# Detailed characterization of the binding site of the lipoprotein lipase-specific monoclonal antibody 5D2

**Shau-Feng Chang,* Berthold Reich,* John D. Brunzell,† and Hans Will[1,*]**

Heinrich-Pette-Institut für Experimentelle Virologie und Immunologie,* Universität Hamburg, Martinistrasse 52, 20251 Hamburg, Germany, and Department of Medicine,† University of Washington, Seattle, WA 98195

**Abstract** **Monoclonal antibody (MAb) 5D2 recognizes lipoprotein lipases (LPL) from different species but not related lipases. This MAb is a unique reagent, used world-wide, because it differentiates between monomeric inactive and dimeric active LPL, inhibits human LPL enzyme activity, and binds to C-terminal LPL sequences involved in interactions with lipoproteins, lipoprotein receptors, and heparin. In this study we have analyzed the fine specificity of the MAb epitope recognition in order to better understand its functional properties and species-specific LPL immune reactivity. In peptide scan assays, MAb 5D2 reacted with all, except two, 13 amino acid-long peptides located between positions 380 and 410. Peptides from the amino terminal end of this region reacted more strongly than those from the carboxyl terminal end. Furthermore, only a peptide from the amino terminal end competed effectively with the binding of MAb 5D2 to native LPL bound to microtiter plates or nitrocellulose. A systematic peptide mutagenesis study indicated that 8 amino acids of the reactive region, mainly located in the amino terminal end, are critical for binding and probably directly interact with MAb 5D2. The experimentally determined antigenicities of species-specific LPL peptides and of the corresponding denatured full-length LPL proteins on immunoblots were consistent with these findings. According to a proposed 3D-model for LPL, only the amino terminal end of the antigenic region is easily surface-accessible. These data combined with 3D-modelling of monoclonal antibody (MAb)–lipoprotein lipase (LPL) protein interaction provide new insight into the known biological effects of MAb 5D2 on LPL and the antigenic determinants that are recognized.—**Chang, S-F., B. Reich, J. D. Brunzell, and H. Will. **Detailed characterization of the binding site of the lipoprotein lipase-specific monoclonal antibody 5D2.** ***J. Lipid Res.*** **1998.** 39: **2350–2359.**

**Supplementary key words** lipoprotein lipase • lipase gene family • lipoprotein metabolism • peptide scan • antigen–antibody complexes

Lipoprotein lipase (LPL, EC 3.1.1.34) plays a pivotal role in lipoprotein metabolism both as a triglyceride hydrolyzing enzyme and as a mediator of interactions of lipoproteins with cell surfaces and receptors (1–3). The fact that human LPL deficiency results in hypertriglyceridemia, a risk factor for atherosclerosis, and the observation that LPL knock-out mice are not viable underscore the importance of this enzyme in physiology (4). The amino acid sequences of the human LPL (hLPL) (5) and a number of sequence polymorphisms as well as clinically relevant mutations are known (1, 6, 7). In addition, the LPL protein sequences of various animal species such as rat (8), bovine (9), sheep (10), pig (11), baboon (12), cat (13), mink (14), mouse (15), guinea pig (16), and chicken (17) have been published. Similar to the situation for human LPL (2, 6, 7), naturally occurring mutations in LPL, which result in hypertriglyceridemia, have also been reported for cat (13) and mink (18). The observation of strong sequence similarities between the coding region and gene organization of LPL, hepatic triglyceride lipase (HL, EC 3.1.1.32), and pancreatic lipase (PL, EC 3.1.1.3) indicates that all three enzymes are members of a gene family (19–22). Based on this information and on the known three dimensional (3-D) structure of PL, a 3D-structure similar to PL was proposed for LPL (23) consisting of N- and C-terminal folding domains.

Enzymatically active LPL appears to be a non-covalently linked homodimer (24–26) with a head-to-tail subunit orientation (27) that rapidly dissociates into inactive monomers (24, 28). However, evidence for enzymatically active monomeric human LPL has also been presented (29). A strong argument for the dimer model is the immune reactivity of native human LPL in a sandwich ELISA with MAb 5D2 (28), provided this MAb recognizes only a single epitope in each monomer of the native dimeric LPL enzyme. As the reactivity of only two short peptide sequences of LPL have been tested to date (30), the existence of additional epitopes recognized by MAb 5D2 is not excluded.

The model of the 3-D structure for LPL has been very useful in the designing of experiments and understanding

Abbreviations: ECL, enhanced chemiluminescence; EPBS, phosphate-buffered saline for ELISA; ELISA, enzyme-linked immunosorbent assay; HL, hepatic triglyceride lipase; HRP, horseradish peroxidase; Ig, immunoglobulin; KLH, keyhole limpet hemocyanin; LPL, lipoprotein lipase; MAb, monoclonal antibody; PBS, phosphate-buffered saline; PL, pancreatic lipase; and TBS, Tris-buffered saline.

[1] To whom correspondence should be addressed.

the data obtained from functional analysis studies performed with naturally occurring and artificially created LPL mutants (31–35). Accordingly, the N-terminal domain (aa 1–312) contains the catalytic center with a covering loop important for interaction with lipid substrates, heparin binding sites, and a binding site for the cofactor apolipoprotein C-II (3). The C-terminal domain (aa 313–448) was shown to contain binding sites for lipoproteins, for the $\alpha_2$-macroglobulin receptor/low density lipoprotein receptor-related protein, and possibly for heparin (36–38). As the sequence similarity between the C-terminal sequences of LPL and PL is much weaker than that between the N-terminal sequences, the folding predictions concerning the C-terminal LPL domain are less certain. In agreement with the proposed folding of the C-terminal LPL domain is the observation that chymotrypsin cleaves human LPL between positions 390/391 and 391/392. In the 3-D model both cleavage sites are located in a surface exposed loop (23, 32). In contrast, the binding site of the LPL-specific MAb 5D2 previously roughly mapped to aa 397–407 at the C-terminal domain (30) is not located at the surface (see below). Therefore, we reasoned that either the proposed 3-D structure does not fully reflect the structure of the native enzyme or the epitope mapped for MAb 5D2 is only accessible in partially denatured LPL. Based on the observation of reduced immune reactivity of LPL mutated in amino acids 390, 394, and 393/394 it has recently been speculated that the epitope recognized by MAb 5D2 may be longer than reported (32). Alternatively, the same authors suggested that these mutations, which are located close to the epitope mapped previously (30), may change the conformation of the epitope and thus abrogate binding of the MAb 5D2 (32).

The LPL-specific MAb 5D2 has several unique features and is one of the most frequently used immunological reagents in LPL research world-wide. Although this MAb was originally produced against bovine LPL, it crossreacts strongly with human LPL and with LPL from all animals (except mice) tested so far (18, 30). No immunological crossreaction has ever been observed with related hepatic lipases or pancreatic lipase. MAb 5D2 was reported to recognize the enzymatically active LPL dimer in a sandwich enzyme-linked assay (ELISA) whereas the inactive monomer is recognized in a sandwich ELISA only when a second LPL-specific antibody is also used (28). Therefore, this MAb is often used to determine the ratio of enzymatically active and inactive LPL as usually present simultaneously in sera and supernatants of transfected cells (32, 34). Binding of MAb 5D2 to native LPL is known to inhibit its hydrolytic activity towards long chain triglycerides (triolein as substrate) but not its esterase activity towards short chain substrates such as tributyrin (33). The inhibition is not due to dissociation of the LPL dimers but presumably due to interference with the function of the second lipid binding site located at the C-terminal domain (33). MAb 5D2 is also often one of the decisive reagents for the determination of the mass of LPL in sandwich ELISAs based on two MAbs including MAb 5D2 (28). This is possible because MAb 5D2 does not only bind to native LPL dimer but also to heat- or GuHCl-denatured LPL (28). Moreover, MAb 5D2 also recognizes nitrocellulose-fixed LPL on immunoblots although the enzyme is boiled and denatured with sodium dodecylsulfate before blotting (28). Taken together, this information clearly indicates that MAb 5D2 has unique binding characteristics. The many known and interesting biological features of MAb 5D2 combined with the wealth of information about the functional domains of LPL as well as the availability of a 3-D model for LPL motivated us to re-investigate and map in detail the epitope recognized by the LPL-specific MAb 5D2. The mapping data obtained indicate that the LPL specific sequences bound by MAb 5D2 are more complex than previously elucidated. Moreover, they provide explanations for the known functions of MAb 5D2 and are consistent with 3-D structure predictions for LPL.

## MATERIALS AND METHODS

### Mapping of hLPL epitopes recognized by MAb 5D2 using cellulose-bound peptides

In total, 232 peptides, each 13 amino acids long with an overlap of 11 amino acids to the next peptide and spanning the whole human LPL protein sequence, were synthesized on cellulose filters using previously reported methods (39, 40) (Jerini Biotools Co., Berlin, Germany). The peptides were designated with P followed by a number indicating the first amino acid of the full length human LPL protein (position one is the first amino acid of the mature protein and position −27 the first amino acid of the signal sequence). The cellulose filters containing the hLPL peptides were incubated with the monoclonal 5D2 antibody (MAb 5D2) (1 μg/ml) in TBS buffer (10 mm Tris-HCl, 150 mm NaCl, pH 7.6). Binding was detected using alkaline phophatase-labeled or horseradish peroxidase (HRP)-labeled anti-mouse antibodies (Dianova, Hamburg, Germany).

### Enzyme-linked immunosorbent assay (ELISA)

Keyhole limpet haemocyanin (KLH)-coupled peptides P384/395 (SDSYFSWSDWWS) and P396/410 (SPGFAIQKIRVKAGE) were dissolved in PBS containing 1% SDS. Microtiter plates (Nunc, Denmark) were coated with the amount of peptides indicated and incubated at 37°C for 2 h. After washing once with EPBS (10 mm $NaPO_4$, 2 mm $KH_2PO_4$, 165 mm NaCl, 3.2 m KCl, pH 7.4) containing 0.1% Tween 20, the plates were blocked overnight with EPBS/2% BSA at 4°C. HRP-labeled anti-LPL MAb 5D2 (70 pg/ml in EPBS/0.1% Tween 20) was then added to the microtiter wells. The plates were then incubated at room temperature for 4 h and washed 5 times with EPBS/0.1% Tween 20. HRP enzyme activity was quantitated using as a substrate 0.5 mg/ml *o*-phenylenediamine in 0.01% $H_2O_2$, 0.1 m citrate–phosphate buffer, pH 5.0, incubated for 10 min. Absorbance was measured at 490 nm.

### Competition ELISA with peptides

Plates were coated with partially purified LPL (kindly provided by U. Beisiegel, Hamburg, Germany, G. Olivecrona, Umeå, Sweden, and H. Jansen, Rotterdam, Holland) diluted in EPBS (50 ng/well) at 8°C overnight. After washing with EPBS, the plate was blocked with EPBS/2% BSA overnight. HRP-labeled MAb 5D2 preincubated for 1 h at room temperature with different concentrations of the peptides were then added to the microtiter wells and incubated for 3 h at room temperature. The plates were finally washed and the HRP enzyme activity was measured as described above.

### Sandwich competition ELISA with two antibodies and peptides

The plates were coated with 50 μl/well of MAb 5D2 (4 μg/ml) overnight at 8°C, and then blocked with EPBS/2% BSA. Partially purified LPL (50 ng/well diluted in EPBS) was then added and the plates were incubated overnight at 8°C. After washing, HRP-labeled MAb 5D2 preincubated with peptides as described above was added to the microtiter plates. After incubation of the plates at room temperature for 3 h, the plates were washed again as described above, and the HRP enzyme activity was determined as described above.

### Immunoblotting

The proteins were either separated by SDS-PAGE and then transferred onto nitrocellulose membrane, or alternatively, the proteins were directly spotted onto the membranes. Non-saturated protein binding sites on the membranes were then blocked with 5% milk powder in $H_2O$ at room temperature for 2 h, and finally incubated for 2 h at room temperature with MAb 5D2 (1 μg/ml in TBS containing 1% milk). After washing the membranes thrice with TBS/0.1% Tween for 10 min and thrice with TBS for 10 min, they were incubated at room temperature for 2 h with HRP-labeled anti-mouse Ab (diluted 1: 50,000 in TBS/1% milk). The membranes were then washed with TBS/0.1% Tween and TBS as described above. Bound antibodies were detected by chemiluminescence using a commercially available kit (ECL-kit, Amersham Buchler, Braunschweig, Germany) and exposure to X-ray films.

### Computer modelling

The 3D-model of lipases of hLPL published previously (23) was drawn using the molecular modeling program SYBYL on a Silicon Graphics INDY workstation. Functional regions and amino acids outlined in color are taken from published data (3, 6, 23, 31). The 3D-structure of the Fab fragment of MAb 59.1 (42) available in the EMBL data bank and known to bind a short peptide (42) was used to outline the likely steric orientation and size dimensions of the hLPL/MAb 5D2 complex. Similarly, the proposed structure of a dodecamer heparin molecule (43) has been aligned rather arbitrarily along the backbone of hLPL in close proximity to the known heparin-binding sequences (23).

## RESULTS

### Definition of LPL-sequences bound by MAb 5D2 using peptide scanning

In order to search systematically for sequences on human LPL that are recognized by MAb 5D2, 232 peptides spanning the whole hLPL sequence, each 13 amino acids long and overlapping with the next by 11 amino acids, were synthesized chemically on a cellulose membrane (see Material and Methods). MAb 5D2 was incubated with the filter and its binding was revealed by alkaline phosphatase-labeled anti-mouse immunoglobulin G (IgG) with a chemiluminescence substrate on photographic films. Thus, immune reactivity of MAb 5D2 was detected with peptides P380 to P388 (for nomenclature see Material and Methods) which all overlap in sequence and cover amino acids 380 to 400 of the LPL protein (**Fig. 1A** and **C**). The immune reactivity was strongest for peptide P384, slightly less for peptides P382 and P386, and much weaker for peptides P380 and P388. These data indicate that MAb 5D2 binds most strongly to amino acid sequence 384 to 396 and that amino acids 388 to 392, which are common to all immune reactive peptides, are crucial for its binding. As we expected to see binding to LPL sequences covering amino acid 396 to 405, previously defined as the epitope recognized by MAb 5D2 (30), we wondered whether the lack of reactivity with the corresponding peptides was due to too low sensitivity of our assay. Therefore, we enhanced the sensitivity of our assay by incubating the filter a second time with MAb 5D2 (2 μg instead of 1 μg) and a second time with alkaline phosphatase-labeled anti-mouse IgG. Photographic exposure of the filter showed strongly increased signals with peptides P380 to P388 as expected, but revealed in addition immune reactivity with peptide P398 (Fig. 1B) which roughly covers the previously mapped epitope sequence (30). Weak signals were also seen with peptides P390 and P396, which are adjacent to both immune reactive regions, as well as with various peptides outside of these regions (Fig. 1B). Because reactivity of the peptides outside of the immune reactive region was not reproducible in a repeat experiment (data not shown) it was considered non-specific. Taken together, these data indicate that MAb 5D2 binds most efficiently to LPL peptide sequences spanning amino acids 380 to 402, and less efficiently to a peptide covering amino acids 396 to 410 (Fig. 1C). Although both epitopes overlap, as defined with our technique, the data clearly indicate that MAb 5D2 can bind to non-overlapping closely adjacent LPL-peptide protein sequences. The overlap of both epitope sequences as well as the weak immune reactivity of peptides P390 and P396 located within the overlapping region may indicate that MAb 5D2 recognizes both ends of a rather long discontinuous LPL epitope extending from amino acid 380 to 410.

### Reactivity of MAb 5D2 with KLH-coupled peptide sequences spanning the antigenic region in ELISA

To test the binding of MAb 5D2 to both ends of the antigenic region in an independent assay, representative peptides (P384/395 and P396/410, numbers correspond to the first and last amino acid position of the human LPL) were synthesized, coupled to KLH, and used as substrate in an ELISA. In this assay, dose-dependent binding of MAb 5D2 to both peptides was observed (**Fig. 2**). However, approximately 1000-fold less of peptide P384/395 compared to P396/410 was required to achieve the same level of Ig-linked alkaline enzyme activity. No significant binding was observed when KLH was used as template in a control experiment (Fig. 2), demonstrating the specificity of the binding of peptides P384/395 and P396/410. These data confirm that MAb 5D2 binds to both epitope-specific sequences and to the N-terminally located epitope more strongly than to the epitope located in the C-terminal domain. This is consistent with the data obtained with the filter binding assay (Fig. 1).

As a further control we also tested in a competition ELISA whether peptides P384/395 and P396/410 inhibit binding of MAb 5D2 to LPL coated onto microtiter plates (**Fig. 3A**), as well as the binding of HRP-labeled MAb 5D2 to LPL held by unlabeled MAb 5D2 in microtiter wells

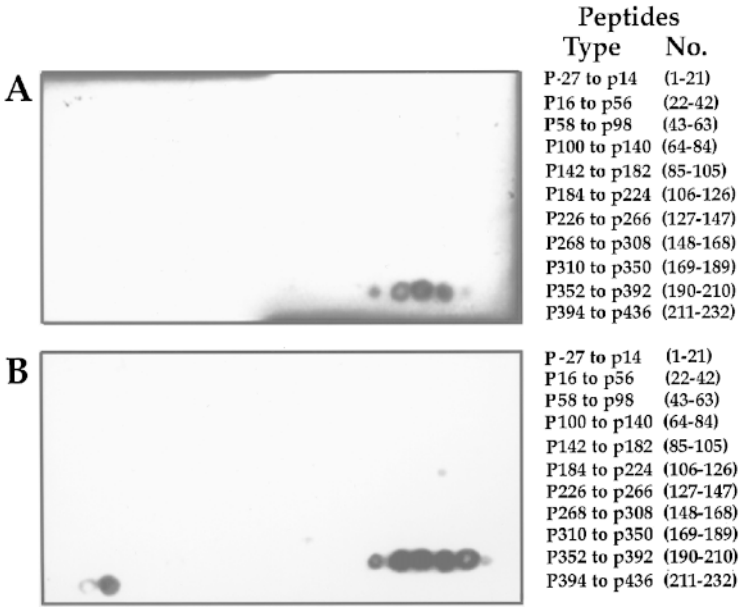


**Fig. 1.** Identification of hLPL-specific peptide sequences immune reactive with MAb 5D2 using peptide scanning. A) Signals obtained after the first incubation of MAb 5D2 with the cellulose filter containng with 232 peptides (each 13 aa long and overlapping by 11 aa) spanning the complete hLPL sequence. Bound MAb 5D2 was detected by alkaline phosphatase-labeled anti-mouse antibodies. B) Signals obtained after incubation of filter shown in A) for a second time with MAb 5D2 and secondary antibodies. The numbers on the right of the filters designate the type and number of peptides synthesized on the cellulose filters. Peptide 1 starts with the first amino acid of the signal sequence of the immature human hLPL (position −27; position 1 corresponds the first amino acid of the mature human LPL) and peptide 232 ends with the last amino acid of the hLPL. C) Sequences of peptides in the immune reactive region of hLPL. The degree of immune reactivity is indicated. The hLPL sequence reported previously recognized by MAb 5D2 (30) is underlined.

C

| 5D2 | Peptides Type | Peptides No. | Peptide Sequences (380 390 400 410) |
|---|---|---|---|
| - | P378 | 203 | LKLKWKSDSYFSW..... |
| ++ | P380 | 204 | ..LKWKSDSYFSWSD..... |
| +++++ | P382 | 205 | ....WKSDSYFSWSDWW..... |
| +++++ | P384 | 206 | ......SDSYFSWSDWWSS..... |
| +++++ | P386 | 207 | .....SYFSWSDWWSSPG..... |
| +++++ | P388 | 208 | .....FSWSDWWSSPGFA..... |
| + | P390 | 209 | .....WSDWWSSPGFAIQ..... |
| - | P392 | 210 | .....DWWSSPGFAIQKI..... |
| - | P394 | 211 | .....WSSPGFAIQKIRV..... |
| + | P396 | 212 | .....SPGFAIQKIRVKA.... |
| +++ | P398 | 213 | .....GFAIQKIRVKAGE.. |
| - | P400 | 214 | .....AIQKIRVKAGETP |

(Fig. 3B). In both assays a dose-dependent competition with peptide P384/395 was observed. Approximately 10 times less peptide P384/395 was required to achieve 50% inhibition when LPL was directly coated to microtiter plates compared to indirect coating (compare Fig. 3A and B). No significant inhibition of MAb 5D2 binding was observed with peptide P396/410 in both assays when compared to a control peptide (Fig. 3A and B). Therefore, we conclude that peptide P384/395 in solution competes efficiently with binding of MAb 5D2 to native LPL whereas P396/410 does not. Consistent with this conclusion, a competition experiment performed with LPL coated onto nitrocellulose filters gave similar results (Fig. 3C). These data suggest that the C-terminally located epitope represented by the P396/410 peptide does not adopt a conformation in solution compatible with binding by MAb 5D2.

### Mutagenesis of the antigenic region for identification of amino acids involved in and essential for MAb 5D2 binding

In order to provide further evidence for the significance of the mapping data obtained and to define the type of amino acids essential for and compatible with binding of MAb 5D2 to human LPL, each amino acid residue of peptides P384/395 and of peptide P398/410 (similar in sequence to P396/410) was replaced individually by all 20 l-amino acids. These peptides and the corresponding mutants thereof were tested for immune reac-

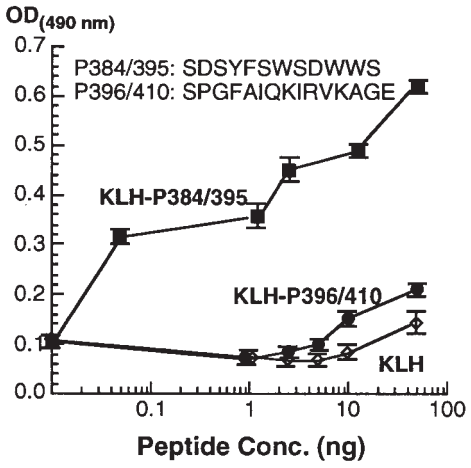


**Fig. 2.** Immune reactivity of KLH-coupled peptides P384/395 and P396/410, corresponding to the N- and C-terminal ends of the antigenic region, with MAb 5D2 as determined by ELISA. Both peptides, but not the control KLH protein, coated to surface microtiter plate wells show dose-dependent reactivity with MAb 5D2. The reactivity of peptide P384/395 is much stronger than that of P396/410.

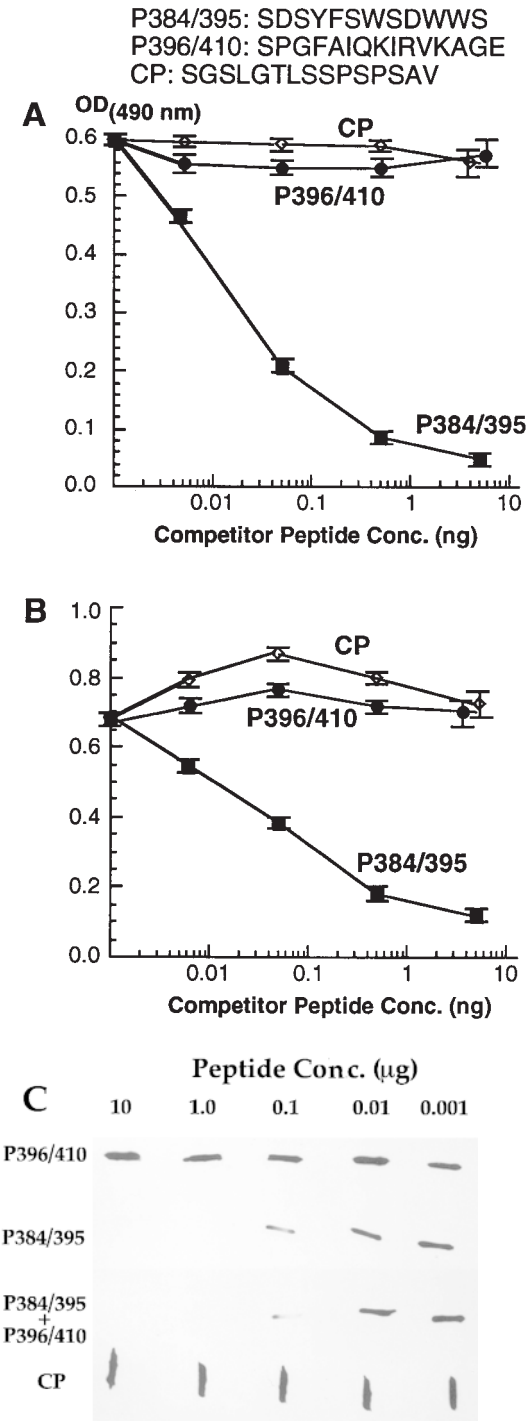


**Fig. 3.** Definition of epitope specificity by competition ELISA with peptides. (A) Effect of LPL-specific peptides P384/395 and P396/410 as well as of a control peptide (CP) on binding of HRP-labeled MAb 5D2 to partially purified hLPL fixed on the surface of microtiter plate wells. A dose-dependent competition with MAb 5D2 binding was only detected when peptide P384/395 was preincubated with the MAb 5D2. (B) Analogous experiment as in panel A with the only difference that the partially LPL protein was fixed to the microtiter plates by coating first unlabeled MAb 5D2. Significant competition is again only observed with peptide P384/395. (C) Reactivity of partially purified hLPL spotted onto nitrocellulose filter with HRP-labeled MAb 5D2 after preincubation with the same peptides as in panels A and B. Significant and dose-dependent competition was observed only with peptide P384/395. A mixture of peptides P384/395 and P396/410 did not compete more effectively than peptide P384/395 alone.

tivity in a cellulose filter-based peptide scan (**Fig. 4**) as described above for peptides covering the full-length LPL. Each spot on the filter represents a single peptide with or without a single mutation as indicated. The analysis performed with peptide P384/395 revealed that amino acids at positions 388 to 391 and 394 are most critical for binding of MAb 5D2 as they could not be substituted by any amino acid other than one similarly charged (position 388 F to W; position 391: S to T) without destroying immune reactivity (Fig. 4, top panel). Amino acids D and S at positions 392 and 395, respectively, are apparently also important for binding but can be substituted by many other amino acids without abrogating binding. In contrast, amino acids in positions 384 to 387, and 393 of peptide P384/395 could be substituted by almost any other amino acid without eliminating the binding. Taken together, these data suggest that side chains of amino acids 389, 390, and 394 of the LPL protein interact directly with amino acid residues in the binding groove of MAb 5D2. Furthermore, as only amino acids S or T at position 391 were compatible with binding and both amino acids are characterized by a hydroxyl group in their side chain, interaction of the binding site of MAb with the hydroxyl group of both amino acids is likely. Similarly, compatibility of amino acids F and W in positions 388 and

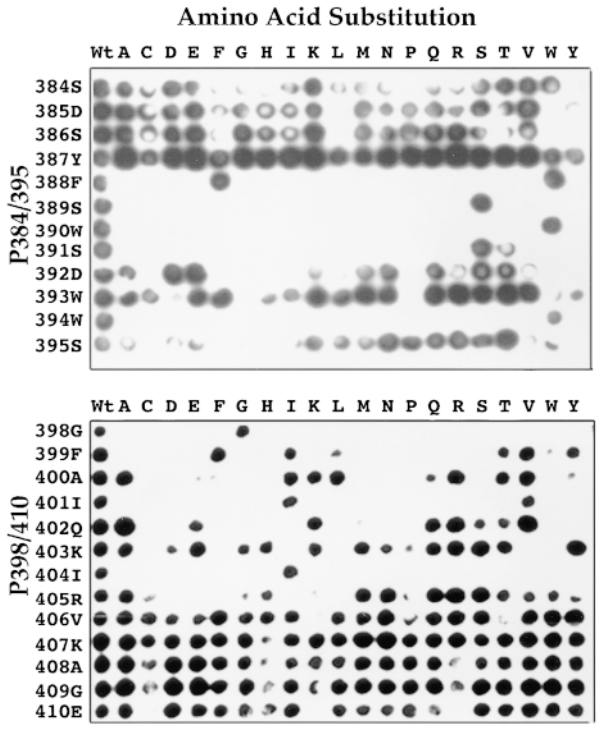


**Fig. 4.** Type of amino acids in peptides of the antigenic region recognized by and compatible with MAb 5D2 binding as revealed by mutant peptide scanning. (A) Immune reactivity of peptide P384/395 and mutants thereof in which each amino acid at each position was substituted by all other 20 amino acids. Immune reactivity was determined by incubation with 1 μg of MAb 5D2 and HRP-labeled mouse antibodies. (B) Analogous experiment with peptides P398/410 derived from the C-terminal region of the antigenic region of LPL.

390, respectively, with binding suggests interaction of the aromatic moieties of the side chains of both amino acids with similar ones in the binding site of MAb 5D2.

The result of the analysis of the mutagenized peptide P398/410 differed in two general aspects from that of P384/395. First, as expected the signal intensity for individual spots was lower when using the same antibody concentration and exposure. Second, almost all amino acid positions that could be mutated in many ways without interference with MAb 5D2 binding were located at the C-terminal end (amino acids 406 to 410; Fig. 4, bottom panel). However, similar as for peptide P384/395, amino acids at two positions (398:G and 404:I) of peptide P398/410 could not be substituted by any other amino acid without abrogating binding of MAb 5D2 (Fig. 4, bottom panel). Amino acid I at position 401 is similarly critical for binding as it could be substituted only by the most similar amino acid V. Amino acids at positions 399, 400, 402, 403, and 405 could be substituted by many other amino acids without abrogating binding. Taken together, these data suggest direct interaction of amino acids 398, 401, and 404 with the binding site of MAb 5D2 and a structural role for all other amino acids, particularly those located at the amino terminal end between amino acid positions 399 to 405.

### Reactivity of MAb 5D2 with native and denatured LPL as well with epitope-specific peptides from different species

The data from the mutagenesis study and the protein sequences available for LPL from different species (**Fig. 5**) predict reactivity or non-reactivity of LPL from different animals and of the corresponding epitope-specific peptides. Accordingly, LPL specific peptides corresponding to the amino terminal epitope derived from human and most animals have a high probability of reacting with MAb 5D2. In contrast, those from rat and mouse LPL (rLPL and mLPL) should not react because amino acids R and P at positions 389 and 401, respectively, are not compatible with binding as seen in our mutagenesis study. The reactivity of the rLPL sequence is the least predictable one because a variant P384/395 peptide with two point mutations as present in rLPL compared to hLPL sequence was not tested by mutagenesis. Therefore, the reactivity of the rat peptide P384/395 or of the full-length rLPL protein needed to be determined experimentally (see below). According to our data of the mutagenesis study, recognition of C-terminal epitope specific peptides from LPL proteins from different species should be more restricted than that of the amino terminal end because five of the 11 LPL proteins have mutations in one or more amino acids incompatible with binding of MAb 5D2 (compare Figs. 4 and 5). The amino acid in position 398 in particular, which could not be substituted by any other amino acid in our mutagenesis study without destroying immune reactivity of the corresponding peptide, is mutated in LPL from rat, mouse, guinea pig, and chicken.

The predictions and hints from the mutagenesis study for the reactivity of the LPL peptides from different species corresponding to peptides P384/395 and P396/410 of human LPL were experimentally tested by probing their immune reactivity. They were chemically synthesized on cellulose filters, incubated with MAb 5D2, and then with HRP-labeled anti-mouse Ig. In these experiments MAb 5D2 recognized P398/410-related LPL peptides derived from human, baboon, bovine, sheep, mink and pig, but not from cat, rat, mouse, guinea pig, and chicken (**Fig. 6A**). Except for the cat-specific peptide, this result is consistent with the expectations from the mutagenesis study. As the cat-specific peptide differs from those tested by mutagenesis by two instead of one amino acid, the presence of both mutations simultaneously probably induced a conformation incompatible with MAb 5D2 binding.

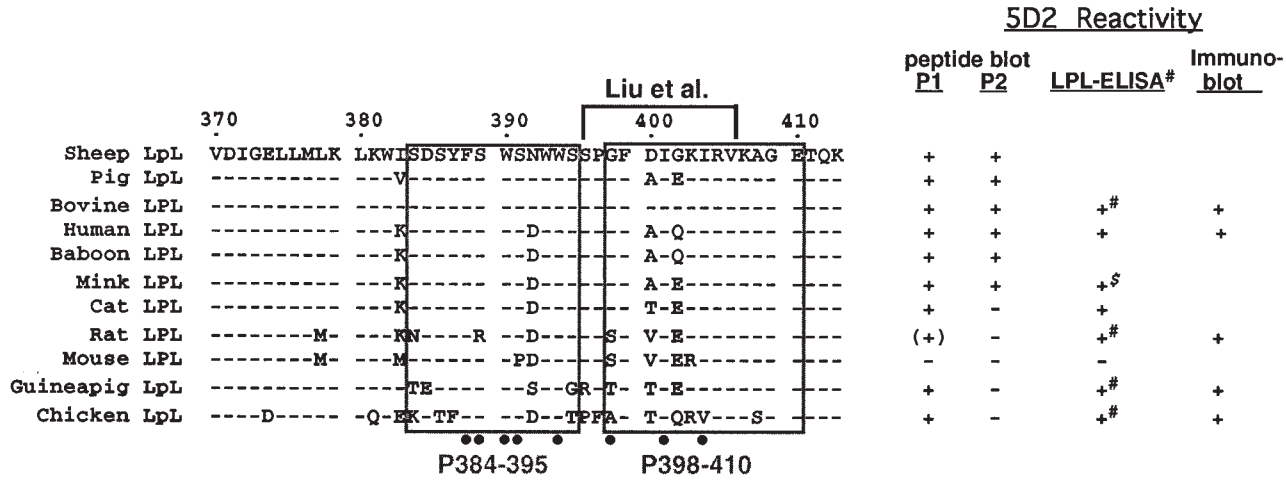


**Fig. 5.** Sequence variation and experimentally determined immune reactivity of LPLs from different species in the N- and C-terminal end of the antigenic region. The antigenic sequence previously shown by Liu et al. (30) to react with MAb 5D2 is indicated. The most likely LPL amino acids interacting directly with amino acids in the binding groove of MAb 5D2 are indicated by dots. LPL-ELISA data are compiled from those published previously (data reported in refs. 30 and 18 are labeled by # and $, respectively) and determined by us; (+/−) indicates borderline immune reactivity. The peptide amino acid positions correspond to the sequence of human LPL. P1 and P2 correspond to peptides derived from the LPL sequence aa 384–394 and 396–410 (see Fig. 6).

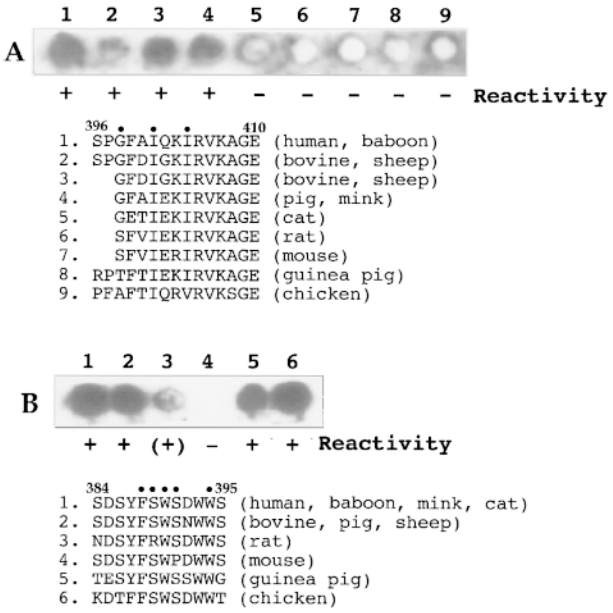


**Fig. 6.** Immune reactivity of MAb 5D2 with peptides derived from LPLs from different species and synthesized onto nitrocellulose filters. A**)** Most of the P398/410- or P396/410-specific peptides are not recognized detectably or only weakly by MAb 5D2. Only the peptides corresponding to human, baboon, bovine, sheep, pig, and mink LPL are efficiently recognized. B) MAb 5D2 does not bind to the mLPL-specific peptide P384/395, binds weakly to that of the rLPL, and binds strongly to all others. All peptide amino acid positions correspond to the sequence of human LPL and are slightly different for some of the corresponding non-human LPL proteins.

Note, two bovine LPL specific peptides corresponding to P398/410 of human LPL were used for the analysis. One (P396/410) differed from the other (P398/410) only by two extra amino acids at the amino terminus and the short peptide reacted more strongly than the longer one (Fig. 6A). This indicates that binding of MAb 5D2 is strongly dependent on sequence context and conformation of the corresponding peptides.

In the analogous experiment with six P384/495 peptides derived from the LPL sequences of 11 species, strong signals were obtained with four peptides, weak signals with the rat peptide, and no signal with that of the mouse (Fig. 6B). This result is consistent with the prediction derived from the mutagenesis study. Note, the rat peptide reacted only weakly in this experiment and has two amino acid changes simultaneously (S384N and S389R) compared to hLPL. The lack of reactivity of a corresponding peptide with mutation S389R only in our previous experiment (Fig. 4) but the presence of reactivity of the peptides with both mutations suggests that a conformational change in the peptide favorable for MAb 5D2 binding is induced by cooperative interaction of both mutations.

Because both the sequence context flanking the epitopes as well as the conformation of peptides seemed to determine whether or not MAb 5D2 reacted, we further tested the immune reactivity of full-length LPL from rat, chicken, and guinea pig. To our knowledge this has not been examined before experimentally with MAb 5D2 by immunoblotting. LPLs were partially purified from sera of the corresponding animals by heparin-Sepharose chromatography and tested by immunoblotting. As visualized in **Fig. 7**, all three LPL proteins reacted strongly and specifically with MAb 5D2. These findings suggest that reactivity of the amino terminal end of the antigenic region alone in these LPL proteins is sufficient for immune reactivity in an immunoblot or that the conformation of nitrocellulose filter-bound LPL exhibits more than one epitope accessible for binding by MAb 5D2. An ELISA performed by us (data not shown), and in part previously by others (30), with LPLs from post heparin sera of mouse, rat, guinea pig, and chicken confirmed the antigenicity of the latter three enzymes with MAb 5D2 and the lack of reactivity of the mouse enzyme.

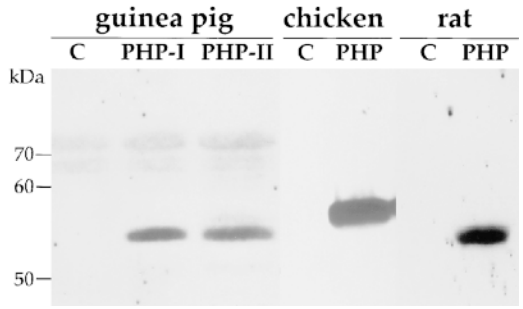


**Fig. 7.** Immune reactivity of LPL partially purified by heparin-Sepharose chromatography from postheparin plasma of animals and tested by immunoblotting with MAb 5D2. LPL from postheparin plasma (PHP) of guinea pig (sera I and II), chicken, and rat immune react in the blot with MAb 5D2. As expected, no LPL was found in proteins isolated by the same procedure from control preheparin plasma (C) taken from the same animals.

## DISCUSSION

In this study we have shown that the LPL-specific MAb 5D2 recognizes each end of a rather long epitope located between amino acids 380 and 410. Eight amino acids were shown to be essential for binding, suggesting that they may directly interact with the binding pocket of MAb 5D2. The binding was highly preferential for the amino terminal end of the epitope which forms an exposed loop in the proposed model for the 3-D structure of LPL. In contrast, most of the carboxyl terminal end of the epitope, which corresponds roughly to the epitope mapped previously, is not surface-exposed in the 3-D structure model of LPL suggesting it may only be exposed in fully or partially denatured LPL. Our mapping data and the sequence variation of the MAb 5D2 specific epitope in LPL from different animals were shown to be consistent with its broad species immune crossreactivity reported in the literature, with new data provided, as well as with the known lack of immune reactivity of the mouse LPL. Taken together, the data indicate that the epitope recognized by MAb 5D2 is more complex than reported previously. Our findings have interesting implications for the understanding of the

biological properties of MAb 5D2 in relation to structure and function of LPL.

In a previous report the epitope for MAb 5D2 was delineated roughly between amino acids 396 and 405 of LPL (30). These mapping data were based on the analysis of a single reactive LPL-peptide and a negative control LPL-peptide, on the comparison of sequence variability and ELISA reactivity of LPL from human, bovine, cat, chicken, as well as known the lack of reactivity of mouse LPL and human hepatic lipase (HL). Our mapping data are largely consistent with this previous report but show that this region is only part of a longer epitope, bound only weakly by MAb 5D2, and surface-exposed probably only in partially or fully denatured LPL (**Fig. 8**). In contrast to a speculation in the same report that alanine at position 400 is critical for immune reactivity and cannot be changed into valine without losing reactivity (30), we have shown by mutagenesis that substitution of alanine at position 400 into valine (Fig. 5) or five other amino acids (Fig. 4) is compatible with the binding of MAb 5D2. These data and the immune reactivity of the corresponding rLPL and mLPL peptides, which both have a valine residue at position 400, clearly indicate that valine at position 400 is not as critical as suggested and is definitely not the reason for lack of immune reactivity of mouse LPL. Moreover, three lines of evidence indicate that it is the change of serine to proline in position 391 of the mouse LPL which prevents binding by MAb 5D2. First, serine at this position is con-

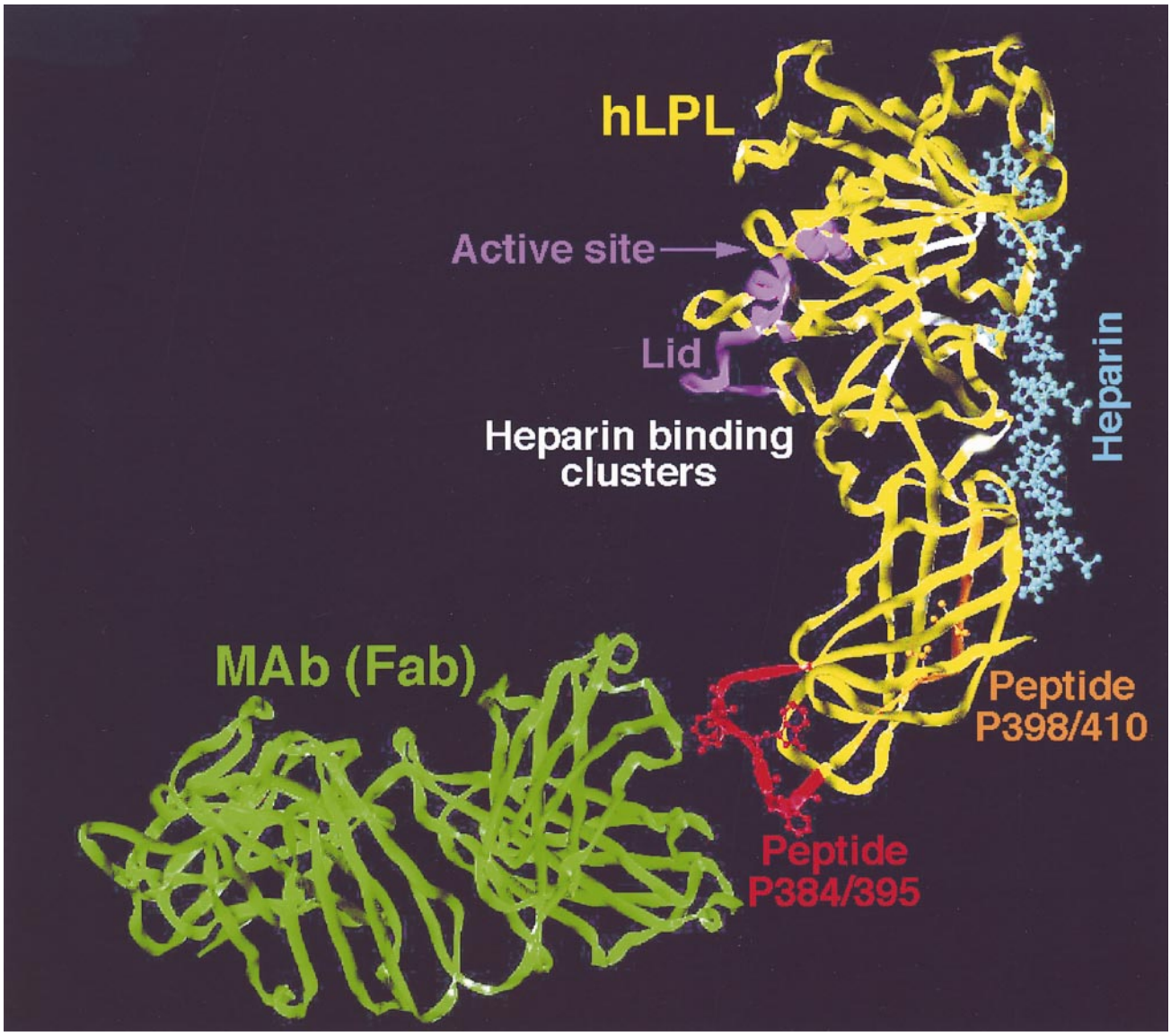


**Fig. 8.** Schematic view of the 3-D model of monomeric hLPL (yellow) with the proposed orientation of a Fab fragment (green) mimicking that of MAb 5D2. The 3-D-structure of hLPL is based on that of van Tilbeurgh et al. (23) and that of MAb 59.1 known to bind a short peptide similar in size to P398/395 from the report of Ghiara et al. (42). The critical serine residue within the active site of LPL and the mobile lid are drawn in purple. The most immune reactive and surface-exposed region of hLPL, identical in sequence with peptide P384/395, is drawn in red whereas the internally buried antigenic region (P398/410) for MAb 5D2 is indicated in brown. The side chains which presumably interact directly with the binding pocket of MAb 5D2 are indicated. A dodecamer of heparin (blue color) is schematically placed in close vicinity to most known heparin binding sequence motifs (white) of hLPL. In this model the binding of MAb 5D2 to hLPL does not interfere with that of heparin.

served in all other known LPL proteins. Second, proline is a known helix breaker and predictably results in an altered conformation of the exposed loop region of the epitope in mLPL as determined by computer-based 3-D structural modeling (data not shown). Third, and most importantly, the mutagenesis study indicated that substitution of serine at position 391 by any other amino acid abrogates binding of MAb 5D2, probably because the side chain of serine 391 is directly interacting with the binding pocket of MAb 5D2. Our mapping data are also consistent with the lack of immune reactivity of MAb 5D2 with LPL-related human hepatic lipase and pancreatic lipase. In neither lipases is there any significant sequence similarity with the LPL antigenic region (data not shown).

The most reactive part of the epitope as mapped here for MAb 5D2 corresponds to an loop exposed at the extreme end of the C-terminal folding domain of LPL (Fig. 8). In fact, the immune reactivity of the full-length rat, guinea pig, and chicken LPL proteins in ELISA as well as on immunoblots is most likely based exclusively on these sequences as the less reactive region (aa 398 to 410) in LPL of other species is non-reactive when tested by peptide ELISA. The highly immune reactive loop contains three tryptophane residues at positions 390, 392, and 394 which were recently shown to contribute to binding of LPL to lipid/water interfaces and possibly also to the productive orientation of the enzyme at the lipid/water interface reference (32). The identification of the same three tryptophanes as part of the high affinity binding site of MAb 5D2 strongly suggests that these functions as well as the long chain triglyceride hydrolytic activity are most likely directly blocked by binding of MAb 5D2 to the C-terminally located lipid binding site.

The knowledge of the overall 3-D structure of some IgG molecules and the proposed location of the epitope of MAb 5D2 in the model of the 3-D structures of LPL provides good suggestions for structure(s) of the LPL–MAb 5D2 complex. Here we present our proposals for the dimeric LPL molecule. Primarily based on suggestions of van Tilbeurgh et al. (23) and recent evidence for the head to tail conformation of the subunits in the dimeric LPL (27), we discuss here the conformation of a hLPL dimer/MAb 5D2 complex in which the monomers are associated side to side such that the active centers and the heparin binding of both monomers are localized oppositely. In our model we positioned the known 3-D structure of the Fab fragment of a MAb with known and similar peptide epitope specificity (42), as mapped here for MAb 5D2, close to the surface-exposed part of the epitope in the native LPL recognized by MAb 5D2 (Fig. 8). This schematic view of the likely arrangement of the monomeric antigen/antibody complex provides obvious explanations for the known biological properties of MAb 5D2 and the corresponding knowledge on functions of the C-terminal domain of LPL. First, MAb 5D2 works well in a sandwich ELISA (28) because the microtiter well-fixed unlabeled MAb 5D2 binds to the C-terminally exposed antigenic loop of one subunit of the LPL dimer which exposes the C-terminal antigenic loop of the other subunit of the LPL dimer on the opposite side where it would be optimally accessible for binding of the HRP-labeled MAb 5D2 used for detection. Second, binding of MAb 5D2 does not interfere with the heparin binding of LPL (33) because they are on opposite sites. Third, binding of MAb 5D2 to LPL dimers does not induce monomerization (33) because the putative contact sites for dimerization, believed to be mainly or exclusively in the amino terminal domain, are sterically separated. Fourth, inhibition of LPL lipid hydrolysis of long chain substrates by MAb 5D2 (33) is due to direct interaction with part of the C-terminally located lipid binding domains in both monomers. Fifth, MAb 5D2 does not inhibit hydrolytic activity of LPL for soluble substrates (33) because the bulky immunoglobulin structure is far from the active site and its mobile covering lid. Further studies on the LPL/LPL dimer concept and exclusion of the existence of epitopes recognized by MAb 5D2, which may have escaped detection by the techniques used so far, are required to experimentally support or modify this attractive model for LPL/MAb 5D2 interaction.

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