidoglycan and catalysing the hydrolysis of the ether bond in N-acetylmuramate 6-phosphate, thus forming N-acetylglucosamine 6-phosphate and D-lactate, [18, 19, 20]. The GKRP sequence (≈ 600 residues), which has about twice the size of the MurQ sequence (≈ 300 residues), contains two regions that are homologous to MurQ. As MurQ is active as a homodimer [14], whereas GKRP is a monomer (it heterodimerizes with GK), it has been suggested that the GKRP structure resembles that of a MurQ dimer [14, 21].

An interesting question is that of the evolution of GKRP, most particularly since there is no obvious connection between muramyl-peptide recycling in bacteria and the allosteric regulation of an enzyme (vertebrate GK) that appeared late in evolution. Are there GKRP homologues in primitive eukaryotes and what is their function? Based on sequence comparisons, Jaeger and Mayer [22] proposed that the gene duplication event that led to present day GKRP pre-dates the separation of bacterial etherases from vertebrate GKRP. One may therefore wonder if the ancestral gene that underwent this duplication event encoded a protein with etherase activity or with another function. The purpose of the present paper was to shine some light on the evolution of GKRP.

EXPERIMENTAL

Reagents

D-lactate dehydrogenase (from *Lactobacillus Leichmannii*) and pig heart glutamate–pyruvate transaminase were from Roche diagnostics (Mannheim, Germany). Glycylglycine, L-glutamate, N-acetylmuramic acid and N-acetyl-D-glucosamine 6-phosphate disodium salt were from Sigma-Aldrich (St. Louis, MO, USA). Molecular biology DNA modifying enzymes and *Pfu* polymerase were from Fermentas GMBH, Germany. *Optimase* polymerase was from Transgenomics, 78990 Elancourt, France. pET expression vectors were from Novagen, Darmstadt, Germany. Recombinant *Haemophilus influenzae* YfeU (or MurQ) [14], xenopus liver GK and GKRP [16] and human beta-cell GK [23] were expressed in *Escherichia coli* and purified as previously described.

Phylogenetic analysis

The *Naegleria gruberi* sequence was placed into an alignment of 2 selected bacterial N-acetylmuramic acid 6-phosphate etherase and 15 eukaryotic GKRP homologues obtained from the NCBI Genebank and TrEMBL databases. A total of 235 unambiguously aligned sites common to all sequences (Supplementary Figure S1) was retained for phylogenetic analysis. Phylogenetic trees were inferred by the Neighbour-joining method [24] as implemented by the program ClustalX version 2 [25] and by Bayesian analysis using MrBAYES version 3.1.2 [26]. The BLOSUM model of evolution and a gamma rate distribution with 4 substitution rate categories fitted best the data. To estimate Bayesian posterior probabilities, Markov Chain Monte Carlo (MCMC) chains were run for 80,000 generations and sampled every 100 generations (burn-in: 20,000 generations). The final dataset for tree construction comprised the following taxa (accession numbers): *N. gruberi* (Naegr1 78308; <http://genome.jgi-psf.org/Naegr1/Naegr1.home.html>), *Xenopus laevis* (Q91754), *Mus musculus* (Q91X44), *Rattus rattus* (Q07071), *Homo sapiens* (Q14397), *Canis familiaris* (XP_532912.2), *Danio rerio* (XP_001334641.2), *Tetraodon nigroviridis* (CAG06348.1), *Nematostella vectensis* (XP_001640085.1), *Branchiostoma floridae* (XP_002249995.1), *Strongylocentrotus purpuratus* (XP_001198229.1), *Monosiga brevicolis* (XP_001750465.1), *Dictyostelium discoideum* (XP_646964.1), *Micromonas pusilla* (EEH52070.1), *Ciona intestinalis* (XP_002123418.1). The sequences of *E. coli* (P76535) and *H. influenzae* (P44862) were used as outgroups.

Overexpression and purification of recombinant proteins

Recombinant proteins were expressed with a C-terminal (rat GKRP and *N. gruberi* GKRP homologue) or a N-terminal (N-acetylglucosamine kinase, N-acetylglucosamine 6-phosphate deacetylase and glucosamine 6-phosphate deaminase/isomerase) 6xHis tag. pBlueScript containing rat GKRP [27] was utilized to PCR amplify the C-terminal end (\approx 300 bp) of rat GKRP, using *Pfu* polymerase. This PCR-amplified product was used to replace the C-terminal end of rat GKRP in the original pBlueScript-GKRP plasmid. After sequencing confirmation, the NdeI/XhoI fragment containing the entire rat GKRP open reading frame without the stop codon (\sim 2000 bp) was excised from pBlueScript and ligated in the pET-24a expression vector. Since *N. gruberi* genes do not have introns, the open reading frame of the GKRP homologue was PCR amplified using genomic DNA from strain DRI using *Optimase*

polymerase. The amplified DNA (1855 bp) without the stop codon was digested with NcoI and NotI and directly cloned in pET-28a. The open reading frames of the genes coding for *Silicibacter pomeroyi* N-acetylglucosamine kinase (YP_167078.1; 890 bp), glucosamine 6-phosphate deaminase/isomerase (YP_167080; 1032 bp) and N-acetylglucosamine 6-phosphate deacetylase (YP_167081; 1146 bp) were PCR-amplified using bacterial genomic DNA and *Pfu* polymerase. The PCR-amplified products containing the ATG codon in a NdeI restriction site and the stop codon flanked by a BamHI (N-acetylglucosamine kinase and glucosamine 6-phosphate deaminase/isomerase) or a BglII (N-acetylglucosamine 6-phosphate deacetylase) restriction site were cloned in pET15b. The sequence of the primers used in the PCR reactions are shown in supplementary Table S1. Following sequence confirmation, the expression plasmids were introduced in *E. coli* BL21(DE3) pLyS. To produce the recombinant proteins, 500 ml of the respective bacterial cultures were grown in Luria-Bertani (LB)-rich medium and the proteins were expressed after addition of 0.4 mM isopropyl-D-thiogalactopyranoside for 18h at 16°C (rat GGRP and *N. gruberi* GGRP homologue) or for 5h (glucosamine 6-phosphate deaminase/isomerase and N-acetylglucosamine 6-phosphate deacetylase) or 18h (N-acetylglucosamine kinase) at 37°C.

Preparation of the bacterial extracts (25 ml) and purification of the 6xHis-tagged proteins on a 1-ml HisTrap column (Ni²⁺ form; GE Healthcare) were done as described previously [28]. Protein elution with imidazole was confirmed by SDS-PAGE (\approx 68 kDa for GKRP, 30 kDa for N-acetylglucosamine kinase, 39 kDa for N-acetylglucosamine 6-phosphate deacetylase and 36 kDa for glucosamine 6-phosphate deaminase/isomerase). The selected fractions were concentrated on 15 ml Vivaspin ultrafiltration columns (Sartorius, Stockport, United Kingdom), and desalted on PD-10 columns (GE Healthcare) equilibrated with 25 mM Hepes, pH 7.4, 10 mM NaCl and 1 mM dithiothreitol. Protein concentration was estimated by measuring A₂₈₀ assuming molar absorptivities of 48,525 (rat GGRP), 39,600 (*N. gruberi* GGRP homologue), 28,210 (N-acetylglucosamine kinase), 18,700 (N-acetylglucosamine 6-phosphate deacetylase) and 12,740 M⁻¹.cm⁻¹ (glucosamine 6-phosphate deaminase/isomerase). From 1 liter of culture we obtained 10, 18, 20, 40 or 8 mg of pure rat GGRP, *N. gruberi* GGRP homologue and *S. pomeroyi* N-acetylglucosamine kinase, N-acetylglucosamine 6-phosphate deacetylase or glucosamine 6-phosphate deaminase/isomerase, respectively. The purified proteins were supplemented with 10% glycerol and stored at -70 °C.

Synthesis of N-acetylmuramate 6-phosphate

N-acetylmuramate 6-phosphate was synthesized by incubating 10 mM N-acetylmuramate for 3 h at 37°C with purified recombinant N-acetylglucosamine kinase (70 µg/ml) in the presence of 50 mM Hepes, pH 7.4, 15 mM ATP-Mg²⁺, 10 mM MgCl₂, 1 mM dithiothreitol and 100 µg/ml bovine serum albumin. To stop the reaction, the mixture was heated at 80°C for 10 min and centrifuged. The supernatant containing the resulting N-acetylmuramate 6-phosphate was purified by anion-exchange chromatography on a 15 ml Dowex 1-X8, 100-200 mesh resin equilibrated with 20 mM Hepes, pH 6.7. N-acetylmuramate 6-phosphate was eluted from the column using a linear NaCl gradient (0–1 M in 150 ml of buffer) and assayed as the inorganic phosphate [29] liberated by alkaline phosphatase. The preparation was stored at -20°C.

Assay for etherase activity

MurQ, xenopus and rat GKRP etherase activities on N-acetylmuramate 6-phosphate were assayed through the formation of D-lactate or N-acetylglucosamine 6-phosphate. The recombinant proteins were incubated for 10 min at 30°C (*H. influenzae* MurQ), or 20 min (xenopus GKRP) or 40 min (rat GKRP) at 37°C in the reaction medium (0.12 ml) containing 25 mM Hepes pH7.4, 10 mM KCl, 1 mM MgCl₂, 2 mM dithiothreitol, 0.5 mg/ml bovine serum albumin, and the indicated concentrations of N-acetylmuramate 6-phosphate, in the presence or in the absence of different phosphoric esters. To stop the reaction, the mixture was heated for 5 min at 80°C and D-lactate was measured in the supernatant after centrifugation at 4°C for 10 min at 16,000 x g. The formation of D-lactate was measured spectrophotometrically at 340 nm using an enzymatic assay based on the oxidation of D-lactate to pyruvate by the stereoisomer specific D-lactate dehydrogenase with reduction of NAD⁺ to NADH+H⁺ in the presence of L-glutamate and glutamate-pyruvate transaminase [30]. The assay was done in a total volume of 0.6 ml of 200 mM glycylglycine-L-glutamate buffer, pH 10, containing 3.5 mM NAD⁺, 7 U of pig heart glutamate-pyruvate transaminase (Roche Applied Science) and 0.1 ml of sample. The absorbance at 340 nm was measured before the reaction was started by adding 15 U of *L. leichmannii* D-lactate dehydrogenase (Roche Applied Science). The change in absorbance at 340 nm, measured after 60 min at 37°C, corresponded to the amount of D-lactate produced during the delactoylation of N-acetylmuramate 6-phosphate.

N-acetylglucosamine 6-phosphate, the second product formed during the etherase reaction, was monitored using a NADP⁺ coupled spectrophotometric assay at 340 nm. N-acetylglucosamine 6-phosphate was first deacetylated by N-acetylglucosamine 6-phosphate deacetylase and the product of the reaction, glucosamine 6-phosphate, was subsequently converted to fructose 6-phosphate by glucosamine 6-phosphate deaminase/isomerase. The fructose 6-phosphate produced was measured spectrophotometrically in an assay mixture (0.6 ml) containing 25 mM Hepes pH 7.4, 10 mM KCl, 1 mM MgCl₂, 2 mM dithiothreitol, 0.5 mM NADP⁺, 5 µl of purified recombinant N-acetylglucosamine 6-phosphate deacetylase (1.2 µg/ml), 3.5 U of yeast phosphoglucose isomerase, and 3.5 U of yeast glucose-6-phosphate dehydrogenase (Roche Applied Science). The reaction was started after 5 min preincubation at 30°C by addition of 15 µl of purified recombinant glucosamine 6-phosphate deaminase/isomerase (2.2 µg/ml) and the increase in A₃₄₀ was followed at 30°C. One unit of etherase activity is the amount of enzyme that converted 1 µmol of N-acetylmuramate 6-phosphate per min under the conditions described. K_m and V_{max} for the etherase activity of the various enzymes were calculated with the Prism 4 software, using the non-linear regression of hyperbola functionality.

Assays of GK and GKRP

GK was assayed at 30°C using a pyruvate kinase/lactate dehydrogenase-coupled assay [2] with the exception that 1 mM DTT was also added. GKRP was assayed in the same assay by its ability to inhibit GK in the presence of 5 (human beta-cell GK) or 3 (xenopus GK) mM glucose and the indicated concentrations of phosphate esters.

RESULTS

Presence of GKRP homologues in primitive eukaryotes

BLAST searches indicated that GKRPs homologues with two MurQ repeats and a sequence length of 550-650 residues are found not only in mammals and amphibians, but also in fishes (*D. rerio*, *T. nigroviridis*), more primitive chordates (*B. floridae*, *C. intestinalis*), invertebrates (*S. purpuratus*, *N. vectensis*), and protists (*M. brevicollis*, *D. discoideum*, *N. gruberi*). No GKRPs homologue with a duplicated MurQ sequence was found in prokaryotes.

It should be noted that fungi also have MurQ homologues with a larger (650-700 residues) size than bacterial MurQ. These putative proteins have a single MurQ repeat fused to a C-terminal domain homologous to N-acetylglucosamine kinase, suggesting that they are bifunctional proteins catalysing both the phosphorylation of N-acetylmuramate to N-acetylmuramate 6-phosphate and the hydrolysis of the ether bond in the latter. They were not included in our further analyses.

Sequence comparisons and structural considerations

GKRPs from rat and xenopus and their homologue from *N. gruberi* are aligned with *H. influenzae* MurQ in Fig. 1. As previously noted for GKRPs, but now extended to more primitive GKRPs homologues, the first ≈ 270 residues of these proteins align with residues 1-222 of MurQ, and the last 300 with residues 25 until the C-terminus of MurQ. In other words, the last ≈ 80 residues are missing in the first MurQ repeat of eukaryotic proteins. Consistent again with what had been observed with GKRPs [14], the residues that are strictly conserved when comparing MurQ with the N-terminal part of the eukaryotic protein (underlined twice in the second MurQ repeat in Fig. 1) are most often (but not always) different from those that are conserved in the C-terminal part of the eukaryotic proteins and reciprocally. This applies also to GKRPs homologues from more primitive eukaryotes.

Phylogenetic analysis (Fig. 2) based on the amino-acid residues shared by GKRPs and MurQ homologues placed the bacterial N-acetylmuramate 6-phosphate etherase sequences and the eukaryotic two-domain etherase/GKRPs sequences into two separate clades. The long branch of *C. intestinalis* must be an artefact because of the presence of some dubious residues in its sequence. All taxa within the eukaryotic clade follow the expected lines of descent, which suggest that the GKRPs was not acquired late in evolution by an event of horizontal transfer from a bacterium, but that most likely a primitive eukaryotic ancestor already acquired a two-domain enzyme.

This evolution of GKRPs can be understood with the help of the three-dimensional structure of *H. influenzae* MurQ (YfeU), a bacterial type N-acetylmuramate 6-phosphate etherase [21]. As previously proposed [14, 21, 22], functional MurQ is a dimer in which two identical subunits assemble to form two catalytic sites at their interface. Each of the catalytic sites is therefore made of residues from the two subunits. Fig. 3 shows the structure of the *H. influenzae* MurQ monomer [21], in which we have emphasized the residues that are strictly conserved in the amino-terminal part of rat GKRPs (Fig. 3.A, residues in green and yellow) or in its carboxy-terminal part (Fig. 3.B, residues in blue and yellow). The yellow residues in both Fig. 3.A and 3.B correspond to those that are conserved in both regions. Strikingly, the residues conserved in the N-terminal “half” of GKRPs form a cluster (Fig. 3.A) that is spatially different from the cluster formed by the residues that are conserved in the C-terminal “half” of GKRPs (Fig. 3.B). Since both clusters are on the same face of the monomer and assuming that the subunits arrange in the MurQ dimer along a two-fold symmetry axis (yellow arrow in Fig. 3), it appears that only one of the two sites of the dimeric bacterial MurQ has been conserved in GKRPs. Thus, GKRPs has a “pseudodimeric” structure, consisting of a first incomplete MurQ repeat linked to a second, nearly complete repeat, through a hinge region that is distal to the conserved catalytic site.

Etherase activity of *H. influenzae* MurQ, rat and xenopus GKRPs and their *N. gruberi* homologue.

Rat GKRPs and its *N. gruberi* homologue were produced with a 6xHis tag added to the C-terminal end of the protein. Xenopus GKRPs [16] and the *H. influenzae* MurQ [14] were produced as unmodified proteins. All four recombinant proteins had the expected size, as determined by SDS-PAGE analysis and were purified to near-homogeneity. Their ability to catalyze the delactoylation of N-acetylmuramate 6-phosphate was determined by measuring the production of D-lactate and N-acetylglucosamine 6-phosphate, which were both assayed spectrophotometrically. As shown in Table 1, *H. influenzae* MurQ and the *N. gruberi* GKRPs homologue showed substantial etherase activities, with comparable catalytic efficiencies. By contrast, rat and xenopus GKRPs showed only a low, yet significant delactoylase activity, with K_{cat} values more than two orders of magnitude lower than those of the bacterial or amoebal enzymes. Rat GKRPs produced only about half as much N-acetylglucosamine 6-phosphate as D-lactate, whereas the three other proteins converted N-acetylmuramate 6-phosphate to stoichiometric amounts of the two products (not shown). We presume that rat GKRPs also produced the alpha-beta unsaturated aldehyde form of N-acetylglucosamine 6-phosphate, a catalytic intermediate of the etherase reaction that is slowly released from the *E. coli* MurQ catalytic site [20].

Modulation of the etherase activity by sugar- and polyol-phosphates

As mentioned in the Introduction, several phosphate esters bind to GKRPs and modulate its interaction with GK [13]. Their binding site might correspond to the catalytic site of GKRPs and we therefore tested their effect on the etherase activity of rat GKRPs and its three recombinant homologues. All tested effectors of rat GKRPs inhibited the etherase activity of this protein (Fig. 4.A and 4.B) and their effects were consistent with what is known on their relative affinities for rat GKRPs based on studies of its interaction with GK [13]. Thus, sorbitol 6-phosphate ($I_{50} = 30 \mu\text{M}$, calculated from plots of $1/v$ versus $[I]$) was a more potent inhibitor of N-acetylmuramate 6-phosphate delactoylation than fructose 6-phosphate ($I_{50} = 130 \mu\text{M}$) (Fig. 4.A), another ligand for rat GKRPs that reinforces GK inhibition. Among the compounds that release the inhibition exerted by rat GKRPs on GK, the most potent etherase inhibitor was fructose 1-phosphate, followed by ribitol 5-phosphate and mannose 6-phosphate and finally ribose 5-phosphate ($I_{50} = 25, 80, 250 \mu\text{M}$, respectively) (Fig. 4.B). Glucose 6-phosphate, which does not affect the interaction between GK and rat GKRPs, did not significantly inhibit the etherase activity, whereas N-acetylglucosamine 6-phosphate, the product of the reaction, exerted a modest inhibition ($I_{50} = 1 \text{ mM}$; Fig. 4.A).

The interaction of xenopus GKRPs with GK is unaffected by fructose 6-phosphate and fructose 1-phosphate [16, 31]. Surprisingly, several of the ligands of rat GKRPs affected the etherase activity of xenopus GKRPs (Fig. 4.C and 4.D), most particularly sorbitol 6-phosphate (Fig. 4.C), which acted with a similar affinity as on rat GKRPs ($I_{50} = 30 \mu\text{M}$), but also mannose 6-phosphate ($I_{50} = 320 \mu\text{M}$) and fructose 6-phosphate ($I_{50} = 1 \text{ mM}$), which were poorer ligands for xenopus GKRPs than for its rat counterpart. Remarkably, fructose 1-phosphate, the best ligand of rat GKRPs [13], did not inhibit the etherase activity of xenopus GKRPs (Fig. 4.D) whereas N-acetylglucosamine 6-phosphate inhibited it ($I_{50} = 130 \mu\text{M}$) more potently than that of the rat protein (Fig. 4.A and 4.C). Thus, unlike what was previously thought, xenopus GKRPs shares some of the ligands of rat GKRPs.

Sorbitol 6-phosphate, ribose 5-phosphate and N-acetylglucosamine 6-phosphate modestly inhibited the activity of the *N. gruberi* GGRP homologue (Fig. 5). Except for N-acetylglucosamine 6-phosphate, none of the modulators of rat GGRP inhibited the activity of *H. influenzae* MurQ (Fig. 5), or of *E. coli* MurQ that was partially purified by DEAE-chromatography from a bacterial cell extract (not shown). These findings indicated that the etherase activities of recombinant rat and xenopus GKRP were authentically contributed by these proteins rather than by a contaminating *E. coli* etherase activity.

Effect of mutations of rat GGRP on its etherase activity

We have also tested the etherase activity of several rat GGRP mutants that had been produced and investigated in previous studies [14, 15]. The etherase activity was markedly reduced in three mutants (R518Q, K514A, T109A, S110A) in which residues that are highly conserved in MurQ/GGRP had been replaced (Table 2). Furthermore, the residual etherase activity of these hypoactive mutants (tested with 1.5 mM N-acetylmuramate 6-phosphate) was insensitive to fructose 6-phosphate, sorbitol 6-phosphate and fructose 1-phosphate (all tested at 5 mM; not shown), consistent with previous findings indicating that these mutants had a markedly reduced affinity for these phosphate esters in assays of GK inhibition. Reciprocally, the etherase activity was essentially unchanged in two other mutants (P446L, G107C) that had not lost their affinity for regulatory phosphate esters (Table 2; Fig. 6). These findings indicated that the catalytic site of the etherase activity and the site regulating the interaction with GK are one and the same.

Studies on the interaction of GKRP and its homologues with GK

Since sorbitol 6-phosphate and mannose 6-phosphate are ligands of xenopus GGRP, we were interested to check whether they were also able to modulate the interaction between GK and xenopus GGRP. Fig. 7 compares the effect of these and other compounds on the inhibition exerted by rat or xenopus GKRP on human islet or xenopus GK. In the case of rat GGRP, all assays were performed either in the absence (Fig. 7.A) or in the presence (Fig. 7.B) of 50 μ M fructose 6-phosphate. The results obtained with the rat protein are consistent with previously published data [13], as they show that sorbitol 6-phosphate and fructose 6-phosphate strongly reinforced the inhibition, whereas mannose 6-phosphate, N-acetylmuramate 6-phosphate and N-acetylglucosamine 6-phosphate alleviated it (Fig. 7.B). The most remarkable observation is that none of these compounds affected the interaction between xenopus GGRP and GK (Fig. 7.C).

We also tested the effect of the *N. gruberi* GGRP homologue or *H. influenzae* MurQ on human and xenopus GK activity. No inhibition was found, even at concentrations of these proteins that were more than 10-fold higher than those at which rat or xenopus GGRP caused 50 % inhibition of GK (not shown).

DISCUSSION

Evolution of MurQ into GGRP

The findings presented in this paper indicate that MurQ underwent a gene duplication and fusion event, which first generated a new form of etherase, later to become GGRP in

vertebrates. The main argument that we provide is that the GGRP/MurQ homologue of the primitive eukaryote *N. gruberi*, while having two MurQ domains and being closer to vertebrate GKRP than to bacterial MurQ, is an efficient etherase, with a K_{cat} that favourably compares with those of its bacterial homologues. A second argument is that both rat and xenopus GKRP display some etherase activity, though this activity is more than two orders of magnitude lower than those of their bacterial or amoebal homologues.

This evolution is schematized in Fig. 8. A dimer comprising two symmetric catalytic sites has been replaced by a monomer with a single catalytic site and a hinge region. Steric hindrance in the hinge region is probably the reason why one of the two catalytic sites has been lost. Further evolution to “authentic” GKRP involved mutations that led (1) to an improved binding of phosphate esters such as fructose 1-phosphate and fructose 6-phosphate, presumably at the expense of a lowering of the catalytic efficiency of the etherase reaction and (2) to the appearance of a binding site for GK.

The “catalytic site” of rat GKRP is indeed responsible for the binding of the allosteric ligands that favour or antagonize its inhibitory effect on GK. This is indicated by the finding that ligands able to modulate the interaction with GK also inhibit the etherase activity of rat GKRP. Moreover, their relative affinity in their effect on the etherase activity is comparable to that observed for the GK/GGRP interaction. Finally, mutations that affect the catalytic activity of rat GKRP also affect its capacity to bind regulatory phosphate esters.

The binding site of GKRP for GK most probably comprises regions that are conserved between the rat and xenopus proteins, but not in bacterial MurQ or in the amoebal etherase. One such region is the N-terminal part, which is well conserved between xenopus and rat GKRP (76 % identity for the first 25 residues, as compared to 59 % identity for the whole sequences), but is absent in the other proteins (Fig. 1). Accordingly, the presence of an N-terminal tag was shown to reduce the affinity of rat GKRP for GK [32], presumably because the tag sterically interferes with the binding of the N-terminal region of GKRP. Another region that has been proposed to play a role in the binding of GKRP to GK is the sequence LSAPfVAG [33]. This sequence, which is largely conserved in xenopus GKRP but less so in the *N. gruberi* GKRP homologue or in bacterial MurQ (see Fig. 1), is close to the N-terminal region in the 3D structure of MurQ [21]. We speculate that this sequence together with the N-terminal extension present in vertebrate GKRP contribute to form the GK binding site. Other regions are probably also involved, since the GK/GGRP interaction is largely hydrophobic [34]. Binding of phosphate esters presumably changes the relative position of these binding regions, thereby favouring or preventing GKRP interaction with GK.

The special case of xenopus GKRP

The effect of xenopus GKRP on the activity of GK has been extensively characterized [16] and found to be insensitive to the classical effectors of rat and human GKRP (fructose 6-phosphate and fructose 1-phosphate), leading to the conclusion that xenopus GKRP was most likely unable to bind these effectors. The effects of sorbitol 6-phosphate, mannose 6-phosphate, fructose 6-phosphate, and ribitol 5-phosphate on the etherase activity of xenopus GKRP all indicate that, on the contrary, this protein is able to bind these phosphate esters. The lack of response in terms of modulation of GK activity suggests that the interaction site with GK is uncoupled from the phosphate ester binding site, in xenopus GKRP and that this protein stays locked in an inhibitory conformation. We may, however, not exclude that ligands other than those we have tested are able to modulate this interaction.

Explanation for the low degree of identity between the two MurQ repeats of GKR

The loss of the second catalytic site in eukaryotic MurQ homologues may explain the intriguing observation (schematized in Fig. 8) that the degree of identity between the two halves of rat GKR (10.6% identity; 14) is significantly lower ($p < 0.0001$ and $p = 0.0165$ by chi square analysis) than for the comparisons between *H. influenzae* MurQ and either the N-terminal or the C-terminal half of rat GKR (25.8 and 18.7 % identity, respectively). The low degree of identity between the two halves of GKR has led Jaeger and Mayer [22] to suggest that the duplication leading to the formation of eukaryotic GKR is a very ancient event, pre-dating the separation between GKR and present day bacterial etherases. If this were true, only one of the two halves of GKR (the one that separated last from the bacterial sequences) should show a higher degree of identity with bacterial MurQs, whereas the other half should show as little identity with bacterial MurQ as with the other half of GKR. This is not the case and we therefore propose an alternative explanation. After the duplication/fusion event, the constraints imposed onto the two MurQ domains were different because only one of the two catalytic sites could be conserved. Thus, several residues that were not “allowed to mutate” in bacterial MurQ could mutate in the N-terminal part of the fusion protein, and a different set of residues could mutate in the C-terminal part, leading to the high degree of divergence between the two MurQ repeats in GKR.

Physiological function of eukaryotic GKR homologues

It is likely that all amoebal homologues of GKR play physiologically the role of N-acetylmuramate 6-phosphate etherases. Amoeba prey on bacteria and it is therefore likely that they have enzymes allowing them to metabolize the muramylpeptide. Interestingly, their genomes encode homologues of anhydromuramate kinase, an enzyme serving to open the internal oside bond of 1,6-anhydro-N-acetylmuramate and phosphorylate the 6th carbon of N-acetylmuramate [35].

At the other extreme of eukaryotic evolution, the rather high K_m and low K_{cat} values of the etherase activity of vertebrate GKRs argues against a role of these proteins in the metabolism of N-acetylmuramate 6-phosphate. This is consistent with the fact that vertebrates do not have homologues of the enzyme that phosphorylates 1,6-anhydro-N-acetylmuramate. An enzyme that could potentially catalyse the phosphorylation of N-acetylmuramate to N-acetylmuramate 6-phosphate is N-acetylglucosamine kinase, most particularly since homologues of this enzyme form bifunctional proteins with MurQ homologues in fungi. However, a partially purified preparation rat liver N-acetylglucosamine kinase did not detectably phosphorylate N-acetylmuramate (Van Schaftingen, unpublished results). All this suggests that the capacity of vertebrates to metabolize N-acetylmuramate 6-phosphate is negligible. This is consistent with *in vivo* data obtained by injecting N-acetylmuramyl peptides to rats, which indicated rather poor and incomplete metabolism of these bacterial products [36].

As for the GKR homologues present in invertebrates, it is difficult to decide about their function. None of these organisms possesses a 1,6-anhydro-N-acetylmuramate kinase, but, as suggested above, N-acetylglucosamine kinase could serve to phosphorylate N-acetylmuramate. Alternatively, the GKR homologues could well have assumed another function, possibly that of controlling the activity of another enzyme than GK.

We do not see indeed any particular reason why GK is regulated by a protein that was originally a N-acetylmuramate 6-phosphate etherase, as there is no obvious link between N-acetylmuramate and GK. It may well be that GKR plays other regulatory roles than that of

controlling the activity and subcellular localisation of GK. Its involvement in the regulation of enzymes involved in lipid metabolism might, for instance, explain the linkage between SNPs in the GKRP gene and triglyceride levels.

ACNOWLEDGEMENTS

We thank J. De Jonckheere for the kind gift of *N. gruberi* DRI genomic DNA and Kate Peel for her expert technical help.

FUNDING

This work was supported by grants from the Belgian Fonds National de la Recherche Scientifique and the Interuniversity Attraction Poles Programme - Belgian Science Policy. Maria Veiga-da-Cunha is *Chercheur Qualifié* of the Belgian Fonds National de la Recherche Scientifique.

REFERENCES

- 1 Van Schaftingen, E. and Vandercammen, A. (1989) Stimulation of glucose phosphorylation by fructose in isolated rat hepatocytes. *Eur. J. Biochem.* **179**, 173-177
- 2 Van Schaftingen, E. (1989) A protein from rat liver confers to glucokinase the property of being antagonistically regulated by fructose 6-phosphate and fructose 1-phosphate. *Eur. J. Biochem.* **179**, 179-184
- 3 Vandercammen, A. and Van Schaftingen, E. (1990) The mechanism by which rat liver glucokinase is inhibited by the regulatory protein. *Eur. J. Biochem.* **191**, 483-489
- 4 Brocklehurst, K. J., Davies, R. A. and Agius, L. (2004) Differences in regulatory properties between human and rat glucokinase regulatory protein. *Biochem. J.* **378**, 693-697
- 5 Agius, L. and Peak, M. (1993) Intracellular binding of glucokinase in hepatocytes and translocation by glucose, fructose and insulin. *Biochem. J.* **296**, 785-796
- 6 de la Iglesia, N., Veiga-da-Cunha, M., Van Schaftingen, E., Guinovart, J. J. and Ferrer, J. C. (1999) Glucokinase regulatory protein is essential for the proper subcellular localisation of liver glucokinase. *FEBS Lett.* **456**, 332-338
- 7 Farrelly, D., Brown, K. S., Tieman, A., Ren, J., Lira, S. A., Hagan, D., Gregg, R., Mookhtiar, K. A. and Hariharan, N. (1999) Mice mutant for glucokinase regulatory protein exhibit decreased liver glucokinase: a sequestration mechanism in metabolic regulation. *Proc. Natl. Acad. Sci. U S A* **96**, 14511-14516
- 8 Grimsby, J., Coffey, J.W., Dvorozniak, M.T., Magram, J., Li, G., Matschinsky, F. M., Shiota, C., Kaur, S., Magnuson, M. A. and Grippo, J. F. (2000) Characterization of glucokinase regulatory protein-deficient mice. *J. Biol. Chem.* **275**, 7826-7831
- 9 Diabetes Genetics Initiative of Broad Institute of Harvard and MIT, Lund University, and Novartis Institutes of BioMedical Research (2007) Genome-wide association analysis identifies loci for type 2 diabetes and triglyceride levels. *Science* **316**, 1331-1336
- 10 Orho-Melander, M., Melander, O., Guiducci, C., Perez-Martinez P, Corella D, Roos C, Tewhey R, Rieder MJ, Hall J, Abecasis G, Tai ES, Welch C, Arnett DK, Lyssenko V, Lindholm E, Saxena R, de Bakker PI, Burt N, Voight BF, Hirschhorn JN, Tucker KL, Hedner T, Tuomi T, Isomaa B, Eriksson KF, Taskinen MR, Wahlstrand B, Hughes TE, Parnell LD, Lai CQ, Berglund G, Peltonen L, Vartiainen E, Jousilahti P, Havulinna AS, Salomaa V, Nilsson P, Groop L, Altshuler D, Ordovas JM, Kathiresan S (2008) Common missense variant in the glucokinase regulatory protein gene is associated with increased plasma triglyceride and C-reactive protein but lower fasting glucose concentrations. *Diabetes* **57**, 3112-3121
- 11 Vaxillaire, M., Cavalcanti-Proença, C., Dechaume, A., Tichet, J., Marre, M., Balkau, B. and Froguel, P.; DESIR Study Group. (2008) The common P446L polymorphism in GCKR inversely modulates fasting glucose and triglyceride levels and reduces type 2

- diabetes risk in the DESIR prospective general French population. *Diabetes* **57**, 2253-2257
- 12 Qi, Q., Wu, Y., Li, H., Loos, R. J., Hu, F. B., Sun, L., Lu, L., Pan, A., Liu, C., Wu, H., Chen, L., Yu, Z. and Lin, X. (2009) Association of GCKR rs780094, alone or in combination with GCK rs1799884, with type 2 diabetes and related traits in a Han Chinese population. *Diabetologia* **52**, 834-843
- 13 Detheux, M., Vandercammen, A. and Van Schaftingen, E. (1991) Effectors of the regulatory protein acting on liver glucokinase: a kinetic investigation. *Eur. J. Biochem.* **200**, 553-561
- 14 Veiga-da-Cunha, M. and Van Schaftingen, E. (2002) Identification of fructose 6-phosphate- and fructose 1-phosphate-binding residues in the regulatory protein of glucokinase. *J. Biol. Chem.* **277**, 8466-8473
- 15 Veiga-da-Cunha, M., Delplanque, J., Gillain, A., Bonthron, D. T., Boutin, P., Van Schaftingen, E. and Froguel, P. (2003) Mutations in the glucokinase regulatory protein gene in 2p23 in obese French caucasians. *Diabetologia* **46**, 704-711
- 16 Veiga-da-Cunha, M., Detheux, M., Watelet, N. and Van Schaftingen, E. (1994) Cloning and expression of a *Xenopus* liver cDNA encoding a fructose-phosphate-insensitive regulatory protein of glucokinase. *Eur. J. Biochem.* **225**, 43-51
- 17 Bateman, A. (1999) The SIS domain: a phosphosugar-binding domain. *Trends Biochem. Sci.* **24**, 94-95
- 18 Jaeger, T., Arsic, M. and Mayer, C. (2005) Scission of the lactyl ether bond of N-acetylmuramic acid by *Escherichia coli* "etherase". *J. Biol. Chem.* **280**, 30100-30106
- 19 Uehara, T., Suefuji, K., Jaeger, T., Mayer, C. and Park, J.T. (2006) MurQ Etherase is required by *Escherichia coli* in order to metabolize anhydro-N-acetylmuramic acid obtained either from the environment or from its own cell wall. *J. Bacteriol.* **188**, 1660-1662
- 20 Hadi, T., Dahl, U., Mayer, C. and Tanner, M. E. (2008) Mechanistic studies on N-acetylmuramic acid 6-phosphate hydrolase (MurQ): an etherase involved in peptidoglycan recycling. *Biochemistry* **47**, 11547-11558
- 21 Kim, Y., Quartey, P., Ng, R., Zarembinski, T. I. and Joachimiak, A. (2009) Crystal structure of YfeU protein from *Haemophilus influenzae*: a predicted etherase involved in peptidoglycan recycling. *J. Struct. Funct. Genomics* **10**, 151-156
- 22 Jaeger, T. and Mayer, C. (2008) The transcriptional factors MurR and catabolite activator protein regulate N-acetylmuramic acid catabolism in *Escherichia coli*. *J. Bacteriol.* **190**, 6598-6608
- 23 Veiga-da-Cunha, M., Xu, L. Z., Lee, Y. H., Marotta, D., Pilkis, S. J. and Van Schaftingen, E. (1996) Effect of mutations on the sensitivity of human beta-cell glucokinase to liver regulatory protein. *Diabetologia* **39**, 1173-1179

- 24 Felsenstein, J.(1981) Evolutionary trees from DNA sequences: a maximum likelihood approach. *J. Mol. Evol.* **17**, 368-376
- 25 Thompson, J.D., Gibson,T.J., Plewniak,F., Jeanmougin,F. and Higgins,D.G. (1997) The ClustalX windows interface: flexible strategies for multiple sequence alignment aided by quality analysis tools. *Nucleic Acids Res.* **25**, 4876-4882
- 26 Huelsenbeck, J. P. and Ronquist, F. (2001) MRBAYES: Bayesian inference of phylogeny. *Bioinformatics* **17**, 754-755
- 27 Detheux, M., and Van Schaftingen, E. (1994) Heterologous expression of an active rat regulatory protein of glucokinase. *FEBS Lett.* **355**, 27-29
- 28 Maliekal, P., Vertommen, D., Delpierre, G. and Van Schaftingen, E. (2006) Identification of the sequence encoding N-acetylneuraminate-9-phosphate phosphatase. *Glycobiology* **16**, 165-172
- 29 Fiske, C.H. and Subarov, Y. (1925) The colorimetric determination of phosphorus. *J. Biol. Chem.* **66**, 375-400
- 30 Gawehn, K. D-(-)-Lactate, in *Methods of Enzymatic Analysis* (vol VI), edited by Bergmeyer, H. U., Bergmeyer, J. and Graßl, M., (3rd ed), VCH Publishers, 1983-1986, pp 588-592
- 31 Vandercammen, A. and Van Schaftingen, E. (1993) Species and tissue distribution of the regulatory protein of glucokinase. *Biochem. J.* **294**, 551-556
- 32 Mookhtiar, K. A., Kalinowski, S. S., Brown, K. S., Tsay, Y. H., Smith-Monroy, C. and Robinson, G. W. (1996) Heterologous expression and characterization of rat liver glucokinase regulatory protein. *Diabetes* **45**, 1670-1677
- 33 Baltrusch, S., Lenzen, S., Okar, D. A., Lange, A. J. and Tiedge, M. (2001) Characterization of glucokinase-binding protein epitopes by a phage-displayed peptide library. Identification of 6-phosphofructo-2-kinase/fructose-2,6-bisphosphatase as a novel interaction partner. *J. Biol. Chem.* **276**, 43915-43923
- 34 Anderka, O., Boyken, J., Aschenbach, U., Batzer, A., Boscheinen, O. and Schmoll, D. (2008) Biophysical characterization of the interaction between hepatic glucokinase and its regulatory protein: impact of physiological and pharmacological effectors. *J. Biol. Chem.* **283**, 31333-31340
- 35 Uehara, T., Suefuji, K., Valbuena, N., Meehan, B., Donegan, M. and Park, J.T. (2005) Recycling of the anhydro-N-acetylmuramic acid derived from cell wall murein involves a two-step conversion to N-acetylglucosamine-phosphate. *J. Bacteriol.* **187**, 3643-3649
- 36 Ono, K., Masayasu, H., Hashimoto, F., Yamada, M. and Takegoshi, T. (1988) Metabolic disposition of ¹⁴C-muroctasin in laboratory animals. *Arzneimittelforschung* **38**, 1009-1014

FIGURE LEGENDS

Figure 1. Alignment of GKRPs and homologous bacterial and amoebal sequences

The GKRPs sequences of *X. laevis* (GKRPL) and *R. norvegicus* (GKRPRn) are aligned with the homologous sequences from *N. gruberi* (MurQNg) and *H. influenzae* (MurQHi1 and MurQHi2). The *H. influenzae* sequence is aligned twice, from residue 1 to 222 (MurQHi1) and from residues 25 to 303 (MurQHi2). The strictly conserved residues are shown in bold. The MurQ residues that are conserved in the N-terminal part of all three eukaryotic proteins are underlined twice in the second MurQ repeat. Those that are conserved in the C-terminal part of the eukaryotic proteins are underlined once in the first MurQ repeat. The LSAPfVAG GK binding motif [33] is underlined in rat GKRPs.

Figure 2. Phylogenetic tree of GKRPs

Phylogenetic tree was estimated from protein sequences using distance matrix neighbour joining (NJ) and Bayesian analysis (MrBayes) methods. Posterior probabilities (MrBayes) or bootstrap support values (NJ) are indicated at the nodes. The horizontal bar represents 20 substitutions per 100 amino-acid residues.

Figure 3. Spatial localization in *H. influenzae* MurQ of the residues that are conserved in the N- and C-terminal halves of GKRPs

The residues of the N-terminal half (A) and the C-terminal half (B) of rat GKRPs that are conserved in *H. influenzae* MurQ are shown as blue or green spheres in the 3D structure of the MurQ monomer [21]. Those that are conserved in both regions are shown in yellow. The vertical line indicates the position of the rotational symmetry axis of the MurQ dimer. The two halves of GKRPs have most likely the same spatial disposition as the bacterial dimer. The orange part in (A) corresponds to the missing amino acid residues at the end of the N-terminal half of GKRPs. This region is fused to the C-terminal half to form a hinge. The position of the residues whose mutations are studied in the present work is also shown.

Figure 4. Inhibition of the etherase activity of rat GKRPs (A and B) and xenopus GKRPs (C and D) by various phosphate esters

The etherase activity of GKRPs was assayed by measuring the formation of D-lactate. Recombinant GKRPs (150 µg/ml) were incubated at 37°C for 40 (rat GKRPs; rGKRPs) or 20 min (xenopus GKRPs; xGKRPs) in presence of subsaturating (350 µM) N-acetylmuramate 6-phosphate (MurNAc6P) and of the indicated concentrations of glucose 6-phosphate (Glc6P; ■), fructose 6-phosphate (Fr6P; ●), sorbitol 6-phosphate (Sol6P; ○), ribose 5-phosphate (R5P; ▲), mannose 6-phosphate (Man6P; ◆), ribitol 5-phosphate (Ri5P; △), fructose 1-phosphate (Fr1P; ◇) or N-acetylglucosamine 6-phosphate (GlcNAc6P; □). Values are means ± SEM (n = 3) and are expressed as percentage of the activity measured in the absence of effectors.

Figure 5. Inhibition of the etherase activity of *H. influenzae* MurQ and *N. gruberi* GGRP homologue by various phosphate esters

The etherase activity of *H. influenzae* MurQ (open bars) or *N. gruberi* GGRP homologue (filled bars) were assayed by measuring the formation of D-lactate. Recombinant purified proteins (0.1 µg/ml) were incubated for 10 min at 30°C in presence of subsaturating (0.2 mM) N-acetylmuramate 6-phosphate (MurNAc6P) and 1 mM of fructose 6-phosphate (Fr6P), fructose 1-phosphate (Fr1P), sorbitol 6-phosphate (Sol6P), ribose 5-phosphate (R5P), glucose 6-phosphate (Glc6P), or N-acetylglucosamine 6-phosphate (GlcNAc6P). Results are means ± SEM (n = 3) and are expressed as percentage of the activity measured in the absence of effectors (100% D-lactate corresponds to approximately 50% delactoylation of the substrate).

Figure 6. Inhibition of the etherase activity of wild type and active mutant forms of rat GGRP by various phosphate esters.

The etherase activity of the indicated rat GGRPs (rGGRP) was tested by measuring the formation of D-lactate in the presence of 350 µM N-acetylmuramate 6-phosphate (MurNAc6P; 100 % of initial activity) with or without 1 mM fructose 6-phosphate (Fr6P), fructose 1-phosphate (Fr1P) or sorbitol 6-phosphate (Sol6P). Values are means ± SEM (n = 3).

Figure 7. Effect of sorbitol 6-phosphate, mannose 6-phosphate, N-acetylglucosamine 6-phosphate and N-acetylmuramate 6-phosphate on the inhibition exerted by rat and xenopus GGRPs on GK .

(A) Human islet GK (hiGK) was assayed in the presence of 5 mM glucose, without or with 5 µg/ml rat GGRP (rGGRP) and with or without 1 mM of the indicated phosphate esters. (B) Same as (A) except that 50 µM fructose 6-phosphate was also present. (C) Xenopus GK (xGK) was assayed in the presence of 3 mM glucose, with or without 0.8 µg/ml xenopus GGRP (xGGRP) and with or without 1 mM of the indicated phosphate esters. The values shown are the ratios (expressed in percent) of the activities observed with and without GGRP and are means ± SEM for n = 3.

Figure 8. Evolution of bacterial MurQ into eukaryotic GGRPs.

In the MurQ dimer, each catalytic site is formed by the two subunits with residues symbolized in black and white. A gene duplication event followed by fusion led to the N-terminal half losing some of the residues involved in the formation of one of the two catalytic sites. Further evolution led to a complete mutational loss of residues forming this site, while the other one was conserved, and became a regulatory site in mammalian GGRP. The asymmetrical mutation of residues in the two halves of GGRP results in their very low degree of identity because of divergent evolution, but maintains a higher degree of identity with bacterial MurQ.

Table 1 Kinetic characteristics of the etherase activity of *H. influenzae* MurQ, *N. gruberi* GKRP homologue and xenopus and rat GKRP

The etherase activity of *H. influenzae* MurQ (MurQHi) or *N. gruberi* GKRP homologue (MurQNg) (0.25 µg/ml) was assayed with variable concentrations (10 to 200 µM) of N-acetylmuramate 6-phosphate (MurNAc6P) by monitoring the formation of N-acetylglucosamine 6-phosphate. The etherase activities of xenopus (xGKRP) and rat (rGKRP) GKRP (150 µg/ml) were measured by monitoring the formation of D-lactate in the presence of 50 µM to 1 mM N-acetylmuramate 6-phosphate. Assays were performed in triplicate and values shown are the means ± standard error.

	K_m (MurNAc6P) (µM)	K_{cat} (sec ⁻¹)	K_{cat}/K_m (sec ⁻¹ .µM ⁻¹)	V_{max} (µmol.min ⁻¹ .mg ⁻¹)
MurQHi	30 ± 1	19.9	0.66	38.3 ± 0.4
MurQNg	134 ± 13	60	0.45	51.5 ± 2.9
xGKRP	674 ± 76	0.118	0.17 x 10 ⁻³	104.3 ± 6 x 10 ⁻³
rGKRP	239 ± 21	0.041	0.17 x 10 ⁻³	36.6 ± 1 x 10 ⁻³

Table 2 Effect of mutations of the catalytic and regulatory properties of rat GKR

The etherase activities were measured by monitoring the formation of D-lactate in the presence of 350 μM to 1.4 mM N-acetylmuramate 6-phosphate (MurNAc6P). Assays were performed in triplicate and values shown are the means \pm standard error. n.d.; not determined.

Rat GKR	K_m (MurNAc6P) (mM)	V_{max} (MurNAc6P) (nmol.min ⁻¹ .mg ⁻¹)	K_d (F6P) (μM)	K_d (Sol6P) (μM)	K_d (Fr1P) (μM)
Wild-type ^a	0.70 \pm 0.11	44 \pm 3	20	5	1
P446L ^b	0.56 \pm 0.20	38 \pm 6	20	5	1
G107C ^a	0.73 \pm 0.07	48 \pm 2	1	0.4	29
R518Q ^b	1.36 \pm 0.64	20 \pm 5	830	80	1600
K514A ^a	2.24 \pm 0.36	13 \pm 1	>2000	n.d.	n.d.
S110A ^a	1 \pm 0.40	11 \pm 2	>2000	300	100
T109A ^a	n.d.	1.33 \pm 0.17 ^c	40	2.9	3.6

inversion of effect

^a The values of K_d (Fr6P), K_d (Sol6P), K_d (Fr1P) are from [14]
^b The values of K_d (Fr6P), K_d (Sol6P), K_d (Fr1P) are from [15]
^c Etherase activity at 1.4 mM MurNAc6P

Accepted Manuscript

Figure 1 (Veiga-da-Cunha et al.)

GKRPX1	MRGTRKYQHVIETPDPGKWE LAGYEESLP ISEKSNPMTRELDKADPSQ-LVQLLRDCDAE	59
GKRPRn	MPGTRKYQHVIETPEPEGEWELSGYEA AVPI TEKSNPLTRNLDKADA EK-IVKLLGQCDAE	59
MurQNg	-----MQRITERTNFITRDIDVVGVDVGMVRLRLRTSDAQ	34
MurQHi1	-----MNDIILKSLSTLITEQRNPNSVDIDRQSALE-IVRLMNEEDKL	42
GKRPX1	IFQEEENLIHYHRLYSESVLKTMGDVAKR VQEV LKNPD---DSL VVLSGCGTSGRLALL	116
GKRPRn	IFQEEGQIVPTYQRLYSESVLTTMLQVAGKVQEV LKEPD---GGLVVL SGGTSGRMAFL	116
MurQNg	MFCG----YEDFPSCFDEDC LGALEKISLRMKLCMQDR LNVGRVGSVVM SGGTSGRIAYF	90
MurQHi1	VP-----LAIESCLPQISLAVEQIVQAFQQ-----GGRLIYI GAGTSGRLGVL	85
GKRPX1	LANSFNGLL KGL-----HKTPCYCYIMS GGD RSIVTSQESSE DNPQLGAQELEKVCEGKK	171
GKRPRn	MSVSFNQLMKGL-----GQKPLYTYLIAGGDRSVVASREQTEDSALHGIEELK KVAAGK	171
MurQNg	CSKNYNYCFRQVFSDRIDNDEC FQFI IAGGVNALVSPQEA AEDKPDIGARDLVNII SNTK	150
MurQHi1	DASECPPTFG-----VSTEMVKGIIAGGEC AIRHPVEGAEDNTKAVLNDLQSIHFSK-	137
GKRPX1	--NVLFIGISCGLSAPFIAGQLDFCMRHLDV-YLPVLVGFNPVSMARNERIEGW HSSFRQ	228
GKRPRn	--RVVVI GIVSGLSAPFVAGQMDYCMDNTAV-FLPVLVGFNPVSMARNDPIEDWRSTFRQ	228
MurQNg	TERGLFIGITCGFSAGYVAGQVQLSEDKSLGFDSVLF GFNPKELARDSPIEGWRDCNIS	210
MurQHi1	--NDVLVGI AASGRTPYVIAGLQYAKS-----LGALTISI ASNS-----	174
GKRPX1	VAERLQTLHDSQKG----FILNPAVGP EGVSGSSRMKGG SATKILLETLLLV AHKAESN-	283
GKRPRn	VAERMQMKQEKQEA----FVLNPAIGPEGLSGSSRMKGGGATKILLETLLLA AHKTVDQ	284
MurQNg	FKDVIENVEKNQ SREDEF FFLNPIVGPESITGSTRMKGG SATKFLLDVAFSSA IMSILDE	270
MurQHi1	-----KSAMAKIAD----IAIETLVGA EVLTGSSRLKSGTAQK MVLNMLTTASMILL...	222
GKRPX1	VPVTEKCLEILRTYERAHKV TYS-----QSK-KIAALMKQTAT SLOKK-----	326
GKRPRn	VVSSQRCLLEILRTFERAHQV TYS-----QSS-KIATLMKQVGISLEKK-----	327
MurQNg	KKKIRDNI IQVLSGF EHTVRSTYDFSSNTFSTNSLKSITPTIEMA ANSLKNGLDERNED	330
MurQHi2	DRQSALEIVRLMNEEDKLVPLAIE-----SCLPQISLAVEQIVQAFQQ-----	68
GKRPX1	--GHLYILGWTLGLV GIMDAVECVPTYQADWRDVRGFI TGGYHSIENKE-----GDLSS	379
GKRPRn	--GRVHLV GQTLGIIAINDGVECIHTFGADFDIRGFLIGDHS DMFNQK-----DELTN	380
MurQNg	QMGHIIYIGAENAGILGF IDSS ECVPTYGAHPNDVRGFIKKGWNDVFTHHNQDINSDFEQ	390
MurQHi2	--GRLIYI GAGTSGRLGVL DASECPPTFGVSTEMVKGIIAGGEC AIRHPV-----EGAED	121
GKRPX1	LGPQFSISHEDFVKNVLP SVSETDTVLLIFTLD----DDL NQIEKLVALVKEKTSNIQVI	435
GKRPRn	QGPQFTFSQDDFLTSILPSL TETDTVVFIFTLD----DNLTEVQALAE RVREKQNIQAL	436
MurQNg	KGSSFGISYDFFKQNILHTVNSRDSVFFLAIEQCIDEBETLADMKEILNKV KERGASVCWI	450
MurQHi2	N-----TKAVLNDLQSIHFSKNDVLVGI AASG----RTPYVIAGLQYAKSLGALTISIA	171
GKRPX1	CHATAGQYLPNSLKKTIP SIIGLTWPLFLFLEYEGAFIQKFQ R---ELSTKWILD TVTSGA	492
GKRPRn	VHSTVGQSLPAPLKKLFPSLISITWPL LFFDYEGTYVQKFQ R---ELSTKWV LNTVSTGA	493
MurQNg	LVTTKGN-----NSLYQTVSELGYPILHHALDSLGPVPFLYGYAELSMK LFLNAITTTGA	504
MurQHi2	SNSKS-----AMAKIADIAIETLVGA EVLTGSSRLKS---GTAQK MVLNMLTTAS	218
GKRPX1	YTLRGKIFRNF MVDFKINNSKLFHRATSVLQRLTGQSQRCTEVLLQSIYGEQTLSEQIR	552
GKRPRn	HVLLGKILQNHMLDLRIAN SKLFWRALAMLQRFSGQSKARCIESLLQAIHFPQPLSDDVR	553
MurQNg	HVLKGMTFGNRMINLKVSNKLFYRAINIVSSIMNVTEELAEKCVLR AIYNDSTP-----	559
MurQHi2	MILLGKCYENLMVDVQASNEK LKARAVRIVMQATDCNKTLAEQTLLEADQNAK-----	271
GKRPX1	NTTIAGHVEAAASQDKVLPVAIVSLLRSCTIQDSRSRINSLSIRS AIESSMNVPGRKR	612
GKRPRn	AAPISCHVQVAHEKEKVIPTALLSLLRCSISEAKARLSAASSVCEVVR SALS GPGQKRS	613
MurQNg	-----ESNLVSLHISHAIKMNKVPVALLASSGGTLTVEQAREMLAKDPVV	606
MurQHi2	-----LAIMMILSTLSKSEAKVLEK HQGLRNALSK-----	303
GKRPX1	AEDSESR-----	619
GKRPRn	TQALEDPACGTLN	627
MurQNg	RQVILKEKKN----	616
MurQHi2	-----	

THIS IS NOT THE VERSION OF RECORD - see doi:10.1042/BJ20090986

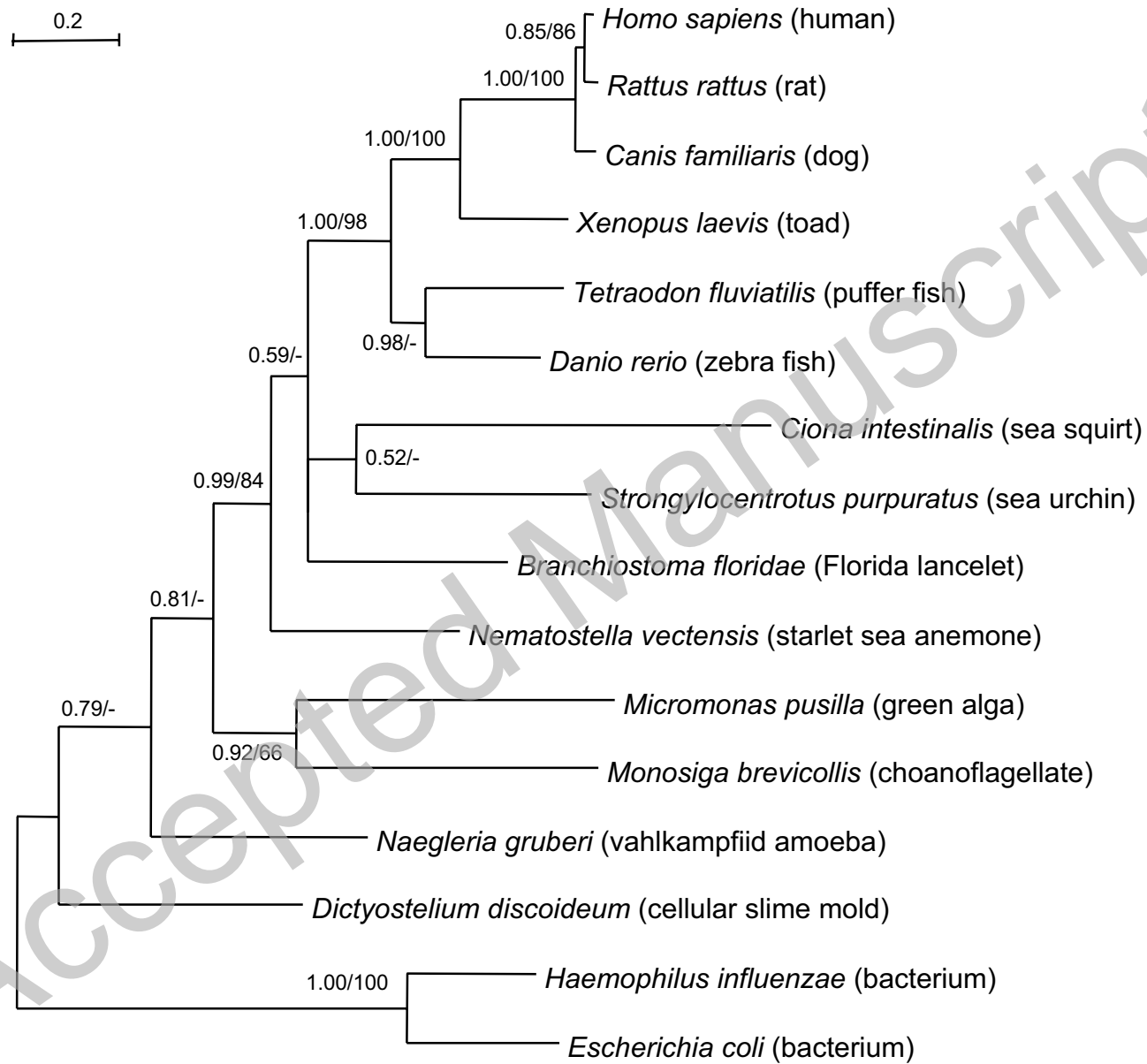


Figure 2 (Veiga-da-Cunha et al.)

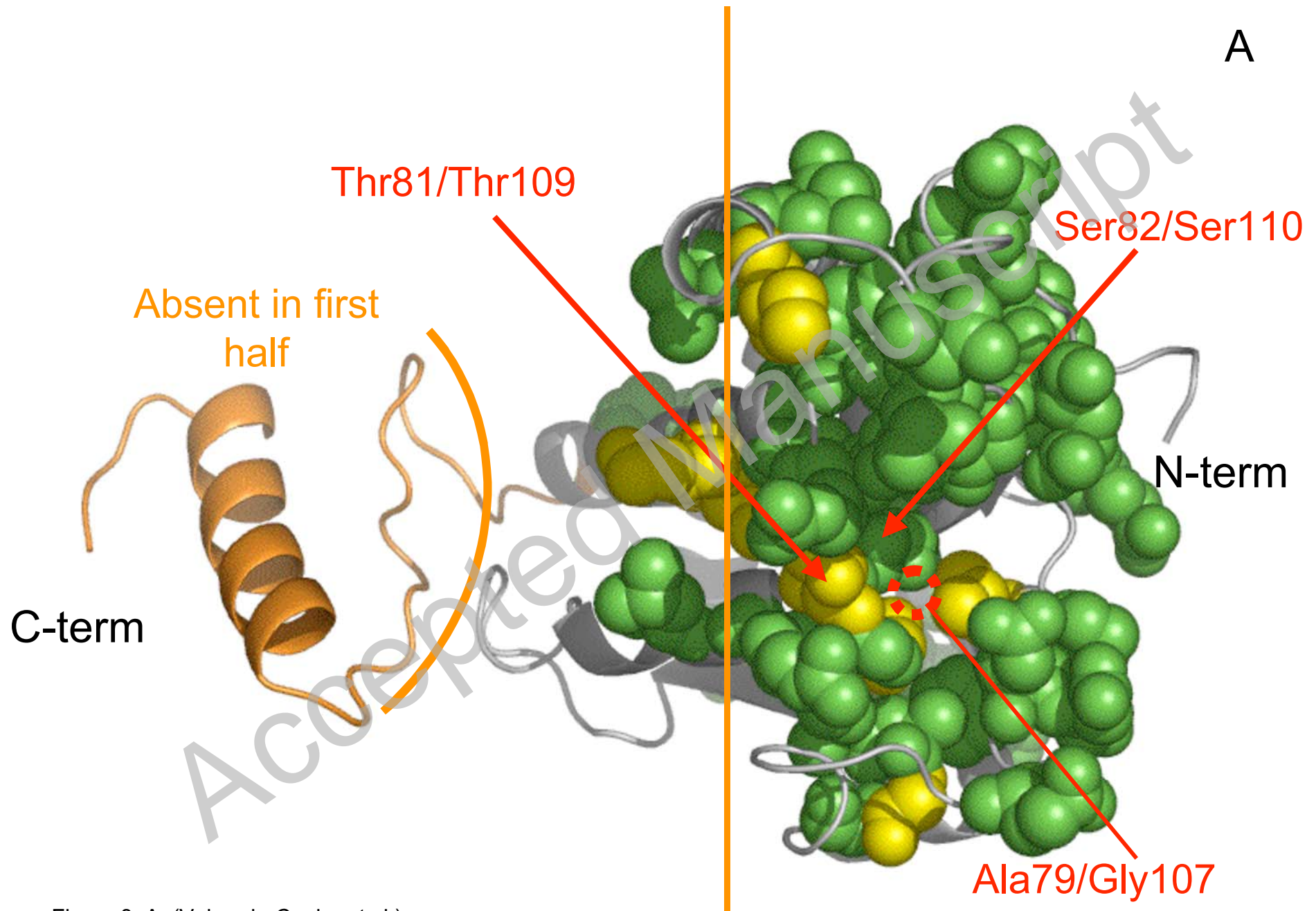


Figure 3. A (Veiga-da-Cunha et al.)

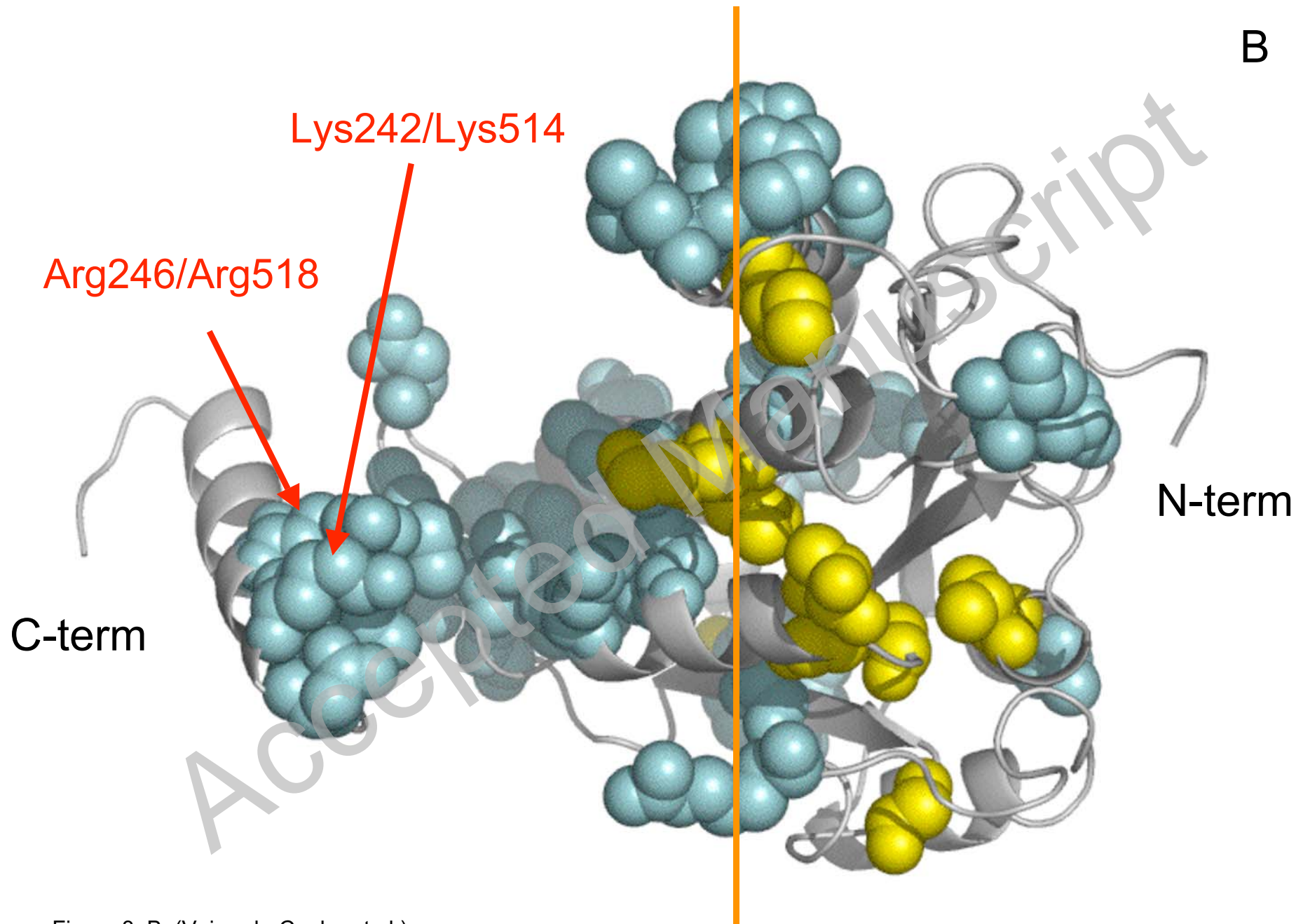


Figure 3. B (Veiga-da-Cunha et al.)

Figure 4 (Veiga-da-Cunha et al)

THIS IS NOT THE VERSION OF RECORD - see doi:10.1042/BJ20090986

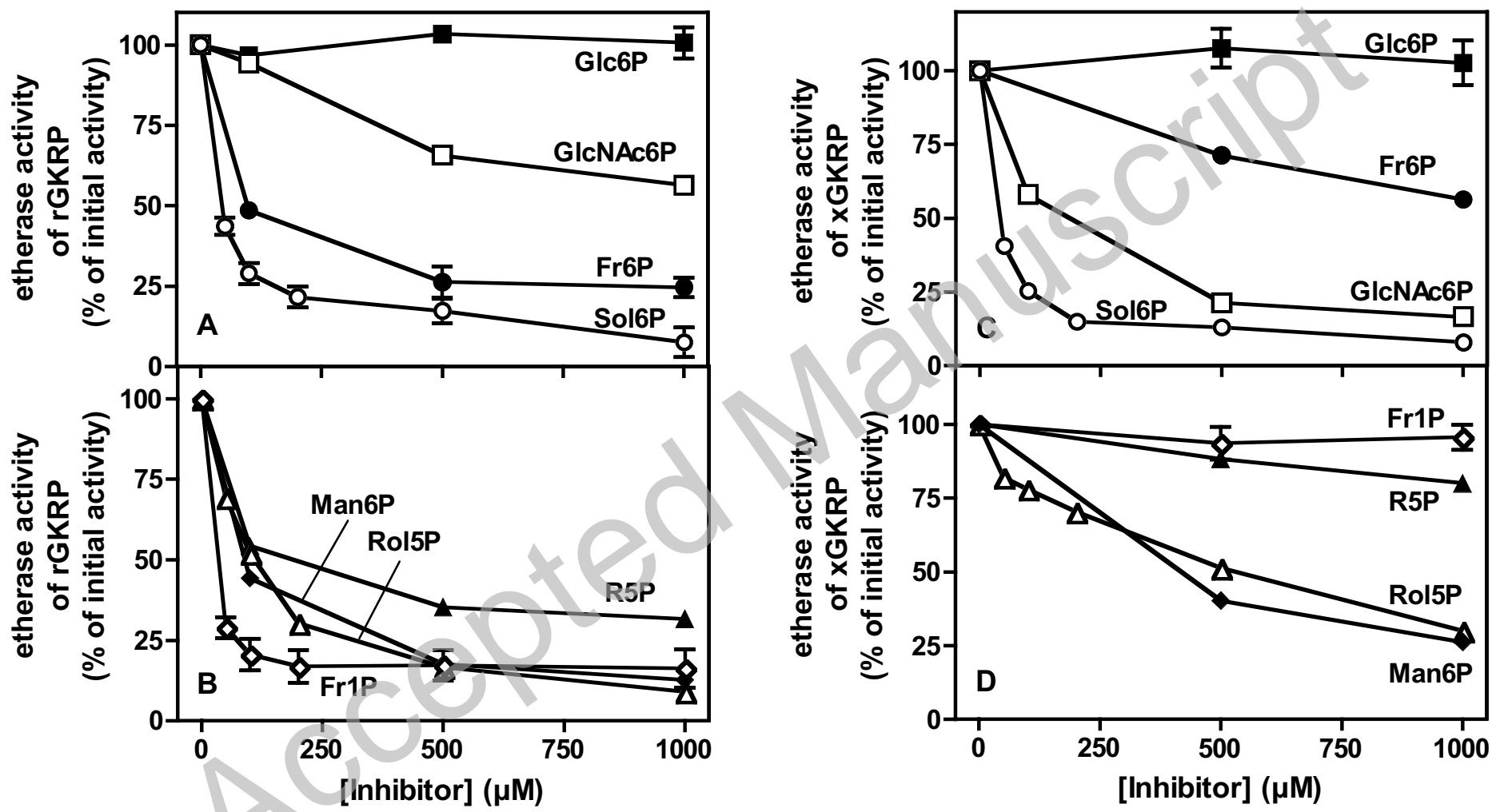


Figure 5 (Veiga-da-Cunha et al.)

THIS IS NOT THE VERSION OF RECORD - see doi:10.1042/BJ20090986

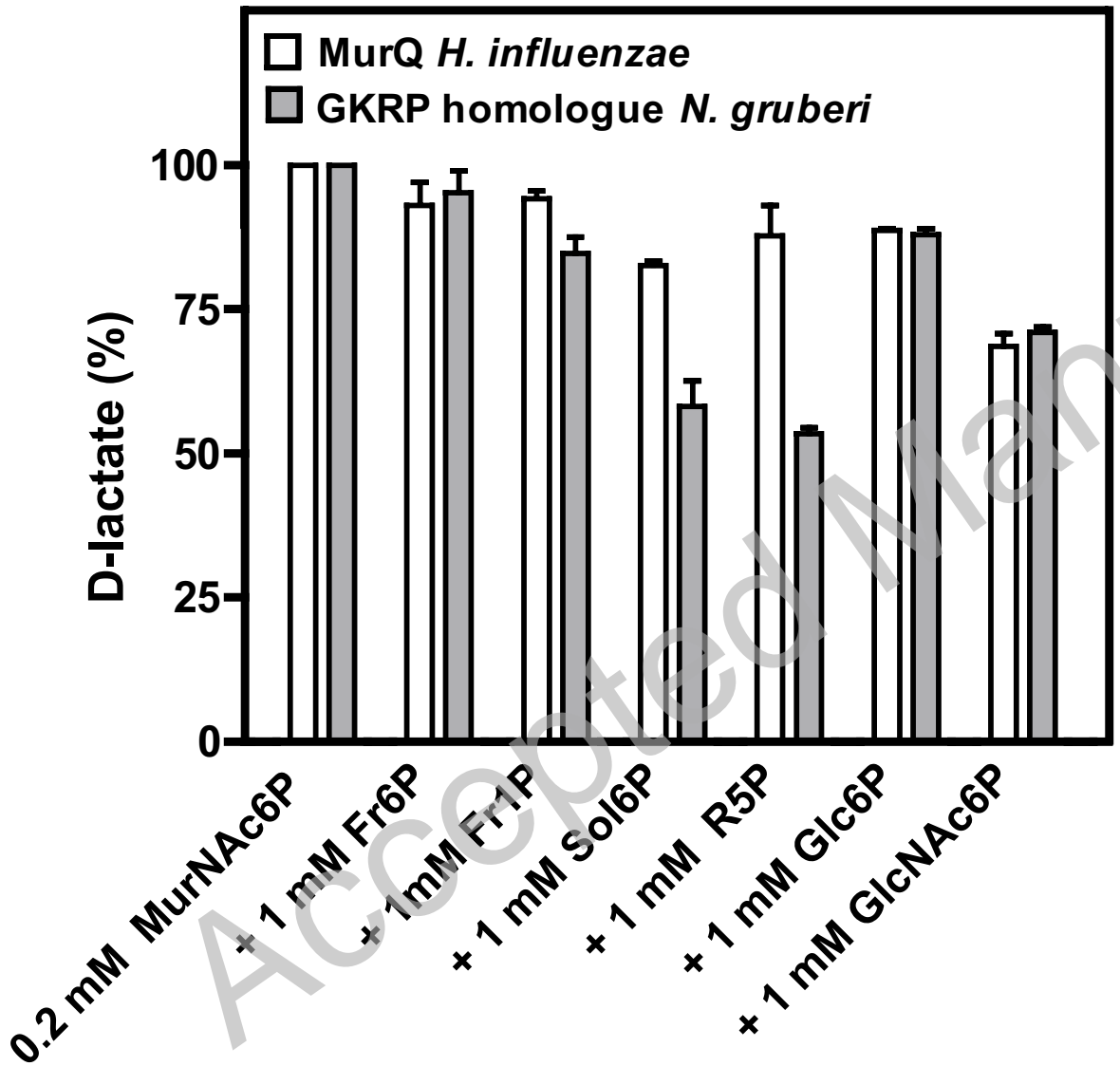
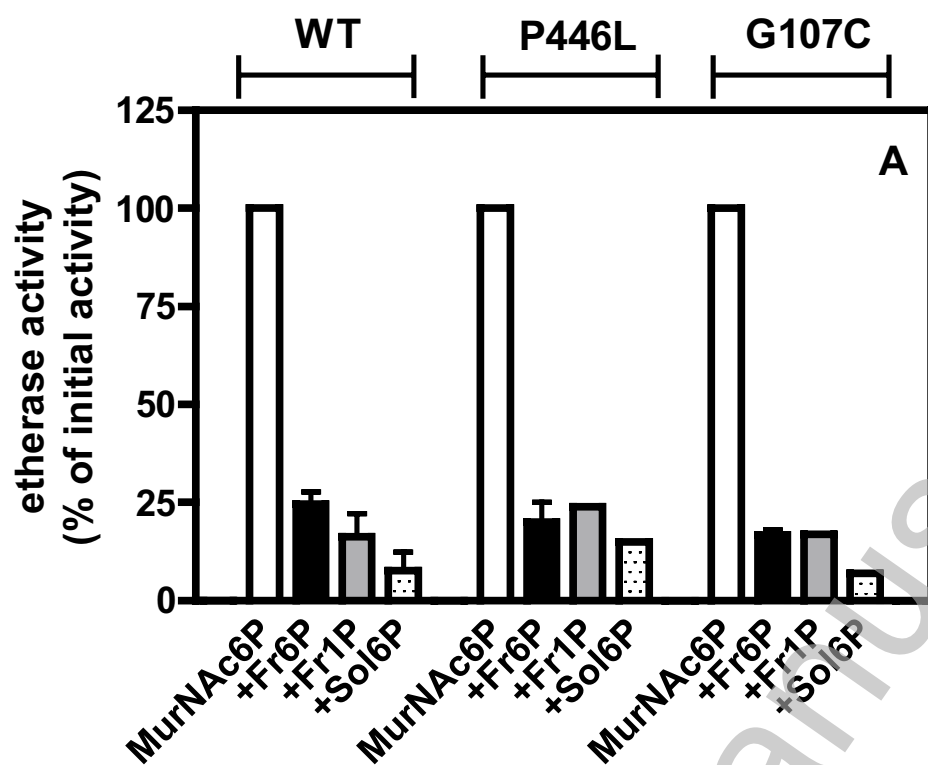


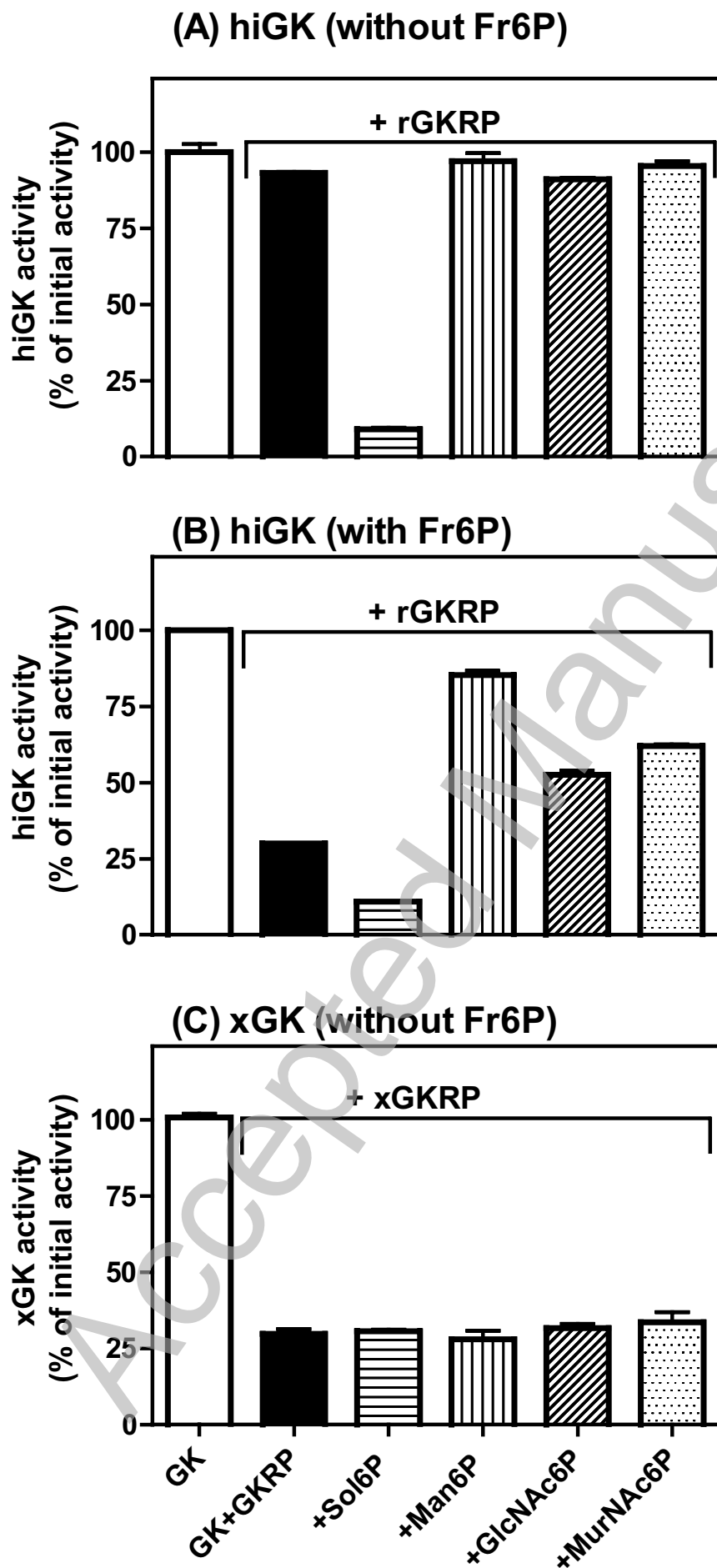
Figure 6 (Veiga-da-Cunha et al)



THIS IS NOT THE VERSION OF RECORD - see doi:10.1042/BJ20090986

Accepted Manuscript

Figure 7 (Veiga-da-Cunha et al)



THIS IS NOT THE VERSION OF RECORD - see doi:10.1042/BJ20090986

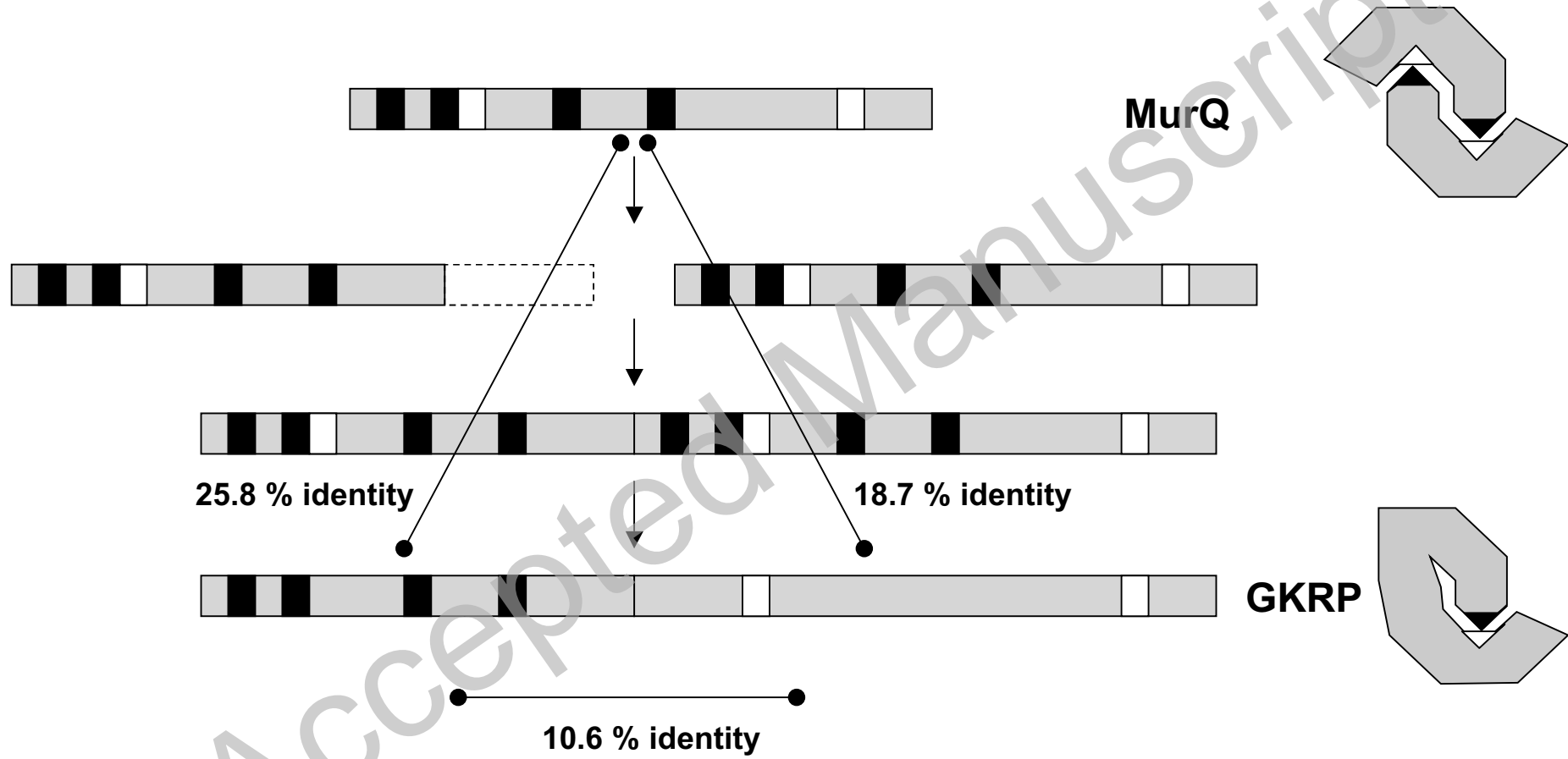


Figure 8 (Veiga-da-Cunha et al.)