INVESTIGATION OF THE MOLECULAR BASIS OF PATIENT DERIVED MONOCLONAL ANTIBODY INTERACTIONS WITH E2 & E3BP LIPOYL DOMAINS OF THE HUMAN PYRUVATE DEHYDROGENASE COMPLEX (HPDC)

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Human pyruvate dehydrogenase multi-enzyme complex (hPDC) catalyses the oxidative decarboxylation of pyruvate, transferring the resultant acetyl group to coenzyme A. It belongs to the family of 2-oxoacid dehydrogenase complexes (2-OADCs) that includes the 2-oxoglutarate dehydrogenase (hOGDC) and branched-chain 2-oxoacid dehydrogenase complexes (hBCOADC). Each assembly consists of multiple copies of three distinct component enzymes termed E1, E2 and E3. HPDC also contains an accessory subunit (E3BP) that mediates stable E3 integration into the E2 'core' of the complex. E2-hPDC consists of the following domains: two tandemly-repeated, amino-terminal lipoyl domains (LDs), an inner lipoyl domain (ILD) and outer lipoyl domain (OLD) to each of which the lipoic acid cofactor is attached covalently in amide linkage via a specific lysine residue; an E1-binding domain and a carboxy-terminal catalytic core domain. (1).

Primary biliary cirrhosis (PBC) is a chronic autoimmune liver disease in which inflammatory infiltration of the intrahepatic bile ducts leads to damage of the biliary epithelial cells followed by fibrosis, cirrhosis and ultimately liver failure. The disease is characterised by the presence of antimitochondrial antibodies (AMA), the production of which occurs at a very early stage of the disease. The major mitochondrial autoantigens have been identified as key constituents of the 2-OADCs, primarily the E2 and E3BP subunits of hPDC. AMA to these polypeptides are present in the serum of more than 95% of patients with PBC (2).

PD1 and PD2 are monoclonal antibodies (mAbs) secreted by individual patient-derived hybridomas (IgG/λ) that interact with a common lipoylation-dependent epitope found on both E2 and E3BP-LDs although the precise antigenic determinant has not been fully defined to date (3,4). These mAbs appear to be representative of the PBC-specific polyclonal antibody population as a whole; therefore, the aim of this study were targeted at determining the precise molecular basis for recognition of E2 and E3BP-LDs of h PDC by mAbs, PD1 and PD2, which is of interest in terms of the aetiology of the disease. To address this issue, three amino acid residues adjacent to the lipoylated lysine residue of the ILD-PDC were mutated systematically to the equivalent residues found in the non-reactive LD

of *Arabidopsis thaliana* plastid E2-PDC, Atpt LD-PDC in an initial attempt to define Ab recognition site of the hILD-PDC. In parallel, the non-reactive LD of hOGDC was mutated at several amino acids around the lipoylated lysine residue to the equivalent residues found in the reactive LDs of E2 and E3BP-hPDC in attempts to restore the level of Ab recognition to that of the hILD-PDC.

Cross reactivity and lipoylation states of wild type (wt) hILD-PDC and its mutant K173Q in the presence and absence of exogenous lipoate

Initially, it was confirmed that the denaturing conditions did not affect the specificity or inhibit the cross reactivity of the antibody as PD1 and PD2 were able to recognize hILD-E2 constructs analysed on SDS-PAGE. The current study also verified previous studies involve that the presence of the covalently-linked lipoate prosthetic group was necessary but not sufficient to induce a positive immune response (5). Thus, Western blot analyses following SDS-PAGE showed that not all lipoylated LDs; Atpt LD-PDC, human LD-OGDC, LD-E3BP, and OLD- and ILD-PDC exhibited cross reactivity with mAbs PD1 and PD2. Western blot analysis of SDS-PAGE and native gels as well as ELISA showed that wt, fully lipoylated ILD-PDC exhibited strong cross reactivity with these mAbs whereas as expected its nonlipoylated ILD K173Q mutant elicited no response.

Interestingly, the expression of wt hILD-PDC in the absence of added lipoate produced approx. 20% octanoylated domain rather than the lipoylated form as this domain was not amenable to methoxy poly (ethylene glycol) maleimide (mPEG maleimide) covalent modification, a thiol group reagent (Mr 5000). This observation was consistent with a previous study reported by Ali *et al* .but it was contrary to a report by Quinn *et al.* who suggested that the apo and holodomains produced under these conditions and resolved by anion-exchange chromatography represented represented nonlipoylated and lipoylated hILD-PDC respectively (6, 7). Q-TOF mass spectrometry has confirmed the presence of octanoylated ILD-PDC in our case. Moreover, this work has demonstrated for the first time using western blot analyses of the native gel that the dithiols of the lipoic acid cofactor were not important in mAb recognition as octanoylated ILD-PDC also displayed strong equivalent cross reactivity.

In order to identify key candidate residues, LD sequences from various enzymes and species were aligned to allow their direct comparison.

HLD-E3BP	G	---	E	I	E	T	D	K*	A	V	--	T	L	D
HILD-PDC	G	---	E	I	E	T	D	K*	A	T		G	F	E
								173						
HOLD-PDC	G-	---	E	V	E	T	D	K*	A	T		G	F	E
LD-Atpt	G-	---	V	V	E	S	D	K*	A	D	--	D	V	E
HLD-OGDC	D	---	E	I	E	T	D	K*	T	S	--	Q	V	P
	33							**43**	**44**	**45**		**47**	**48**	**49**

Sequence comparison of the various LDs (reactive and non-reactive) around the lipoyl lysine (*) attachment site indicated that a central block of 8 highly-conserved amino acids is a common feature of all cross-reacting domains. Interestingly, there is a valine substitution at the C-terminal end of this central core in the weakly cross-reacting lipoyl domain, hLD-E3BP. In addition, two non-conserved residues (threonine and serine) lie at the C-terminal end of this block in the non-reactive LD, hLD-OGDC. Three altered residues were observed in the non cross-reacting LD of Atpt LD-PDC, a valine residue situated at the N-terminus of this block, a serine situated two residues to the N-terminal side of the lipoylation site and an aspartic acid situated at the C-terminal end of this central core. A second valine residue near the N-terminus of this block in Atpt LD-PDC was excluded from the comparison, since the same residue occurs in the reactive LD, hOLD-PDC. These differences highlighted the potential involvement of these residues as important constituents of the antigenic determinant recognised by PD1 and PD2.

Cross reactivity and lipoylation states of hILD-PDC mutants

A QuickChange TM Site Directed Mutagenesis Kit (Stratagene), was employed to convert the reactive hILD-PDC to be equivalent to the sequence of the non-reactive LD of Atpt PDC around the lipoyl-lysine residue as single, ILD-PDC **E168V**, **T171S**, and **T175D**; double **E168V:T171S**, **E168V:T175D** and **T171S:T175D**; and triple **E168V:T171S:T175D** mutants. Western blot analysis and ELISA were used to assay the cross reactivity between hILD-PDC mutant constructs and mAbs. Native gel analysis permitted us to study the lipoylation status of these mutants through the separation of lipoylated and nonlipoylated domains. In addition, the lipoylation status of the various mutants was confirmed by subjecting all mutants to modification using mPEG maleimide.

Threonine residue situated one residue from the C-terminal end of the central core of hILD-PDC (Thr-175) as well as the other threonine residue situated two residues to the N-terminal side of the lipoylation site (Thr-171) play a major role in Ab recognition. Thus although the **T175D** was approx. 50% lipoylated and **T171S** was fully lipoylated there was a dramatic loss of cross reactivity with mAbs PD1 and PD2. In contrast, glutamic acid residue situated situated at the start of 8-residue central block (Glu-168) partially affects lipoylation but has no direct effect on the extent of Ab recognition (Fig 1).

Cross reactivity and lipoylation states of hLD-OGDC mutant constructs

In a parallel study, the non-reactive domain, hLD-OGDC was mutated in a stepwise fashion in attempts to restore the level of Ab recognition to that of hILD-PDC. A series of 12 mutant domains were produced systematically in hLD-OGDC using site-directed mutagenesis to determine their involvement in the mAb PD1 and PD2 response. The same techniques used in

hILD-PDC work was performed to check mAb responses to hLD-OGDC mutant constructs and to assess their lipoylation status.

Initially, in order to verify the importance of the central block of 8 amino acids immediately adjacent to the lipoyl-lysine residue in Ab recognition, the two distinctive C-terminal amino acids in the non-reactive hLD-OGDC central region were replaced by the corresponding residues in the reactive LD, ILD-PDC as single and double mutations. However, it was found that only negligible cross activity was restored in **S45T** even though it was fully lipoylated whereas no cross reactivity appeared with other mutations although their lipoylation status was similar to wt hLD-OGDC (70%). Thus it was realised for the first time that the major epitope recognised by mAbs PD1 and PD2 did not solely involve this highly-conserved block of 8 amino acids. Following this result, our search was extended outside this block to focus on additional conserved residues within 10 amino acid residues of the lipoylation site which could potentially be accommodated within the Ab binding site. It was noted that two additional highly conserved residues located on the N- and C-terminal sides of the reactive hILD-PDC were altered in hLD-OGDC, namely Asp-33 and Pro-49. It was found after replacing these residues by the equivalent one in the reactive hILD-PDC as single and double mutations that slight cross reactivity (< 5%) was restored with **P45E** and **D33G:P49E** mutants even though they were fully lipoylated. This encouraging result prompted us to study the cumulative effect of these four mutations around the lipoyl-lysine residue on Ab recognition. A series of double, **S45T:P45E**; triple, **T44A:S45T:P49E** and **D33G:T44A:S45T**; and quadruple, **D33G:T44A:S45T:P59E** mutations were produced. Approx. 10% reactivity was restored with the fully lipoylated **S45T:P45E**, **T44A:S45T:P49E** and **D33G:T44A:S45T:P59E** constructs (Fig 2).

These findings directed our attention to the C-terminal side of the lipoylation site of hLD-OGDC since the replacement of Asp-33 by Gly alone or in combination with the two residues in the central block had no detectable effect on reactivity. Two further residues were identified to be highly conserved in the reactive human ILD- and OLD-E2 located four and five residues to the C-terminal side of the lipoylation site. It was found that partial recognition (approx 40%) was restored with **T44A:S45T:Q47G:P49E** and 100% with **T44A:S45T:Q47G:V48F:P49E** compared to wt hILD-E2 (Fig 2).

Conclusions

Several conclusions can be drawn from this study: (a) mAbs PD1 and PD2 recognise a contiguous epitope involving the lipoic acid prosthetic group and a region of primary sequence approx 9-11 amino acids in length located C-terminal to the lipoylation site; (b) apart from the lipoyl-lysine itself, no specific amino acid has been identified as essential to Ab recognition; thus, both loss and reappearance of cross-reactivity require the presence of two or

more mutations; (c) significant restoration of Ab cross-reactivity with hLD-OGDC was achieved only after generation of constructs containing Gln-47 and Val-48 replacements; however, these mutant constructs contained a total of 5 substitutions overall so it is likely that final reconstitution of the antigenic determinant stems from a cumulative effect of multiple substitutions; (d) the epitope is confined to this region of primary sequence which can be inserted into different LD backgrounds, and (e) this work has demonstrated for the first time that the dithiols of the lipoic acid cofactor were not important in mAb recognition.

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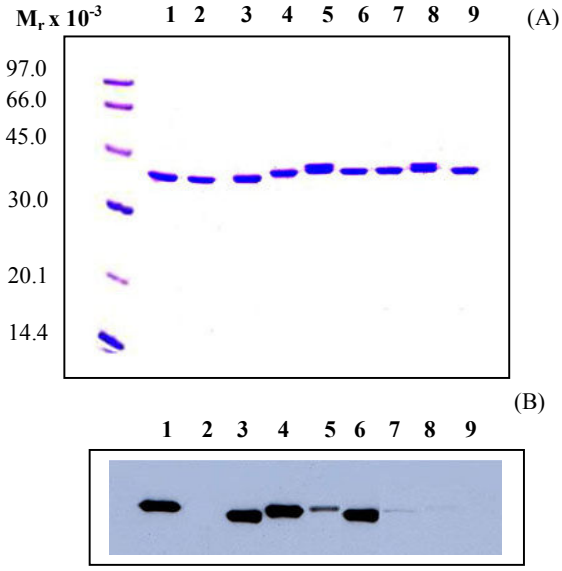

Figure 1 Immunoblotting of the recombinant ILD-PDC mutants with mAb PD2
Panel (A): Purified mutated ILD-GST fusion proteins (5µg) were separated by 12% SDS/polyacrylamide gel electrophoresis and stained with Coomassie Brilliant Blue.

Panel (B): Purified mutated ILD-GST fusion proteins (1µg) probed with mAb PD2 (1 in 500 dilution).

Lanes 1 and *2*: ILD-PDC and its K173Q mutant as positive and negative controls respectively; *lanes 3, 4* and *5*: single mutations, E168V, T171S and T175D respectively; *lanes 6, 7* and *8*: double mutations, E168V:T171S, E168V:T175D and T171S:T175D respectively; *lane 9*: the triple mutation, E168V:T171S:T175D.

M: low molecular mass markers are shown to the left

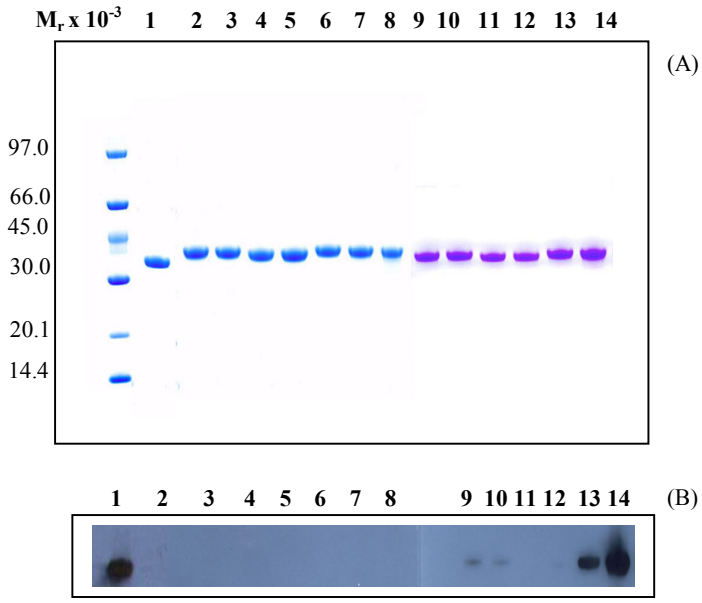

Figure 2 Immunoblotting of the recombinant LD-OGDC mutant constructs with mAb PD2
Panel (A): Purified mutated LD-OGDC GST fusion proteins (5µg) were separated by 12% SDS/polyacrylamide gel electrophoresis and stained with Coomassie Brilliant Blue. In addition, ILD-PDC (5µg) was used as a positive control.

Panel (B): Purified mutated LD-OGDC-GST fusion proteins (1µg) were probed with mAb PD2.

Lane 1,wild type ILD-PDC as a positive control; *lane 2*, wild type LD-OGDC; *lanes 3, 4* and *5*, T44A, S45T and T44A:S45T respectively; *lanes 6, 7* and *8*, D33G, P49E and D33G:P45E respectively; *lane 9, 10, 11* and *12*, S45T:P49E, T44A:S45T:P49E, D33G:T44A:S45T and D33G:T44A:S45T:P49E respectively; *lanes 13* and *14*, T44A:S45T:Q47G:P49E and T44A:S45T:Q47G:V48F:P49E.

M: low molecular mass markers are shown to the left of the gel

Two gels are joined; the first gel involves lanes 1-8 whereas the second gel involves 9-14.