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Enhanced detection of lipoprotein lipase by combining immunoprecipitation with Western blot analysis

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Abstract This manuscript describes the problems inherent in combining immunoprecipitation of lipoprotein lipase (LPL) with its detection by Western blot, and how these problems can be circumvented by the preparation of suitable immunoreagents. These reagents used during the immunoprecipitation step, include Fab fragments of the primary antibody (chicken anti-bovine LPL), and a covalently linked immunomatrix of the secondary antibody (rabbit anti-chicken IgG). The use of these reagents in conjunction with Western blot detection virtually eliminates the problem of non-relevant protein detection when analyzing LPL from complex biological samples. Moreover, this approach can be adapted to detect any protein with the same inherent problems as LPL, such as hepatic lipase.—**Doolittle, M. H., O. Ben-Zeev, and V. Briquet-Laugier.** Enhanced detection of lipoprotein lipase by combining immunoprecipitation with Western blot analysis. *J. Lipid Res.* 1998. **39:** 934–942.

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Western (or immuno) blotting integrates in one method the resolving power of electrophoresis with the specificity and sensitivity of immunochemical detection. It is an excellent choice for the estimation of steady-state protein levels, and provides the ability to examine protein molecular weight and posttranslational modifications. Nevertheless, the procedure is constrained by the abundance of the specific protein of interest, and the specificity and sensitivity of the antibody used for detection. For example, non-specific signals can occur in any immunochemical technique if other proteins in the sample express cross-reacting epitopes. The appearance of such non-specific signals can be exacerbated in Western blotting due to: *i*) the denaturation of proteins prior to SDS PAGE that may expose cross-reacting epitopes that are otherwise buried; and *ii*) the formation of high local concentrations of proteins on the membrane that permits interaction of antibodies with low affinity, cross-reacting epitopes (1). Thus, Western blotting by itself is often not adequate to specifically detect a relevant protein unless sensitivity and specificity can be enhanced. In this regard, these factors can be greatly improved by combining immunoprecipitation with Western blotting; immunoprecipitation concentrates and partially purifies the relevant protein, and Western blotting provides the resolving power and added specificity so that the relevant protein becomes the predominant signal detected. Moreover, unlike Western blotting, immunoprecipitation permits antibody–antigen interactions to occur in solution, where the antibody can be allowed to react with native (non-denatured) proteins (if desired), and where high local concentrations of cross-reacting epitopes generally do not exist.

This paper describes the utilization of immunoprecipitation combined with Western blot analysis to detect lipoprotein lipase (LPL), a secretory asparagine-linked glycoprotein that is synthesized by a variety of tissues (2, 3). The analysis of LPL protein by techniques such as Western blotting is particularly important, as its maturation to an active lipase is dependent on co- and posttranslational steps that can be most easily monitored by examining changes in migration of the protein during SDS PAGE (4, 5). Also, direct examination of the LPL protein is required in domain-exchange and mutational approaches that are used to assign functional properties to enzyme subdomains and to assess the ef-

Abbreviations: SDS PAGE, sodium dodecyl sulfate polyacrylamide gel electrophoresis; LPL, lipoprotein lipase; Staph A, preparation of insoluble *Staphylococcus aureus* membranes; IgG, immunoglobulin G; Pro5, a control Chinese hamster ovary cell line; Lec1, a mutant Chinese hamster ovary cell line deficient in *N*-acetylglucosaminyltransferase activity; Lec23, a mutant Chinese hamster ovary cell line deficient in α-glucosidase I activity; SC-2, *Drosophila melanogaster* Schneider cells.

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fect of induced and naturally occurring mutations on characteristics such as specific activity, secretion, and subunit structure (6, 7). In our experience, the use of immunoprecipitation to enrich and concentrate LPL prior to its detection by Western blotting is far superior to detection by immunoblotting alone. Nevertheless, the use of immunoprecipitation creates technical difficulties that must be overcome before the subsequent detection of LPL by Western blotting. Here we describe the source of these problems and their solution through the use of suitable immunoreagents that permit the specific detection of LPL protein, with little background contamination, from even non-abundant sources.

MATERIAL AND METHODS

Materials

Materials were purchased from the following sources. Sigma (St. Louis, MO): Freund's complete and incomplete adjuvant; purified bovine LPL; lyophilized cell powder of *Staphyloccus aureus* (Cowan strain); dithiothreitol; Triton X-100; ethanolamine; deoxycholate (sodium salt); *N*-lauroyl sarcosine (sodium salt); β-mercaptoethanol; Tween-20. Promega (Madison, WI): EGGstract™ yolk purification system. Pharmacia (Uppsala, Sweden): cyanogen bromide (CNBr)-activated Sepharose 4B. Pierce (Rockford, IL): immobilized papain; rabbit anti-chicken IgG and biotinylated rabbit anti-chicken IgG (ImmunoPure™); dimethyl pimelimidate·2HCl; PVDF membrane; SuperSignal® chemiluminescent substrate. Bio-Rad (Hercules, CA): acrylamide and *N,N′*-methylene-bis-acrylamide; TEMED; ammonium persulfate; extra thick filter (blotting) paper. Amersham (Arlington Heights, IL): Rainbow® colored protein molecular weight markers, high molecular weight range; Hyperfilm-ECL photographic film. ICN Biomedicals (Aurora, OH): casein hydrolysate. Gibco BRL (Gaithersburg, MD): horseradish peroxidase (HRP)-conjugated streptavidin.

Solutions

Fab dialysis buffer: 20 mm sodium phosphate buffer, pH 7.0, 10 mm EDTA; *Fab digestion buffer* (prepare fresh before use): 20 mm sodium phosphate buffer, pH 7.0, 10 mm EDTA, 20 mm cysteine·HC1; *concentrated cysteine·HCl solution* (prepare fresh before use): 20 mm sodium phosphate buffer, pH 7.0, 10 mm EDTA, 70 mm cysteine·HCl; *SDS wash buffer*: 0.1 m Tris·HCl, pH 7.2, 2% SDS, 20 mm dithiothreitol; *Triton wash buffer*: 0.1 m Tris·HCl, pH 7.2, 3% Triton X-100, 0.1% *N*-lauroyl sarcosine; *sample lysis buffer*: 50 mm NH_4OH·HCl buffer, pH 8.0, 0.2% deoxycholate (sodium salt), 10 U/ml heparin; *immuno wash buffer*: 50 mm Tris·HCl, pH 7.5, 3% Triton X-100, 0.3% SDS, 0.1% *N*-lauroyl sarcosine (sodium salt), 0.15 m NaCl; *dissociation buffer*: 50 mm sodium phosphate buffer, pH 5.7, containing 0.5% SDS; *glycerol concentrate*: 50% glycerol, 5% β-mercaptoethanol, 0.01% bromophenol blue; *Western transfer buffer*: 25 mm Tris base, 0.2 m glycine; *Western blocking buffer*: 50 mm Tris·HCl, pH 7.5, 2% casein hydrolysate, 0.1% Tween-20 (or Triton X-100); *PBS-T*: PBS (10 mm sodium phosphate, pH 7.2, 0.15 m NaCl) containing 0.1% Triton X-100; *Western wash buffer*: 0.01 m Tris·HCl, pH 7.5, 1.0 m NaCl, 0.5% Triton X-100, 0.5% *N*-lauroyl sarcosine (sodium salt), 0.05% SDS, 1 mm EDTA.

Cell lines

The CHO cell lines used in this study were Pro5, Lec1, and Lec23. Pro5 and Lec1 were obtained from ATCC (Rockville, Maryland); Lec23 was a generous gift from Pamela Stanley. Lec1 and Lec23 are mutant cell lines with deficiencies in *N*-acetylglucosaminyltransferase and α-glucosidase I activity, respectively (8, 9), and Pro5 cells serve as the unaffected control. For transfection of these cell lines, human LPL was subcloned into the *Not I* site of pRC/RSV (Invitrogen, San Diego, CA), an expression vector containing an RSV LTR promoter and neomycin resistant gene. After calcium–phosphate transfection, the cells were selected against the neomycin analogue G418 (800 μg/ml); G418-resistant clones were picked and screened for LPL activity. Over 10% of these clones expressed significant LPL activity, and the clones expressing the highest activity were used in the present study.

The *Drosophila melanogaster* Schneider (SC-2) cell line (10) was a generous gift from Mitch Kronenberg. Human LPL was subcloned into the *Nco I/Sal I* site of pRmHa3, a vector containing the insect metallotheonein promoter (11). SC-2 cells were co-transfected by the calcium–phosphate procedure with LPL-pRmHa3 and phshsneo (12), and selected against the neomycin analogue G418 (1.5 mg/ml). As SC-2 cells are non-adherent, individual G418-resistant clones were not picked, but instead multiple passages in G418 were carried out to enrich for resistant cells. The expression of LPL under the regulation of the metallotheonein promoter was induced by adding 1 mm copper sulfate to the media 48 h prior to harvesting.

LPL antibody

Laying hen chickens were immunized subcutaneously with 100 μg of bovine LPL denatured in 0.1% SDS in PBS and emulsified in Freund's complete adju-

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vant. Four weeks later, the chickens were boosted with the same amount of denatured LPL in Freund's incomplete adjuvant (secondary immunization). Eggs were collected starting 3 days before the first immunization, and throughout both the primary and secondary immunization periods until 5 weeks after the boost. Egg yolks diluted 1:2 in PBS were assayed for the presence of LPL antibody by an ELISA using denatured bovine LPL as the capture antigen and biotinylated rabbit anti-chicken IgG as the detecting antibody. We found that the peak immune response occurred 10–15 days after the boost. Total IgG was isolated from the combined yolks from this period by using the EGGstract™ yolk purification system and instructions from the manufacturer. Denatured bovine LPL was coupled to CNBr-activated Sepharose and used to affinity purify LPL-specific antibodies from total IgG as described (13).

Production of chicken Fab fragments

One mg of affinity-purified chicken IgG (in 1 ml) was dialyzed overnight against Fab dialysis buffer at 4°C. After dialysis, 0.25 ml of the concentrated cysteine·HCl solution was added followed by the addition of 0.5 ml of immobilized papain. Prior to its use, the immobilized papain was washed 3 times by adding 4 ml Fab digestion buffer, carefully stirring the slurry, and separating the papain gel by centrifugation at 4°C for 5 min at 500 *g*. Digestion of the chicken IgG by papain was allowed to occur overnight (16 h) at 37°C on a rotating vertical wheel, and the reaction was stopped by adding 10 μl of 1 m Tris·HCl, pH 7.5. The immobilized papain was removed by centrifugation (5 min at 500 *g*). Complete digestion was assessed by observing the disappearance of the 66 kDa IgG heavy chain and the appearance of 20–25 kDa bands representing the Fab and Fc portions of the chicken IgG. The resulting Fabs were stored at −70°C.

Preparation of Staph A

Staph A was prepared from the lyophilized cell powder of *Staphyloccus aureus* (Cowan strain) as described (14). Briefly, the powder was resuspended in SDS wash buffer (as a 10% w/v solution), placed in a boiling water bath for 10 min, and centrifuged at 12,000 *g* for 5 min at room temperature. The resulting pellet was again resuspended in SDS wash buffer, boiled, and centrifuged. This process was repeated 5–7 times until very little protein was released from the Staph A. The pellet was then washed in Triton wash buffer an additional three times (without boiling), and the washed pellet was suspended in PBS as a 10% v/v slurry. One ml of the preparation bound 1 mg of rabbit IgG. The Staph A was stored at −70°C.

Preparation of immunomatrix

One mg of rabbit anti-chicken IgG was incubated with 1 ml Staph A for 60 min at 4°C with gentle mixing. After centrifugation at 12,000 *g* for 5 min, the Staph A pellet (with bound IgG) was washed 3 times by resuspension in 10 ml 0.2 m sodium borate buffer, pH 9.0, and finally resuspended in 10 ml 0.2 m sodium borate buffer, pH 9.0. Solid dimethylpimelimidate·2HCl was added to the suspension to a final concentration of 20 mm, and the solution was re-adjusted to pH 9.0 with NaOH. After a 90-min incubation at room temperature with gentle mixing, the cross-linking reaction was stopped by adding ethanolamine to a final concentration of 20 mm. The cross-linked immunomatrix was washed once with 10 ml of water, and then resuspended in 10 ml of 0.1 m glycine·HCl, pH 2.5, to remove any non-crosslinked antibody. Immediately after resuspension, the solution was centrifuged (12,000 *g*, 5 min) and the pellet washed two times with 0.1 m Tris·HCl, pH 8.0, and twice with 10 ml of PBS. The final pellet was resuspended in 1 ml PBS (final concentration of IgG is about 1 mg/ml of slurry). The immunomatrix was stored at −70°C.

LPL immunoprecipitation

Cells were sonicated in sample lysis buffer, and centrifuged at 12,000 *g* for 5 min at 4°C to remove nuclei and insoluble debris. LPL activity was assayed as described (15) and total protein was determined by the BCA method (Pierce, Rockford, IL). A volume of lysate or medium equivalent to 10–20 mU of activity was routinely used for immunoprecipitation (1 mU = 1 nmol fatty acid released/min).

SDS was added to the lysate so that the SDS:protein weight ratio was at least 2:1; the sample was placed in a boiling water bath for 2 min, and then cooled to room temperature. To ensure that the antibody added for immunoprecipitation would not be damaged by denaturation, the SDS in the lysate was sequested by addition of Triton X-100 so that the Triton X-100:SDS weight ratio was at least 7:1; NaCl was also added to a final concentration of 1.0 m. After mixing, 2.5–5.0 μg anti-LPL Fabs was added, and the mixture was incubated overnight (16–20 h) at 4°C.

To precipitate the LPL-Fab immune complexes, 25 μl of the rabbit anti-chicken immunomatrix was added, and the sample was incubated for 2 h at 4°C with constant gentle mixing. The sample was centrifuged at 12,000 *g* for 2–5 min, and the supernatant was removed. The pellet was washed twice with 1 ml of immuno wash buffer, followed by a wash with 1 ml of water. After removing the final wash, the pellet was resuspended in 35 μl of dissociation buffer, and placed

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for 2 min in a boiling water bath. The sample was then centrifuged for 5 min at 12,000 *g*, and 30 μl of the supernatant was mixed with 20 μl of glycerol concentrate before loading onto a polyacrylamide gel.

Western blot analysis

The samples were electrophoresed on a 7% or 9% SDS polyacrylamide gel, and electro-blotted over to a PVDF membrane. The membrane was incubated in Western blocking buffer for 30 min at room temperature. After blocking, affinity-purified LPL antibody diluted to 0.3–0.4 μg/ml in PBS-T was added, and the membrane was incubated overnight at 4°C with gentle shaking. All other manipulations were done at room temperature. The membrane was rinsed 4 times with water, and then incubated in Western wash buffer for 5 min with gentle shaking. This rinse and wash cycle was repeated for a total of 4 times. The membrane was then incubated with biotinylated rabbit anti-chicken IgG diluted 1:5,000 in PBS-T for 30 min, washed as above, and finally incubated in HRP-streptavidin diluted to 1:5,000 in PBS-T for 10 min. The membrane was washed again as above, and incubated with chemiluminescent substrate for 5 min before exposing to photographic film. Exposure times generally varied between 1–30 sec.

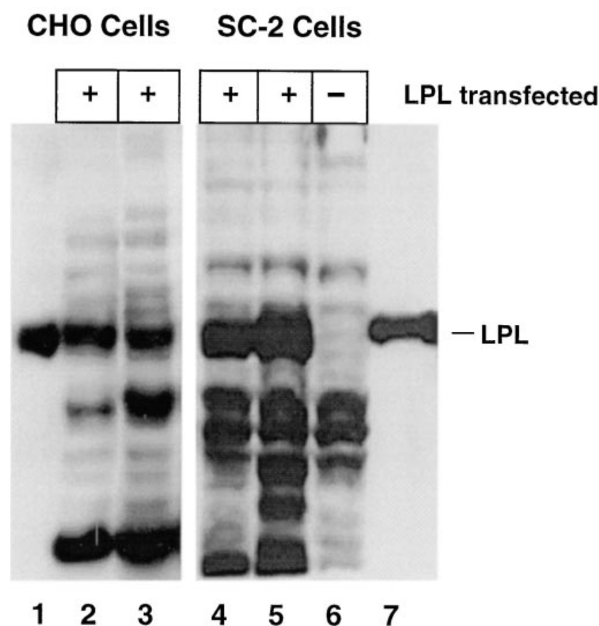

Fig. 1. Western blot analysis of cell lysates from mammalian and insect cell lines. A mammalian cell line (CHO cells) and an insect cell line (SC-2 cells) were transfected with human LPL. Cell lysates containing 100 μg protein were subjected to Western blot analysis using an affinity-purified bovine LPL antibody. Purified bovine LPL (5 ng) was used as a standard (lanes 1, 7). The CHO cell lines used were: Pro5, lane 2; Lec23, lane 3 (for characteristics of cell lines, see text).

RESULTS

Figure 1 indicates that direct Western blot detection of LPL from cell lysates was confounded by the presence of numerous, non-specific bands that partially obscured the LPL signal and severely compromised the utility of this technique as an analytical tool. As shown in this figure, this problem did not appear to be inherent to any particular cell line. Clearly, many non-specific proteins were detected by affinity-purified LPL antibody, as shown by the similar distribution of bands present in untransfected cells (lane 6) compared with LPL transfected cells (lanes 4, 5). We concluded that the majority of these non-specific bands were abundant proteins expressing low-affinity epitopes, and that immunoprecipitating the sample prior to Western blot analysis would help eliminate most of these non-relevant proteins.

The first step in developing a technique using LPL immunoprecipitation in conjunction with Western blotting was to determine the pattern of band(s) detected when these two methods were combined. As suitable antibodies for LPL are produced in chicken, and chicken IgG does not bind to Protein A, LPL was immunoprecipitated by a three-step protocol. First, affinity-purified chicken anti-bovine LPL antibody (primary antibody) was used to bind LPL in cell lysates; second, an affinity-purified rabbit anti-chicken IgG antibody (secondary antibody) was utilized to bind the primary antibody; and third, an insoluble preparation of *Staphylococcus aureus* membranes (Staph A) was used to bind the secondary antibody and thus remove LPL immune complexes from the solution. The LPL samples used for immunoprecipitation were lysates of CHO cells stably transfected with human LPL. Pro5 are wild-type CHO cells that produce LPL with a molecular mass of approximately 57 kDa (4). Lec1 are mutant cells that produce LPL with a slightly lower molecular mass, resulting from the inability of these cells to process the two glycan chains of LPL past the truncated $Man_5 GlcNAc_2$ stage (8).

The two cell lines were solubilized in deoxycholate-containing lysis buffer and a sample containing 10 mU lipase activity (equivalent to about 6–10 ng LPL) was used for immunoprecipitation. As a negative control, immunoprecipitation of a sample containing lysis buffer was also carried out. The immune complexes were released from Staph A by SDS treatment, which fees both the antigen (i.e., LPL) and immunoglobulins from the Staph A pellet. The SDS-solubilized immunoprecipitates were electrophoresed and transferred over to a PVDF membrane for Western blot detection.

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Figure 2 shows the resultant Western blot developed by incubating the membrane with primary antibody, followed by biotinylated secondary antibody and streptavidin-conjugated horseradish peroxidase. A chemiluminescent substrate was used to visualize the immuno-reactive bands. As expected, immunoprecipitated LPL in Pro and Lec1 was detected as a band that migrated with, or just below, the purified bovine LPL standard at 57 kDa, respectively (compare lanes 2 and 3 to lane 4). However, three additional bands were also detected, one at 66 kDa and two more at about 50 kDa and 25 kDa. The 66 kDa and 50 kDa bands flanked, and partially obscured, the LPL bands in Pro5 and Lec1. All three bands were also seen in the negative (buffer) control (lane 1), indicating that they did not originate from the cell lysates. The molecular weights of these contaminating bands suggested that they resulted from the IgG used for LPL immunoprecipitation.

To confirm that these contaminating bands were IgG, primary and secondary antibodies were electrophoresed either directly or after immunoprecipitation with Staph A. After transfer to PVDF membranes, identical blots were developed either with both primary and biotinylated secondary antibodies or with biotinylated secondary antibody alone (**Fig. 3**). The 66 kDa and 25 kDa bands were thus identified as the heavy and light chains of chicken IgG, respectively, detected by the biotinylated secondary antibody as expected (lanes 1 and 5). (A band in the primary antibody sample migrating just above the 46 kDa was also detected by the biotinylated second antibody; it is faintly visible in lanes 1 and 5 and more clearly seen in lane 8. This 46 kDa band represents a breakdown product of the heavy chain of chicken IgG.) The 50 kDa band was identified as the heavy chain of rabbit IgG, that, while not detected by its biotinylated counterpart (lanes 6 and 7), was surprisingly detected by the chicken anti-bovine LPL antibody (lanes 2 and 3). The inclusion of Staph A to precipitate the secondary antibody alone (lanes 3 and 7) or to immunoprecipitate the combined primary/secondary immune complex (lanes 4 and 8) did not change the pattern of contaminating bands. None of the various IgG bands were detected when the blots were developed with streptavidin-conjugated horseradish peroxidase alone (data not shown). Based on these results, immunoprecipitation combined with Western blotting could not be used unless chicken and rabbit IgG heavy chains could be eliminated from the gel samples.

As a means of removing chicken heavy chain contamination at 66 kDa, the affinity-purified LPL antibody was subjected to papain cleavage to produce a trun-

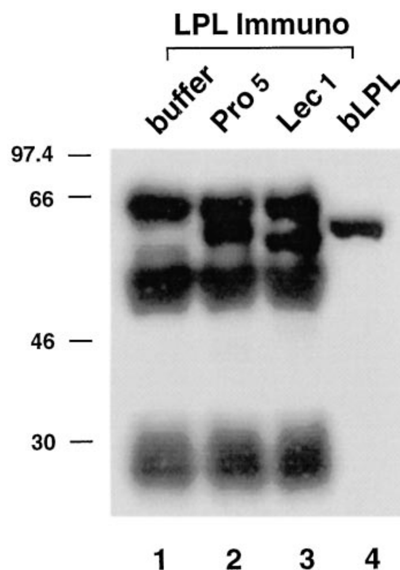

Fig. 2. LPL immunoprecipitation using chicken anti-LPL and rabbit anti-chicken IgG. The samples subjected to immunoprecipitation were: lysis buffer alone (lane 1); lysates of Pro 5 (lane 2), a wild type CHO cell line; lysates of Lec 1 cells (lane 3), a mutant CHO cell line expressing a slightly lower molecular weight form of LPL. Both cell lines were stably transfected with human LPL. The immunoprecipitates were electrophoresed on a 9% gel, and the resulting Western blot was probed with chicken anti-bovine LPL followed by biotinylated rabbit anti-chicken IgG. A purified bovine LPL standard was included (lane 4). The position of molecular weight standards (in kDa) is indicated.

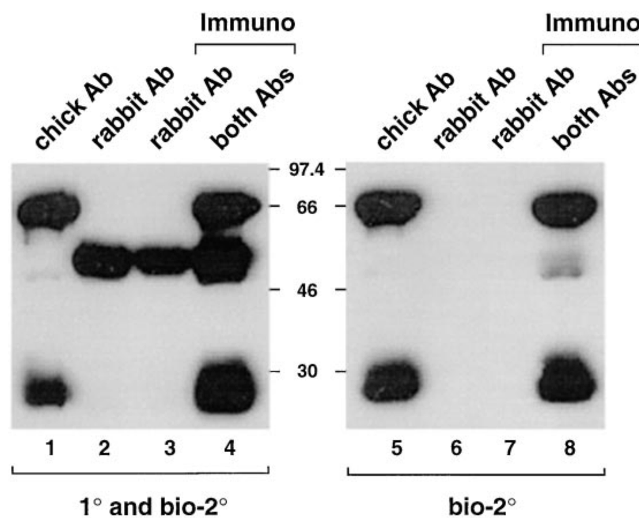

Fig. 3. Antibodies used for LPL immunoprecipitation are detected during Western blot analysis. Antibodies were either used directly (lanes 1, 2 and 5, 6) or immunoprecipitated with Staph A (lanes 3, 4 and 7, 8) before electrophoresis on 9% gels: chick Ab, chicken anti-bovine LPL; rabbit Ab, rabbit anti-chicken IgG; both Abs, chicken and rabbit antibodies combined. After Western transfer, the blots were probed with either chicken anti-bovine LPL followed by biotinylated rabbit anti-chicken IgG (1° + bio-2°), or with biotinylated rabbit-chicken IgG only (bio-2°).

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cated antigen binding fragment (Fab). Upon reduction, the truncated heavy chain of the resulting Fab was reduced from 66 kDa to about 25 kDa, well below the LPL band migrating at 57 kDa. When medium of Pro5 cells was subjected to immunoprecipitation with Fab fragments, LPL was seen free of the 66 kDa chicken IgG heavy chain that had partially obscured its detection (**Fig. 4**). Figure 4 also shows that the Fab fragments efficiently immunoprecipitate LPL; when 40 ng of purified bovine LPL was immunoprecipitated by the addition of 5 μg Fab fragments, greater than 90% was recovered.

A similar strategy to eliminate the rabbit IgG at 50 kDa could not be used, as papain detaches the Fc portion, containing the crucial Staph A binding site, from the antigen binding site. Consequently, an "immunomatrix" was used that was comprised of the second antibody covalently cross-linked to Staph A. The cross-linking prevents dissociation of the secondary antibody from Staph A; thus, the antibody remains bound to the Staph A pellet after treatment with SDS. Therefore, when both chicken IgG Fabs and immunomatrix were used in conjunction, both the 66 kDa and 50 kDa IgG heavy chain bands were eliminated, and the region surrounding the immunoprecipitated LPL band was free of heavy chain contamination (**Fig. 5**).

It was also anticipated that the use of Fabs and immunomatrix would prevent LPL band distortion that was

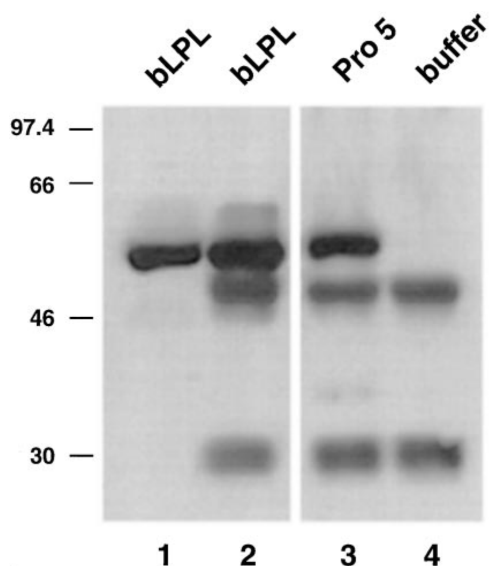

Fig. 4. LPL immunoprecipitated with Fabs and rabbit anti-chicken IgG. The samples immunoprecipitated with 5 μg anti-LPL Fab fragments were: lane 2, purified bovine LPL (40 ng); lane 3, medium of Pro5 cells stably expressing human LPL (a total of 25 mU LPL activity was used, equating to about 25 ng LPL); lane 4, lysis buffer alone. Lane 1 represents purified bovine LPL (20 ng) that was directly loaded onto the gel. After samples were electrophoresed on a 7% gel and transferred to PVDF, the resulting blot was probed with chicken anti-LPL antibody and biotinylated rabbit anti-chicken IgG. Densitometric scanning of lanes 1 and 2 was used to calculate the efficiency of LPL immunoprecipitation, which was 93%.

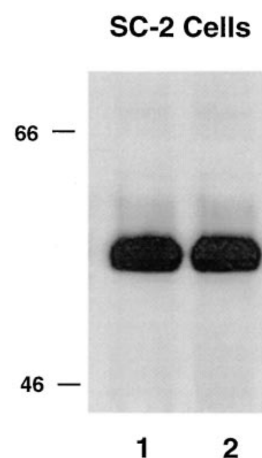

Fig. 5. LPL immunoprecipitation using Fabs and immunomatrix. LPL was immunoprecipitated from *Drosophila* SC-2 cell lysates (300 μg protein) using Fabs and immunomatrix (lanes 1, 2). The samples used for immunoprecipitation correspond to the identical samples used for direct Western blot analysis in Fig. 1 (lanes 4, 5). The 25–30 kDa bands originating from the chicken IgG light chain and papain-cleaved heavy chains seen in Fig. 4 are not present in lanes 1 and 2 because the blot was trimmed just below the 46 kDa molecular weight marker. The immunoprecipitates were electrophoresed in a 9% gel.

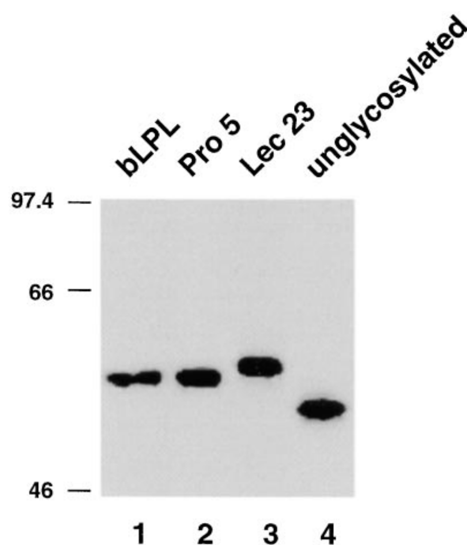

Fig. 6. Slight variations in LPL molecular weight are readily resolved by this technique. LPL was immunoprecipitated from Pro 5 cell lysates (lane 2) or Lec 23 lysates (lane 3). Unglycosylated LPL (lane 4) was produced by treating the LPL immunoprecipitate in lane 3 with endoglycosidase H. Samples were electrophoresed in a 7% gel.

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sometimes encountered when high concentrations of IgG heavy chains migrated close to LPL during electrophoresis. Band distortion can severely compromise the ability to resolve distinct forms of LPL that are distinguished only by slightly different migration rates. To demonstrate that small differences in LPL migration rate would be readily detected using the techniques described herein, LPL from Lec23 and Pro5 cells was immunoprecipitated with Fabs and immunomatrix. Lec23 cells cannot process core high mannose glycan chains ($Glc_3Man_9GlcNAc_2$) due to a deficiency in glucosidase I activity (9). As a consequence, these unprocessed chains cause the migration rate of LPL to be slightly retarded (4). **Figure 6** shows that the small difference between LPL from wild-type and Lec23 cells was clearly resolved (compare lane 3 with lanes 1 and 2). In addition, unglycosylated LPL was also analyzed (lane 4). This LPL was obtained by treating the Lec23 immunoprecipitate with an endoglycosidase that removes all $Glc_3Man_9GlcNAc_2$ chains. This unglycosylated form, migrating at about 51 kDa, would have been totally obscured by the 50 kDa rabbit IgG heavy chain if the latter was not eliminated by use of immunomatrix.

DISCUSSION

This study describes a method of incorporating immunoprecipitation with Western blot detection to obtain strong LPL signals with low background. The utility of immunoprecipitation is obvious; it can be used to enrich and concentrate LPL from cell lysates, plasma samples, or tissue culture medium that is otherwise replete with non-specific proteins. Without immunoprecipitation, non-specific proteins not only limit the amount of sample that can be placed on a gel, but create background bands ("noise") that can obscure the specific signal (Fig. 1). Thus, when LPL is nonabundant and/or signal-to-noise ratios are low, Western blot detection by itself is usually not satisfactory. As shown in this study, this limitation is overcome when immunoprecipitation is used prior to Western blot detection.

The inherent problem in analyzing LPL immunoprecipitates by Western blot detection is the presence of large amounts of heavy chain IgG migrating close to LPL on reducing SDS polyacrylamide gels; on a molar basis, to quantitatively precipitate LPL with our antibody, chicken and rabbit heavy chain are 100 and 1000 times more abundant than LPL, respectively. The large amount of chicken anti-LPL antibody used for immunoprecipitation was expected to be detected on the blot by the biotinylated secondary antibody (rabbit anti-chicken IgG); indeed, this was the source of the 66 and 25 kDa bands seen in Figs. 2 and 3. Somewhat surprising was the detection of the 50 kDa rabbit heavy chain by the primary antibody (see Fig. 3). The most likely explanation is that rabbit heavy chain IgG has an epitope(s) that is recognized with low affinity by the chicken anti-LPL antibody. With the high amounts of concentrated rabbit heavy chain on the blot, even low affinity sites (10^6 mol^{-1}) can be detected (16).

In addition to the method presented in this paper, several other strategies were used in an attempt to solve the problem of IgG heavy chain contamination. The simplest strategy was to analyze LPL immunoprecipitates on SDS gels in the absence of reducing agent (i.e., without β-mercaptoethanol). Under these conditions, IgG does not dissociate into heavy and light chains but remains as a 200 kDa complex. We found this to be unsatisfactory, however, as most "non-reduced" preparations of antibody contained significant amounts of reduced IgG heavy chain. In addition, in the absence of reducing agent, some LPL did not enter the separating gel, most likely due to aggregation. A second strategy was the use of biotinylated primary (chicken anti-LPL) antibody for Western detection. This approach eliminated the need to use biotinylated rabbit antibody, which detected the chicken IgG heavy chain. However, overall sensitivity was significantly decreased, and detection of the 50 kDa rabbit IgG heavy chain was still evident. As a third strategy, chicken anti-LPL and rabbit anti-chicken IgG were cross-linked to each other and to Staph A to produce a "primary" immunomatrix that was used directly for LPL immunoprecipitation. While this approach eliminated contamination of IgG from both species, the efficiency of LPL immunoprecipitation was reduced as the primary antibody was bound and not free in solution. Consequently, larger amounts of primary antibody (chicken anti-LPL) were needed to quantitatively bind LPL. Because chicken anti-LPL antibody is difficult to obtain and is needed for many applications, this was not a satisfactory solution. We chose instead to use an immunomatrix comprising only the secondary antibody, which is commercially available and affordable.

We found that the most successful strategy to eliminate chicken heavy chain contamination at 66 kDa was to use Fab fragments for immunoprecipitation (see Fig. 4). Proteolytic digestion with papain was found to be suitable for generation of Fabs from chicken IgG, although it has been reported that pepsin can be used as well (17). The principal sites of papain cleavage are located to the amino-terminal side of the disulfide bonds that hold the two heavy chains together. Thus, papain digestion releases two antigen binding domains and one Fc fragment. Under the reducing conditions used for SDS PAGE, the Fabs dissociate into a light chain

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(about 20 kDa) and a heavy chain fragment (about 25 kDa); the Fc portion breaks up into two fragments (about 25 kDa each). As all these fragments, including the reduced Fc portion, are much smaller than LPL, it is not necessary to further purify the Fab domain away from the Fc fragment after papain treatment.

An immunomatrix comprised of rabbit anti-chicken IgG and Staph A was utilized to remove the contaminating 50 kDa band (see Fig. 5). While Staph A was used to prepare the immunomatrix, Protein A-Sepharose can be used as well. Regardless of the immunoabsorbant used, the cross-linking of the IgG to a solid phase greatly decreases the dissociation of IgG when treated with SDS. In fact, it is recommended that the efficiency of cross-linking of newly prepared immunomatrix should be determined by examining the amount of heavy chain IgG that is removed from the Staph A pellet before and after addition of cross-linking agent; greater than 95% of the IgG should remain with the matrix after treatment with SDS. An alternative to a cross-linked immunomatrix is the use of biotinylated antibody with streptavidin-conjugated Sepharose (or agarose). The biotin-streptavidin bond has a very high affinity constant and remains intact when treated with SDS (18).

The optimal amount of sample to be used for immunoprecipitation can be estimated by LPL activity. Based on reported values for LPL specific activity (19, 20), 10 mU equates roughly to 6 ng LPL. Generally this amount is readily detected by Western blot techniques using chemiluminescent substrates. However, sensitivity is also a function of antibody affinity. For example, we could detect sub-nanogram amounts of bovine LPL compared to nanogram quantities of human LPL because the antibody was raised against the former species. Moreover, sensitivity is also affected by the number of binding sites; thus, by using biotinylated secondary antibody, as opposed to biotinylated primary antibody, sensitivity was also increased.

The procedure described here would also work well to immunoprecipitate LPL from biosynthetically labeled cells or tissues. The use of Fabs and immunomatrix as opposed to intact soluble IgG will prevent band distortion that can affect resolution in the region of the gel containing LPL. Moreover, using this technique, total LPL mass from biosynthetically labeled cells can be estimated by Western blot detection, and the label incorporated into the mass can be detected on the same blots by autoradiography or phosphorimaging. Thus, in tracer kinetic studies, this approach would permit a relative assessment of LPL radiospecific activity. Indeed, the method described here is applicable to all proteins with molecular masses in the 45–70 kDa range where IgG heavy chain contamination can be a problem.

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