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3D mapping of glycogenesis-causing mutations in the large regulatory alpha subunit of phosphorylase kinase.

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This article is dedicated to the memory of our colleague Nicolas Boisset, who passed away on January 4, 2008.

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Abbreviations: **PhK** Phosphorylase Kinase, **XLG** X-linked Liver Glycogenesis, **CBL** Calcineurin B-Like, **GH** Glycoside Hydrolase, **HCA** Hydrophobic Cluster Analysis

Abstract

Mutations in the liver isoform of the Phosphorylase Kinase (PhK) α subunit (PHKA2 gene) cause X-linked liver glycogenosis (XLG), the most frequent type of PhK deficiency (glycogen-storage disease type IX). XLG patients can be divided in two subgroups, with similar clinical features but different activity of PhK (decreased in liver and blood cells for XLG-I and low in liver but normal or enhanced in blood cells for XLG-II).

Here, we show that the PHKA2 missense mutations and small in-frame deletions/insertions are concentrated into two domains of the protein, which were recently described. In the N-terminal glucoamylase domain, mutations (principally leading to XLG-II) are clustered within the predicted glycoside-binding site, suggesting that may have a direct impact on a possible hydrolytic activity of the PhK α subunit, which remains to be demonstrated. In the C-terminal calcineurin B-like domain (domain D), mutations (principally leading to XLG-I) are clustered in a region predicted to interact with the regulatory region of the PhK catalytic subunit and in a region covering this interaction site. Altogether, these results show that PHKA2 missense mutations or small in-frame deletions/insertions may have a direct impact on the PhK α functions and provide a framework for further experimental investigation.

1. Introduction

Phosphorylase Kinase (PhK; EC 2.7.11.19) is a large hexadecameric complex made of four different subunits ($\alpha\beta\gamma\delta$)₄. It plays a key role in the regulation of glycogenolysis, by catalyzing the phosphorylation and activation of glycogen phosphorylase [1].

The catalytic activity of PhK is conferred by the γ subunit, and is modified by the phosphorylation state of the large regulatory α and β subunits, which together, account for approximately 4/5 of the mass of the holoenzyme and probably arose from gene duplication [2]. The PhK γ subunit binds an intrinsic molecule of calmodulin, which constitutes the δ subunit of the multimeric assembly. PhK has a widespread tissue distribution and the α , β and γ subunits have several tissue-specific isoforms. Some of isoforms are encoded by distinct genes, whereas other results from differential splicing of the same gene [3, 4]. Two genes (PHKA1 and PHKA2) encode the PhK α subunit, one gene (PHKB) the PhK β subunit, two genes (PHKG1 and PHKG2) the PhK γ subunit, whereas three genes (CALM1, CALM2 and CALM3) encode for identical calmodulin proteins (δ subunit).

Deficiency of PhK results in glycogen-storage disease (GSD) type IX. It is the most frequent inherited disorder of glycogen metabolism, accounting for approximately 25 % of all cases of GSDs (approximately 1/100,000 births) [4]. Mutations of the liver isoforms of PhK α or γ subunits (PHKA2 and PHKG2 genes) cause liver-specific glycogenoses [3, 5, 6]. X-linked liver glycogenosis (XLG) is the most frequent type of PhK deficiency [7] and is caused by mutations in the PhK α L isoform (PHKA2 gene) [3, 5]. Mutations in the PhK α M isoform (PHKA1 gene) cause X-linked muscle specific PhK deficiency [8-11], whereas mutations in the ubiquitously expressed PhK β subunit (PHKB gene) cause combined liver and muscle PhK deficiency [12, 13].

XLG (caused by mutations in the *PHKA2* gene) can be divided in two subtypes, XLG-I and XLG-II, with patients having very similar clinical symptoms (hepatomegaly and growth retardation during childhood) but different *in vitro* enzyme deficiency. Patients with XLG-I have indeed a deficiency in PhK activity in peripheral blood cells and liver [7], whereas patients with XLG-II have normal PhK activity in peripheral blood cells and variable activity in liver [14]. Interestingly, it has been suggested that most of the XLG-I mutations, principally inducing truncation or disruption of the protein, may lead to an absence of the α subunit and/or to an unstable PhK complex. In contrast, XLG-II mutations, which are missense mutations or small in frame deletions and insertions, may directly affect the function of the α subunit and lead to *in vivo* deregulation of the holoenzyme [3, 15] (**Table 1**).

In the past, the sequences of the PhK α and β subunits have not provided much insight into their possible functions. However, a few years ago, Pallen predicted that their N-terminal extremities correspond to glucoamylase-like domains (family 15 of glycoside hydrolases [16]), suggesting a previously overlooked amylase activity [17], which however still remains to be experimentally demonstrated. More recently, we reported that the C-terminal domains (that we called domains C and D) of PhK α and β can be related to calcineurin B-like (CBL) proteins [18]. These proteins, which are members of the EF hand family, are involved in the regulation of kinases of the CIPK/PKS family, and relieve autoinhibition of their target kinases by binding to their regulatory region [19]. The prediction that we made of CBL-like domains in the large regulatory subunits of PhK provided new perspectives for understanding the mechanism by which the “CBL-like” domain D of PhK α might regulate the activity of the γ subunit, through a direct interaction with its regulatory C-terminal region [20].

Interestingly, missense mutations (and small in-frame deletions/insertions) affecting the PHKA2 gene are concentrated in either the GH15-like domain and the CBL-like domain D of the protein (**Table 1**). In this study, we considered 3D structure models of these two domains of PhK α , in order to predict the potential impact that these mutations may have on its structure and/or function(s). The 3D model of the GH15-like domain of PhK α was constructed on the basis on a refined alignment that we made here of the PhK α sequence with a bacterial glucoamylase template, whereas the 3D model of the PhK α CBL-like domain D originated from our previous analysis [18].

2. Material and Methods

Models of the three-dimensional structures of the PhK α and β GH15-like domains and CBL-like domains D were constructed as described in **Supplementary data 1**. Three-dimensional structures were visualized using Swiss-PdbViewer [21].

3. Results and discussion

3.1. *The GH15-like domain of PhK α and β : Modeling and analysis of the putative active sites.*

The GH15-like domains of the PhK α and β subunits were modeled on the basis of the alignment shown in **Figure 1**, using the experimental structure of the glucoamylase from *Clostridium thermosaccharolyticum* as template (pdb 1LF9, see **Supplementary data 1** for details of the procedure). Glucoamylases adopt a characteristic $(\alpha/\alpha)_6$ barrel structure (also known as six-helical hairpin toroids) [22] (**Figure 2**). Helical hairpins are arranged into a two-layered toroid (with helices of the inner and outer layers designated, in **Figure 1** and **2**, “int” and “ext”, respectively). Long loops connect external (outer) with internal (inner) helices, forming at one end of the toroid a narrow pocket in which the substrate binds. In contrast,

connecting loops on the other end of the toroid (thus connecting internal with external helices) are relatively short, and do not protrude from the toroid core. GH15-like domains of the PhK α and β subunits share these features, with a particular good sequence conservation of loops connecting external with internal helices, forming the walls of the active site (**Figure 1**). Noteworthy is that the small β -strands or extended regions existing in these loops are particularly well conserved. Only the loop linking helix ext1 with helix int1 is smaller in the PhK α and β subunits than in the 1LF9 template (**Figure 1**). The loop linking helix int3 with helix ext4 is also smaller (with the two helices being probably smaller), but this region, which is the most difficult to align with accuracy, is located opposite to the predicted active site (**Figure 1**).

In glucoamylases, two acidic residues (E438 and E636 in 1LF9) directly participate in the hydrolysis of the glycosidic bond of the non-reducing end of polysaccharides through a general acid-base mechanism [16]. As shown on **Figure 2** and **Table 2**, the GH15-like domain of the PhK α subunit conserves not only these two catalytic residues, but also most of the residues contacting the substrate in the active site (black circles up to the 1LF9 sequence on **Figure 1**), among which several aromatic amino acids can be found. This suggests, as already hypothesized by Pallen [17], that this domain might have a catalytic activity, even though to our knowledge, this activity has not yet been experimentally demonstrated. It is interesting to note that the aromaticity of the substrate-binding pocket is likely increased in the PHK α and β GH15-like domains, as three residues of the bacterial glucoamylase template (1LF9 G392, L602 and E605) are substituted by aromatic residues (H132, F331 and Y334 in PHK α ; H169, F368 and Y371 in PhK β ; highlighted with the symbol “+” in **Figure 1** and colored salmon in **Figure 2**). The glucoamylase (1LF9) L652, which directly contacts the substrate (**Figure 2** and **Table 2**), is also substituted by an aromatic residue in PhK (H400 in PHK α and F401 in PhK β).

In contrast to PhK α , the GH15-like domain of the PhK β subunit lacks some of the active site residues, in particular a glutamic acid (E), which would assist the hydrolysis by acting as a base. This one is replaced in PhK β by a lysine (K408, **Figures 1 and 2, Table 2**). These features suggest that the PhK β subunit might be devoid of catalytic activity and thereby, might rather play a regulatory role relative to the PhK α subunit.

3.2. Mapping of disease-causing mutations (missense and small in-frame deletions/insertions)

Most of the missense mutations (amino acid substitutions) and small deletions (del), duplications (dup) and insertions (ins) in the PHKA2 gene are concentrated within the GH15-like domain and the CBL-like domain D (**Table 1**). Indeed, only four missense mutations and an in-frame deletion of eight amino acids were described outside these domains.

3.2.1. The GH15-like domain of PhK α

We mapped the known PHKA2 missense mutations in the GH15-like domain (**Table 1**) on the corresponding 3D model (**Figures 1** (black stars) and **3**). Remarkably, all of them are located in the long loops connecting the external with the internal helices, and are located in close proximity, if not corresponding to the residues contacting the substrate in the predicted active site. Indeed, R186 and R295 are aligned with 1LF9 E439 and R575 (**Table 2, Figures 1 and 3**), whose side chain atoms make close contacts with acarbose in the experimental structure of glucoamylases [22]. C91Y/Y116D/ H132, K189/G193, D299 and P399 are located in close proximity of the substrate-binding pocket residues of the ext2-int2, ext3-int3, ext5-int5 and ext6-int6 loops, respectively (**Figure 3**). H132 corresponds to one of the « new » aromatic residue of the substrate-binding pocket (see before) and is located two residues downstream the 1LF9 W390/PhK α W130 (**Figure 1**).

Thus, most of the missense mutations observed in the GH15-like domain of PhK α lie within its predicted substrate-binding site. Only C91Y, Y116D and P399 are located a little farther (**Figure 3**). However, one might expect that the mutation of P399 in a serine would lead to a local perturbation of the conformation of the loop and consequently of the two residues located one and three positions downstream (H400 and W402), which directly contact the substrate. C91 and Y166 are also located farther, but always make part of the large surface displayed at one end of the α/α barrel core. Thus, they may be also involved in substrate recognition, although they are more distant from the active site. In this respect, it is worth noting that the substitutions of C91, Y116 and P399 are the missense mutations in the GH15-like domain that are exclusively associated with XLG-I (and not XLG-II; R295H being associated with XLG-I and XLG-II). This suggests that in contrast to other mutations which might impair the predicted domain function, these three last mutations might rather affect its structure.

Two small deletions of one and two amino acids (T251del [15] and K189-T190del [3], respectively) and a duplication (Y116-T120dup [23]), are also associated with XLG-II, in addition to the most of the missense mutations reported above. The two amino acid deletion involves one amino acid (K189), which is also the target of a missense mutation (see above). Interestingly, Y116-T120 and T251 are located in the ext2-int2 and ext4-int4 loop, respectively, thus also within the predicted substrate-binding pocket (**Figure 3**). In contrast, the deletion of F141 is associated with XLG-I. Accordingly, this amino acid is localized buried with helix int2 (**Figures 1 and 3**), and it is likely that its mutation impairs the folding or the fold stability of the GH15-like domain, rather than its predicted function.

Finally, we completed this mutation mapping by reporting on the 3D models the missense mutations observed in PHKA1 (X-linked muscle-specific PhK deficiency). In contrast to

PHKA2, only a few mutations have been reported for PHKA1. There is to our knowledge only two missense mutations in PHKA1, corresponding to the D299V [9] and G223R [24] substitutions. Interestingly, the mutation of the same D299 in glycine in PHKA2 leads to XLG-II [25] (see above). G223 is also located in the long loops connecting the external with the internal helices, but farther from the substrate-binding site (**Supplementary data 2, left view**).

In PHKB (autosomal recessive PhK deficiency of liver and muscle), there is, to our knowledge, only two missense mutations (A118P and M185I) in the GH15-like domain [12, 23]. Interestingly, both residues are located in close proximity, buried within the $(\alpha/\alpha)_6$ toroid core of the PhK β GH15 model (A118 within helix ext2, M185 at the end of helix int2, **Supplementary data 2, right view**). This observation suggests that mutation of these amino acids would disturb the folding and stability of the domain. Thus, in contrast to what is observed for PHKA1 and PHKA2, these PHKB missense mutations do not likely directly affect a potential function of this subunit, but rather its structure.

3.3.2 The CBL-like domain of PhK α

The six missense mutations that have been reported in the CBL-like domain D of PHKA2 principally lead to XLG-I (**Table 1**). This contrasts with missense mutations associated with the GH15-like domains, which mainly lead to XLG-II. Interestingly, the mapping of the six mutated amino acids on the 3D models we previously made of the CBL-like domain D [18] led to observe that they are also clustered in a same region, making part of the hydrophobic groove (M1113, T1114, E1125) and in the C-terminal extension shielding the hydrophobic groove (P1205, G1207, G1210) (**Figure 4**). The in-frame insertion of two amino acids (R1111_E1112insTR), as well as and the trinucleotide deletion leading to the replacement of two amino acids by one new residue (L953-N954I) also occur within the hydrophobic groove

(**Figure 4**). By analogy with the known CIPK/CBL system [26-28], this hydrophobic groove might be able to interact with the regulatory domain of the kinase subunit (γ subunit), which would take the place occupied in the uncomplexed state by the C-terminal extension of the domain. It is thus likely that mutations in this hydrophobic groove or in the C-terminal extension covering this groove when not interacting with the γ subunit, dramatically impair the interaction with the kinase and thus the PhK function. The small in-frame deletion of one amino acid (Δ R1070, Table 1) lies outside this active site, but it is likely that this deletion alters either the domain fold (as R1070 is located within a regular secondary structure (helix F1[18])) or the function of the large insertion included in this domain and containing the multiphosphorylation sites.

3.2.3 The other domains of PhK α

Out of the remaining mutations (Table 1), two missense mutations (P869R, R916W) and the eight amino acid deletion (Q818_Y825del) are located into domain C, which was predicted to also have a CBL-like architecture [18]. The modeling of this domain is however more difficult to perform given the very low levels of sequence identities with domain D and with CBL proteins. Sequence analysis however predicted that P869 and R916 are included within helices (buried in helix E3 and solvent exposed in helix E4, respectively), without being involved in a hydrophobic groove, as for domain D (data not shown). The eight amino acid deletion (Q818_Y825del) might lead to the absence of helix F1 (data not shown), and thus to a disrupted domain structure. The two last missense mutations (P498L, I566N) are included in domain B, which remains to be deciphered.

3.3. Conclusions

In contrast to XLG-I mutations, which result in a truncated or disrupted α subunit and/or in an unstable holoenzyme, XLG-II mutations are thought to have a minor impact on the structure of the protein. It has been hypothesized that several of them may be involved in the regulation of PhK by phosphorylation of the α subunit [15]. Although such an hypothesis can not be completely ruled out, we clearly show here that the known missense mutations leading to XLG-II are principally clustered into the predicted active site of the PhK α GH15-like domain. They thus might have a direct impact on the predicted hydrolytic activity of the α subunit, which however remains to be demonstrated. The likelihood of such an activity is otherwise reinforced by the present mapping of glycogenesis-inducing mutations. Missense mutations associated with the PhK α CBL-like domain D, which principally lead to XLG-I, are clustered in the region predicted to interact with the regulatory domain of the catalytic subunit and in a region covering this interaction site. Therefore, they probably lead to an unstable or less regulated holoenzyme, as the α - γ interaction has been suggested to modulate and/or increase the affinity of the catalytic subunit for calmodulin [18].

Altogether, these findings open new perspectives for understanding the molecular mechanism(s) by which the PhK α subunit may regulate the holoenzyme activity and may lead, when mutated, to PhK deficiencies.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at....

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Legends to tables and figures

Table 1: Known missense mutations or small deletions/insertions in PhK α L subunit (*PHKA2* gene), which lead to XLG-I and/or XLG-II. The small in-frame deletions (del), insertions (ins) and duplications (dup) are indicated in italics.

Table 2: Amino acid residues contacting the substrate in the active site of GH15 (1LF9) and in the potential active site of GH15-like (PhK α and β) domains. The GH15 experimental structure (pdb 1LF9) has been solved in complex with acarbose, a potent glucoamylase inhibitor. The residues contacting the substrate are indicated with a black circle up to the 1LF9 sequence in Figure 1. The glutamic acids (E) acting (or predicted to act) as the catalytic acid (ext3-int3) and base (ext6-int6) are underlined.

Figure 1: Alignment of the sequences of the GH15-like domains of the PhK α and β subunits (*PHKA1* (KPBB1), *PHKA2* (KPBB2) and *PHKB* (KPBB)) genes) with the GH15 domain of the *Clostridium thermosaccharolyticum* glucoamylase, whose 3D structure is known (pdb 1LF9, [22]). The observed secondary structures of the glucoamylase are reported up to its sequence, with external and internal helices labeled « ext » and « int », respectively. This alignment was refined using Hydrophobic Cluster Analysis (HCA), as described in the **Supplementary data 1**. Sequences identities are shown white on a black background, whereas similarities between hydrophobic amino acids (VILFMYWW) or amino acids which can substitute them in a context-dependent way (ACTS for aliphatic residues, or H for aromatic residues) are shaded grey. Other similarities are boxed. Amino acids participating in the 1LF9 glucoamylase active site are numbered and highlighted with black circles. Amino acids mutated in the *PHKA2* gene are numbered and highlighted with black

stars. G223 and D299 mutated in the PHKA1 gene (G223R and D299V, see text) are indicated with grey stars, whereas the amino acid substitutions in the PHKB gene are indicated with a white stars. Small deletions and an insertion (duplication) in the PHKA2 gene are underlined and highlighted with pentagons.

Figure 2: The active site of GH15 and predicted active-sites of GH15-like domains (PhK α and β subunits). On top are shown two orthogonal views of the 3D structure of the *Clostridium thermosaccharolyticum* glucoamylase GH15 domain (ribbon representation pdb 1LF9, [22]), in complex with acarbose (in green). Amino acids participating in the glucoamylase active site are shown in atomic details. At bottom is shown the comparison of the active sites of the experimental structure of the *Clostridium thermosaccharolyticum* glucoamylase GH15 domain (1LF9) with the predicted ones in the 3D models of the PhK α (liver isoform, PHKA2 gene) and β (PHKB gene) GH15-like domains. The two catalytic glutamate residues are depicted in red, and the acarbose molecule in green. Amino acids that are not strictly conserved in PhK α or β relative to 1LF9 are underlined and labeled in purple. The four aromatic residues increasing the aromatic character of the predicted substrate-binding site of PhK α and β are colored salmon.

Figure 3: Missense mutations and small in-frame deletions/duplication within the GH15-like domain of the PhK α subunit (PHKA2 gene). Top: the predicted active site is viewed in the same orientation as in figure 2, with the active site residues depicted in atomic details. Amino acids that are the targets of missense mutations or small in-frame deletions (T251del, F141del) are labeled and shown in a van der Waals representation. The position of Y116-T122 duplication (dup) is shown as a purple ribbon. Bottom: two orthogonal views of the location of the same missense mutations or small in-frame deletions within the context of the whole domain (shown in a ribbon representation).

Figure 4: Missense mutations and small in-frame deletions/insertion within the CBL-like domain D of the PhK α subunit (PHKA2 gene). Molecular surface of the model of the CBL-like domain D of PhK α , at the exception of the C-terminal extension of this domain, shown in a ribbon representation (green). This C-terminal extension shields the hydrophobic groove, which likely interacts with the regulatory domain of the target kinase (the PhK γ

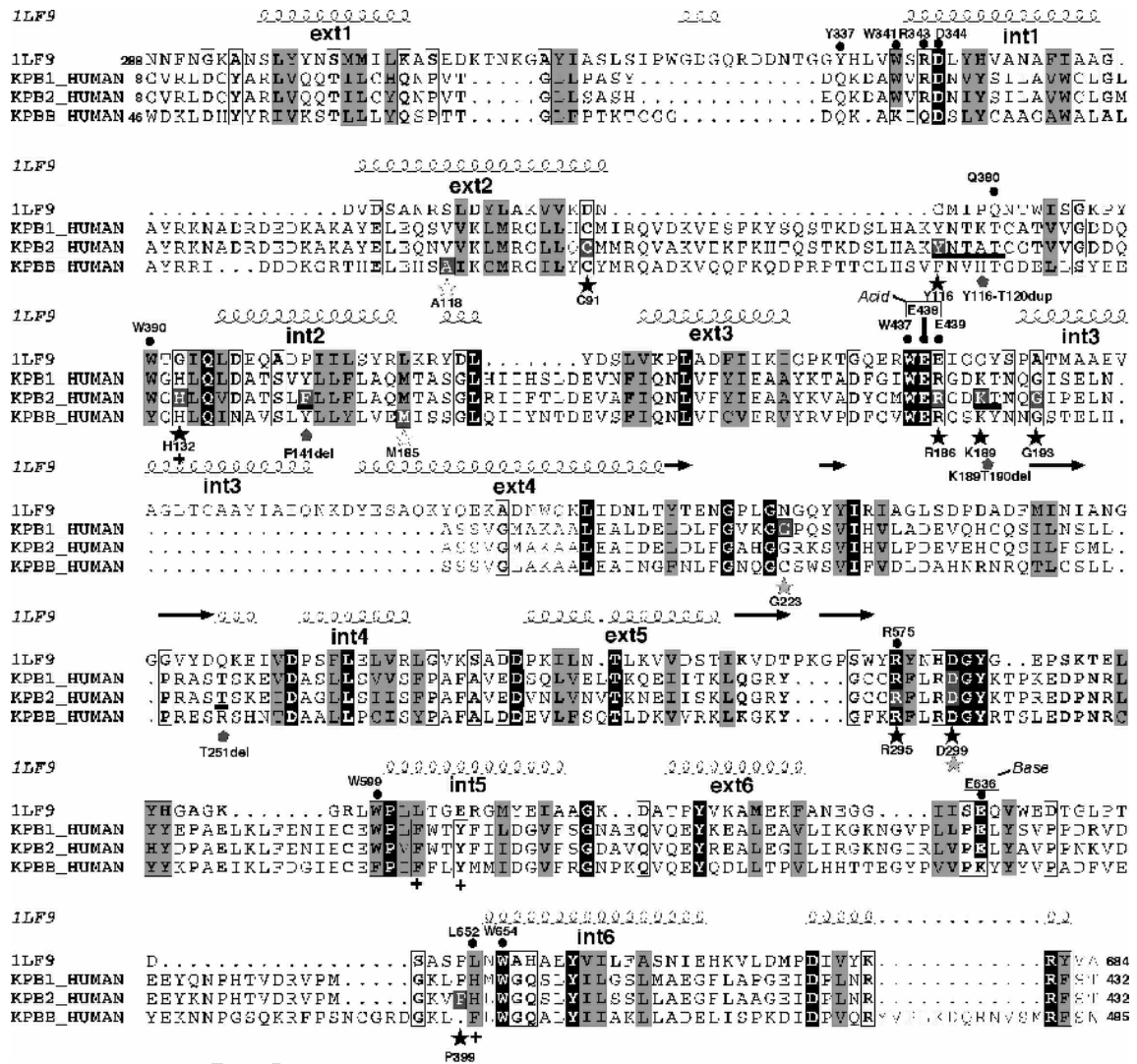
subunit). The six known missense mutations are colored red (the three located in the groove) and orange (the three lying in the C-terminal extension). The solvent accessible surface of R1111, after which a two amino acid insertion occurs (R1111_E1112insTR), and of N953/L954, which are replaced by an isoleucine after a trinucleotide deletion (N953/L954I) are shown in pink. The side chain of R1070 (R1070del) is shown in blue. The large insertion occurring between helices α B and α C, which has not been modeled, is symbolized with a star.

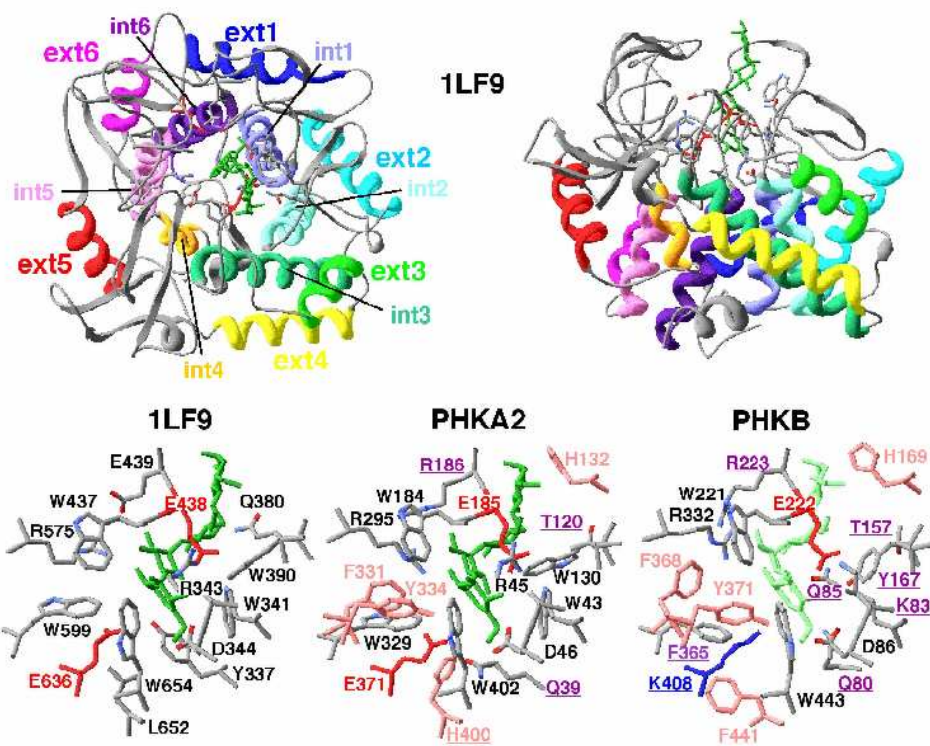
Table 1

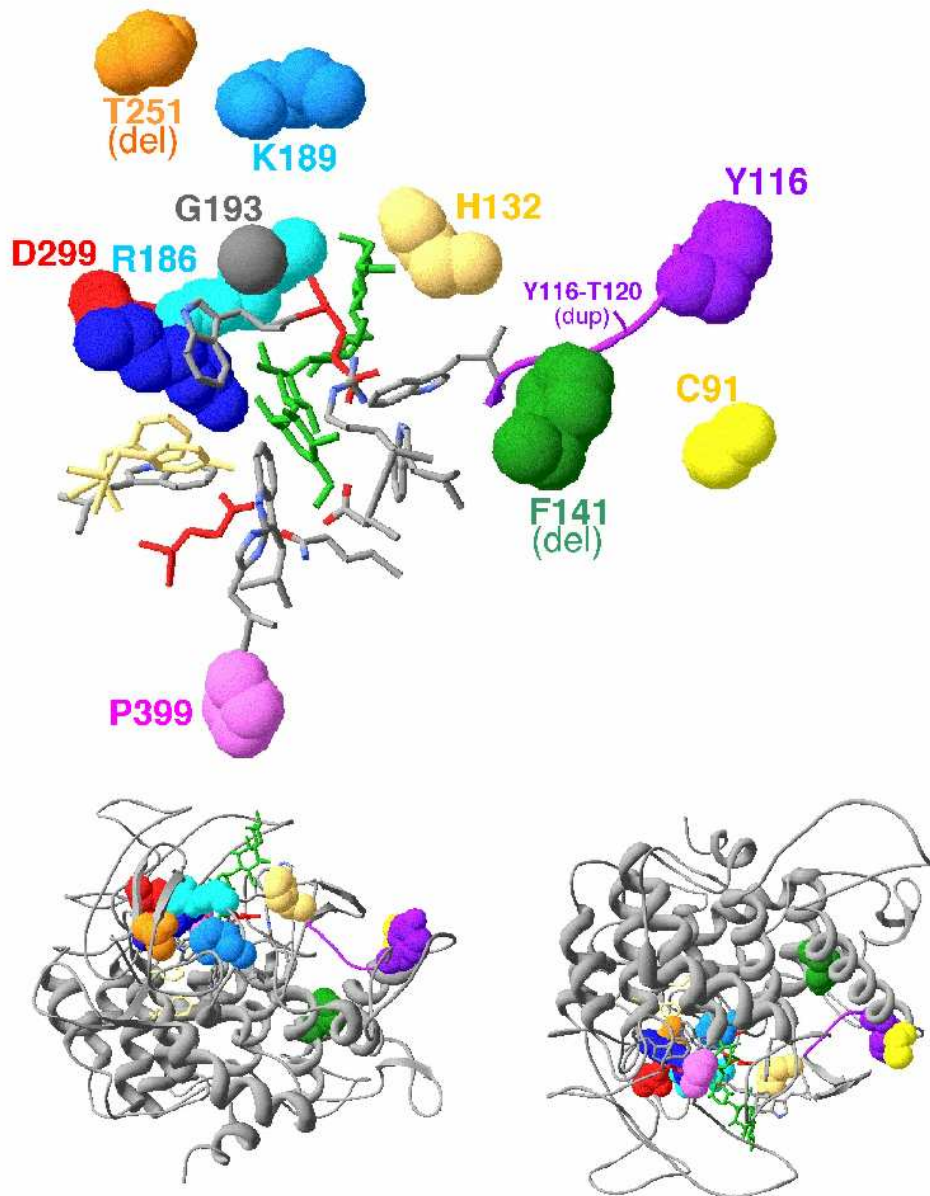
Missense mutation	Type of XLG	Reference
GH15-like domain		
C91Y	XLG-I	[29]
Y116D	XLG-I	[30]
<i>Y116-T120dup</i>	<i>XLG-II</i>	<i>[23]</i>
H132Y	XLG-II	[25]
H132P	XLG-II	[25]
<i>F141del</i>	<i>XLG-I</i>	<i>[31]</i>
R186C	XLG-II	[3, 15]
R186H	XLG-II	[25]
K189E	XLG-II	[5]
<i>K189_T190del</i>	<i>XLG-II</i>	<i>[3]</i>
G193V	XLG-II	[32]
<i>T251del</i>	<i>XLG-II</i>	<i>[15]</i>
R295H	XLG-I/ XLG-II	[3]
R295C	XLG-II	[33]
D299G	XLG-II	[25]
P399S	XLG-I	[5]
CBL-like domain D		
<i>N953/L954I</i>	<i>XLG-I</i>	<i>[5]</i>
<i>R1070del</i>	<i>XLG-I</i>	<i>[23]</i>
<i>R1111_E1112insTR</i>	<i>XLG-II</i>	<i>[15]</i>
M1113I	XLG-I	[23]
T1114I	XLG-II	[15]
E1125K	XLG-I	[3]
P1205L	XLG-I	[25, 30, 31]
G1207W	XLG-I	[5]
G1210E	XLG-I	[29]
Other domains		
P498L	XLG-II	[23]
I566N	XLG-I	[34]
<i>Q818_Y825del8</i>	<i>XLG-I</i>	<i>[5]</i>
P869R	XLG-I	[23]
R916W	XLG-I	[23]

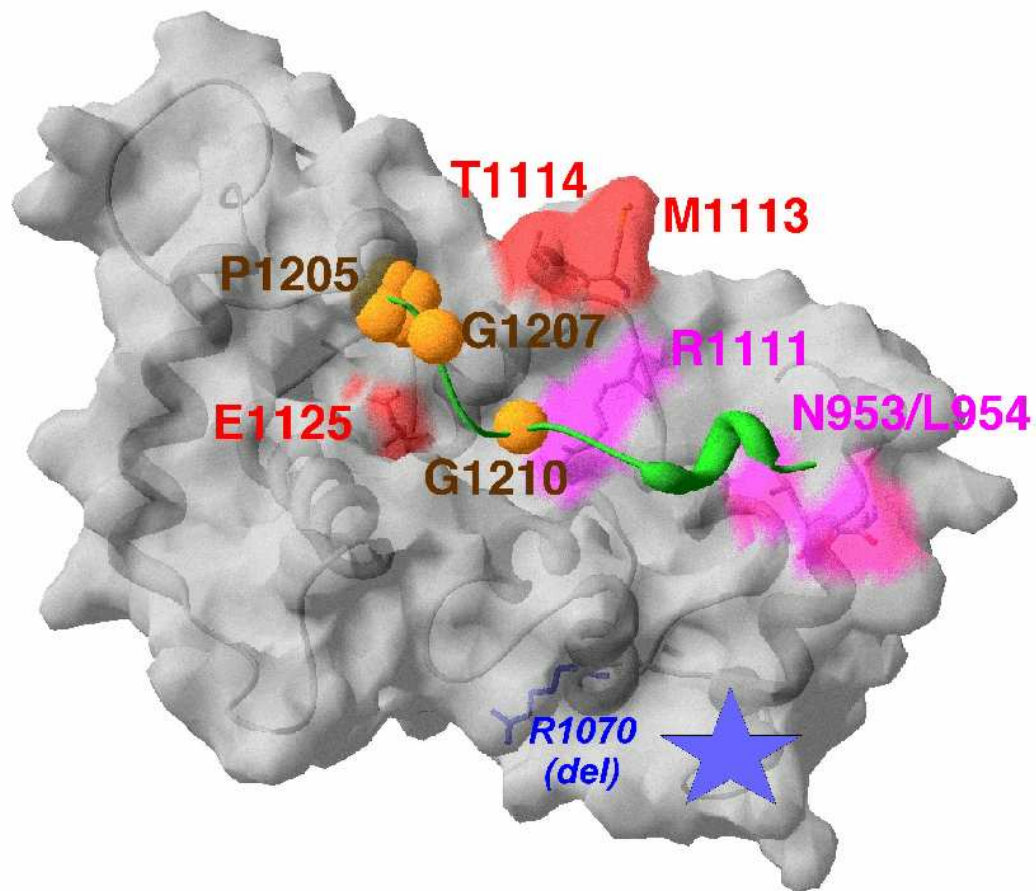
Table 2

	1LF9	PHKA1	PHKA2	PHKB
Ext1-Int1	Y337	Q39	Q39	Q80
	W341	W43	W43	K83
	R343	R45	R45	Q85
	D344	D46	D46	D86
Ext2-Int2	Q380	T120	T120	T157
	W390	W130	W130	Y167
Ext3-Int3	W437	W184	W184	W221
	<u>E438</u>	<u>E185</u>	<u>E185</u>	<u>E222</u>
	E439	R186	R186	R223
Ext5-Int5	R575	R295	R295	R332
	W599	W329	W329	F365
Ext6-Int6	<u>E636</u>	<u>E371</u>	<u>E371</u>	K408
	L652	H400	H400	F441
	W654	W402	W402	W443









ACCEPT