Expression of rat hepatic lipase in heterologous systems: evidence for different sites for interface binding and catalysis

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Abstract Rat hepatic triglyceride lipase was expressed as a bacterial fusion protein and as a secreted protein in eukaryotic cells. The bacterial fusion construct coded for seven amino acids at the N-terminus which are not present in the hepatic lipase cDNA, but otherwise consisted of only the complete mature lipase sequence. Fusion protein was isolated as an insoluble product which did not have lipase or phospholipase activities; it was, however, active as an esterase when solubilized after preparative gel electrophoresis. The fusion protein was used to raise polyclonal antibodies that recognize native rat hepatic lipase and inhibit its activity. For eukaryotic expression, a full-length rat hepatic lipase cDNA clone was inserted into the metallothionein promoter expression vector pMTSV40polyA·Bam. Transfected CHO cells, induced with ZnSO₄, secreted an immunoreactive protein of $M_r \sim 57,000$. A lipase-producing clonal cell line was isolated and used to characterize the enzyme. The protein was purified from serum-free medium by heparin-Sepharose and DEAE-Trisacryl M column chromatography. It was apparently identical to native rat hepatic lipase, with the exception of the conformation of the linkage of the sialic acids which form part of the N-linked carbohydrate complexes. The bacterial fusion protein, the CHO-produced lipase, and native rat hepatic lipase were all inhibited by phenylmethylsulfonyl fluoride, implying that they function catalytically as serine esterases. ■ Substrate competition studies indicated that the esterase and lipase activities use the same active site; thus, the major defect in the fusion protein was probably in triglyceride substrate binding. These results suggest that interface binding and catalysis occur at different sites in the protein. — **Komaromy, M. C., and M. Reed.** Expression of rat hepatic lipase in heterologous systems: evidence for different sites for interface binding and catalysis. *J. Lipid Res.* 1991. **32:** 963–975.

Supplementary key words fusion protein • CHO cells • serine esterase • active sites • lipid binding

It is generally believed that hepatic lipase functions in the metabolism of circulating lipoproteins (1). The enzyme is synthesized only in the parenchymal cells of the liver, but is also present in the adrenal gland and the ovaries (2–8). The protein is synthesized as a precursor containing a 22 amino acid signal sequence, and, after processing, the mature glycoprotein is secreted from the hepatocytes and presumably transported to the surfaces of hepatic, ovarian, and adrenal endothelial cells (2, 3, 7, 9, 10). The presumed function of the enzyme is the hydroly-sis of triglycerides in intermediate density lipoproteins (IDL) and chylomicron remnants, as well as of phospholipids in high density lipoproteins (HDL) and in low density lipoproteins (LDL) (11). The broad substrate specificity of hepatic lipase, with the ability to hydrolyze phospholipids and a wide variety of glycerides, is consistent with these potential functions.

Hepatic lipase from rat liver has been purified to homogeneity (12, 13). The enzyme has an apparent molecular weight of 57,000 and contains approximately 8% carbohydrate. Its activity is characterized by salt resistance, an alkaline pH maximum, and binding to heparin. The cDNA for the rat enzyme has been previously isolated and sequenced (14). We have used a cDNA clone consisting of only the mature protein coding region and the 3'-untranslated region to produce a fusion protein in *E. coli*. Investigation of the activity of the fusion protein indicates that it is not able to catalyze hydrolysis of lipid substrates; however, it is able to act as an esterase when assayed on water-soluble substrates. In addition, we have constructed a eukaryotic expression vector to express the enzyme in high yield in Chinese hamster ovary (CHO) cells and have isolated the recombinant protein for further characterization. Our results suggest that the active site is functional and distinct from the lipid binding site.

EXPERIMENTAL PROCEDURES

Materials

Restriction endonucleases and T₄ DNA ligase were purchased from Pharmacia-LKB (Piscataway, NJ). All enzymes were used according to the supplier's instruc-

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Abbreviations: IDL, intermediate density lipoproteins; LDL, low density lipoproteins; HDL, high density lipoproteins; CHO, Chinese hamster ovary; PBS, phosphate-buffered saline; TBS, Tris-buffered saline; PNPB, *p*-nitrophenyl butyrate.
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tions. Adaptor linkers were obtained from New England Biolabs (Beverly, MA). Radioisotopes were obtained from DuPont NEN (Boston, MA). The CHO-K1 cell line was obtained from the ATCC (Rockville, MD). Dulbecco's modified Eagle's medium (high glucose) (DMEM) and fetal bovine serum were purchased from GIBCO BRL (Grand Island, NY), and Coon's F-12 medium was purchased from Irvine Scientific (Santa Ana, CA). Heparin-Sepharose was purchased from Pharmacia-LKB and DEAE-Trisacryl M was purchased from IBF Biotechnics (Savage, MD). All other biochemicals, reagents and enzymes were purchased from Sigma (St. Louis, MO). Antiserum raised against native rat hepatic lipase was a kind gift from Drs. Howard Wong and Michael Schotz (Los Angeles, CA).

Methods

Construction of expression vectors. For production of a bacterial fusion protein, the rat hepatic lipase cDNA from clone C141.10 (14) was subcloned into the EcoR I site of M13mp19. After digestion with Kpn I and BamH I, the exonuclease III was used to generate a series of unidirectional deletion mutants (15). The reaction was performed at 22°C to remove an average of 50 bases per min. A number of deletions were sequenced in order to find one that no longer contained either the 5'-untranslated region or the 66-base region encoding the precursor region. This mutant, D75.2-73, was recloned into the EcoR I site of pUC18, using a linker to regenerate the deleted EcoR I site. After transformation into *E. coli* RR1 (16), ampicillin-resistant colonies were immunoscreened with anti-hepatic lipase antibody.

For eukaryotic expression, full-length hepatic lipase cDNA (containing a 15-base 5' untranslated region, and the entire leader, coding a 3' untranslated regions) was excised from the plasmic C141.10 (14) with EcoR I and recloned into the BamH I site of the vector pMTSV40polyA·Bam using oligonucleotide adaptors. The sequence of the ends of several positive clones was established by double-stranded dideoxy sequencing (17) using primers from within the insert.

Isolation of fusion protein. Bacteria were grown in tryptone–yeast extract at 37°C to an A_{600} of 0.5, induced by the addition of isopropyl-β-D-thiogalactoside to a concentration of 0.35 mM and shaken at 37°C for an additional 4–8 h. Cells were pelleted, resuspended in TEN (50 mM Tris-HCl pH 8.0, 25 mM Na₂EDTA, 0.2 M NaCl), and lysed by the sequential addition of lysozyme (150 μg/ml for 20 min, 25°C) and Nonidet P-40 (to 0.1% w/v). Samples were homogenized in a Polytron for 1 min at the maximum setting and centrifuged for 10 min at 12,000 g at 4°C. The pellet was washed three times with water and with 20 mM Tris-HCl, pH 7.6, 1 mM EDTA. Samples were resuspended in urea–SDS buffer (10 mM NaPO₄, pH 7.2, 1% v/v β-mercaptoethanol, 1% w/v SDS, and 6

M urea). Preparative denaturing polyacrylamide gel electorphoresis was done as described (18), using a Hoefer SE 600 vertical slab gel apparatus containing a 10%, 1.5 mm-thick gel with a single slot. Gels were stained without fixing with cupric chloride (19). Protein was electroeluted from gel slices with an ISCO Model 1750 sample concentrator, into 20 mM Tris-HCL, pH 8.4, 0.15 M glycine, 0.01% SDS. SDS was removed using an Extracti-Gel column (Pierce, Rockford, IL). In a typical experiment, 1 mg of total insoluble bacterial protein was layered on a gel, and approximately 50–100 μg of fusion protein was recovered (final yield).

Cell culture, transfection, and induction. CHO-K1 cells were grown in an equal-volume mixture of DMEM and Coon's F-12 supplemented with 10% fetal bovine serum at 37°C and 5% CO_2 as described (20). Plasmids were co-transfected with pSV2neo (21) at a molar ratio of 10:1 into CHO cells by the calcium phosphate precipitation method with glycerol shock as described (22). At 24 h post-transfection, the antibiotic G418 was added (in fresh medium) to a final concentration of 400 μmg/ml. The cells were washed with phosphate-buffered saline (PBS) every 2 days, and fresh G418-containing medium was added. Cell death in mock-transfected plates was seen at day 6 of G418 addition, and selection was stopped after 2 weeks.

Isolation of lipase-producing clones. Cells were plated in 96-well microtiter dishes at a titer of 3–5 cells per well. After cell growth to approximately 90% confluence wells were washed once with pBS and 100 μl of induction medium (DMEM:Coon's F-12 1:1 containing 30 μM ZnSO₄ plus 10 μg/ml sodium heparin) was added to each well. After a 24-h incubation, the medium was removed to a fresh microtiter dish and the cells were overlaid with DMEM:Coon's F-12 with 10% fetal bovine serum and returned to the incubator. Fifty-microliter aliquots of conditioned medium were applied to nitrocellulose paper [Nitroplus 2000, MSI, Westboro, MA, pore size of 0.45 μm, prewashed with Tris-buffered saline (0.01 M Tris-HCl, pH 8.0, 0.15 M NaCl) (TBS)] in a dot-blot apparatus (Bio-Rad), using a slight vacuum. The wells were washed twice with 200-μl aliquots of TBS. Filters were air-dried for 30–60 min and developed with rabbit antibody to native hepatic lipase followed by alkaline phosphatase-conjugated goat and anti-rabbit second antibody (Promega). Lipase producing cells were cloned by two additional rounds of end-point dilution, growth, and rescreening.

Purification of CHO recombinant lipase. Cells were grown in flasks or in roller culture in DMEM:Coon's F-12 (1:1) plus 10% fetal bovine serum until just subconfluent and the medium was replaced with induction medium. This medium was harvested and replaced every 24 h for up to 2 weeks with little decrease in the yield lipase. Trasylol was added to 300 units/ml and the medium was spun at 5,000

g for 10 min and stored at $-80°C$ until used. Lipase was purified on heparin-Sepharose and DEAE-Trisacryl M columns as described (13, 23). Fractions containing lipase activity were concentrated with Centricon-30 filters and stored in liquid nitrogen.

Enzyme assays. Triglyceride lipase assays were done by the method of Nilsson-Ehle and Schotz (24), using a [³H]triolein sonicated emulsion in the presence of 1 M NaCl. Monoglyceride lipase and phospholipase assays were done as described (25, 26). For determination of the positional specificity of the phospholipase activity, the substrates 1,2-di[1-¹⁴C]palmitoyl-*sn*-3-glycerophosphocholine and 1-palmitoyl,2-[1-¹⁴C]palmitoyl-*sn*-3-glycerophosphocholine were used. Assays of hydrolysis of short-chain triglycerides were done using a Dosimat 412 (Metrohm, Switzerland), except for triacetin, which was assayed by the method described by Chapus et al. (27). Esterase assays, using the substrates *p*-nitrophenyl acetate or *p*-nitrophenyl butyrate, were done by the method of Quinn et al. (28), in 1-ml reactions of 0.1 M sodium phosphate, pH 7.25, containing 1.0 M sodium chloride, with the addition of fatty acid-free bovine serum albumin (Miles, Naperville, IN) to keep the protein concentration constant at 50 μg/ml. The reactions were done in a jacketed 1-cm path-length cuvette (Hellma, Jamaica, NY) and monitored at 400 nm in a Beckman DU-62 spectrophotometer. Temperature was maintained at 37°C using a circulating water bath. Reactions were prewarmed to 37°C for 10 min before the addition of PNPB and enzyme. Background values were determined from a no-enzyme blank reaction. The concentration of the released *p*-nitrophenol was determined using a molar extinction coefficient of 16,230 (28). All assays were run in triplicate. Inhibition of hepatic lipase in the presence of SDS was done as described (29). Inhibition of esterase activity by phenylmethylsulfonyl fluoride (PMSF) was done by preincubation of enzyme extracts in 1 mM PMSF for 15 min, followed by substrate addition and assay of hydrolysis.

Production of antisera. Anti-fusion protein antibodies were raised in rabbits. The initial injections used a slurry of the insoluble bacterial lysate, resuspended in SDS-urea (see above) and injected with Freund's complete adjuvant. Approximately 1 mg of total insoluble protein was injected per rabbit. Boosts were done at 4-week intervals with gel-purified fusion protein (50 μg/injection) and Freund's incomplete adjuvant. In order to generate antipeptide antibodies, peptides were derivatized with PPD (Cambridge Research Biochemicals, Valley Stream, NY) and injected subcutaneously into rabbits that had been sensitized with BCG vaccine. Boosts were done at 4-week intervals. All peptide injections used 100 μg of peptide conjugate.

Other methods. SDS-polyacrylamide gel electrophoresis was done as described (18) using a minigel apparatus

(Hoefer Scientific). Gels were silver-stained by the method of Wray et al). (30). Immunoblots were done as described by Doolittle et al. (2). Blots were developed using a 1:1000 dilution of the indicated antibodies followed by visualization using an alkaline phosphatase-based kit (Promega). Determination of protein concentrations was done using the bicinchoninic acid (BCA) protein assay reagent method (31) (Pierce, Rockford, IL). Isoelectric focusing was done using a Rotofor focusing apparatus (Bio-Rad, Richmond, CA) in a 2% carrier ampholyte solution (pH 3 to 10) containing 10 mM NaPO₄, pH 7.0, at 12 watts for 6 h. Immunobeads were purchased from Bio-Rad and linked to IgG and used in immunoprecipitations according to product instructions. Native rat hepatic lipase was released from rat livers by heparin–saline perfusion and purified as described (13) and modified (23).

RESULTS

Production of hepatic lipase bacterial fusion protein

Hepatic lipase cDNA was fused to a DNA segment encoding the N-terminal three amino acids of the α-peptide of *E. coli* β-galactosidase in pUC18 (Met Thr Met). The construct also contained, between the β-galactosidase and the N-terminal amino acids of the mature hepatic lipase, four amino acids encoded by the linker sequence (Ile Thr Asn Ser), for a total protein coding region of 479 amino acids, including the 472 amino acids of the mature hepatic lipase. Transcription of the fusion was under the
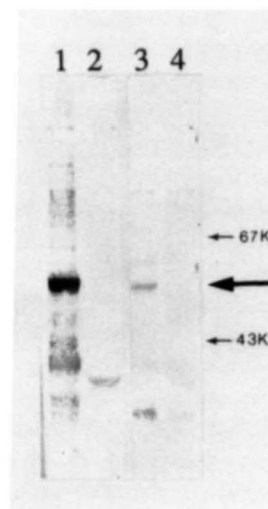
Fig. 1. Western blot of bacterial fusion protein. Extracts from induced *E. coli* RR1 containing either D75.2-73 (lanes 1 and 3) or pUC18 (lanes 2 and 4) were run on 8% SDS-PAGE and blotted as described in the text. Lanes 1 and 2 were developed with antiserum raised against native rat hepatic lipase, and lanes 3 and 4 were developed with antiserum raised against a synthetic peptide (see text). The large arrow indicates the migration position of the fusion protein. Small arrows indicate marker sizes, in kilodaltons.
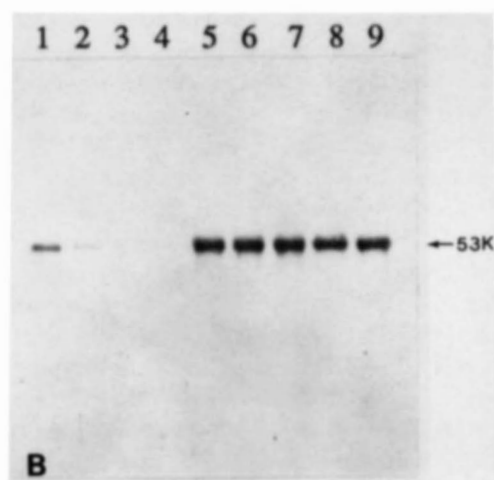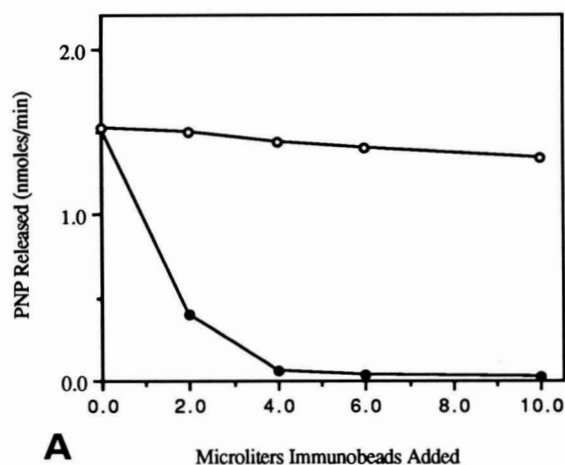
Fig. 2. Esterase activity in crude lysates. One microgram of purified fusion protein was incubated with varying amounts of IgG-linked Immunobeads for 60 min at 4°C. The beads were removed by centrifugation and the supernatants were assayed for esterase activity and also run on SDS-PAGE for immunoblot analysis. The results from the esterase assays are shown in panel A. Open circles show the esterase activity remaining in the supernatant using preimmune beads, while filled circles show the activity using anti-peptide beads. Panel B shows the results from the Western blot. The blot was developed with polyclonal antibody to native rat hepatic lipase. Tracks 1–4 in the blot show the supernatants from incubations with increasing amounts of anti-peptide beads (2 μl, 4 μl, 6 μl, and 10 μl, from left to right). Tracks 5–8 show the supernatants from incubations with increasing amounts of preimmune beads (2 μl, 4 μl, 6 μl, and 10 μl, from left to right). Track 9 contains untreated purified fusion protein. The protein size, in kilodaltons, is indicated by the arrow.

control of the *lac* promoter and could thus be induced with isopropyl-β-D-thiogalactoside. Aqueous-insoluble proteins from induced cells containing this construct were solubilized in SDS-urea, run on an SDS-polyacrylamide gel, transferred to nitrocellulose paper, and developed with antisera raised against either native rat hepatic lipase or a synthetic peptide corresponding to amino acids 209–238 in the rat hepatic lipase sequence (**Fig. 1**). Both antibodies recognized a single protein band in the expected size range of the fusion protein (52 kDa), whereas no similar-sized immunoreactive band was present in lysates from cells which did not contain the fusion vector. There were a number of other immunoreactive bands present in the fusion-containing lysate (Fig. 1); however, these were not present in the control lysate, indicating that they were derived from the fusion construct, and represent proteolytic fragments or other related species. Despite the presence of seven additional amino acids, the major fusion protein species migrated with a slightly higher electrophoretic mobility on SDS-PAGE than did native rat hepatic lipase (data not shown), presumably because it was not glycosylated. The immunoreactive band was partially purified by preparative SDS-PAGE and recovered by electroelution. Fusion protein purified by this method was stable and soluble even after removal of SDS, and could thus be used in subsequent assays.

Catalytic activity of fusion protein

No lipolytic activity was detected when purified fusion was used in a triolein emulsion assay. The absence of activity was not due to the presence of an interfering substance (such as the low level of SDS remaining in the purified fusion protein after Extracti-Gel removal of detergent) or to an endogenous inhibitor, since the addition of purified fusion protein to assays containing native rat hepatic lipase did not inhibit lipase activity. Since native hepatic lipase is able to utilize a number of different substrates, assays were performed in which the length of the fatty acid carbon chains in water-insoluble substrates was varied from six carbons (tricaproin) to twenty (triarachidin). In addition, the degree of saturation of the fatty acid chain was varied, and monoolein and phosphatidylcholine were also assayed. The fusion protein failed to hydrolyze any of these water-insoluble substrates; however, esterase activity was detected when the assays contained the water-soluble substrates *p*-nitrophenyl acetate (PNPA), *p*-nitrophenyl butyrate (PNPB), or triacetin. Hydrolysis of water-soluble esters has previously been observed for native rat hepatic lipase (32), suggesting that the fusion protein retains the esterase activity of the native enzyme.

Origin of esterase activity

There was detectable esterase activity in lysates of the host bacteria, as well as in bacteria containing the vector pUC18; however, this was not responsible for the esterase activity that corresponded to the fusion protein. This was demonstrated in two ways. First, electroelution of the 52 kDa region of SDS-PAGE gels of lysates from vector-containing bacteria had no detectable esterase activity (data not shown). Second, IgG-linked Immunobeads were used to precipitate gel-purified fusion protein. Two types
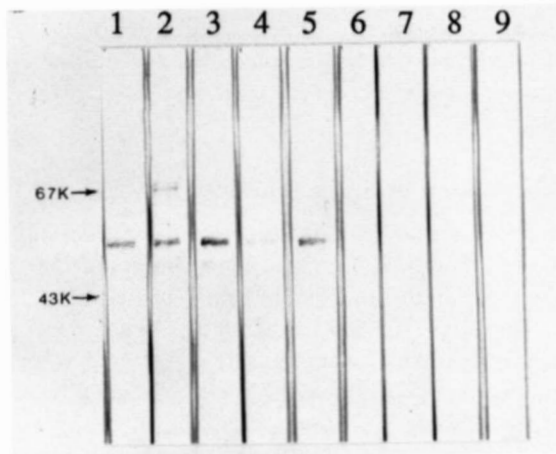
Fig. 3. Binding of anti-fusion antisera to native rat hepatic lipase. 8% SDS-PAGE gels were run and blotted as described in Materials and Methods. Blots were developed using 1:500 dilutions of crude rabbit serum, followed by binding of goat anti-rabbit second antibody conjugated to alkaline phosphatase and development with nitro blue tetrazolium and 5-bromo-4-chloro-3-indolyl phosphate. All lanes contained 30 ng of partially purified native rat hepatic lipase. Lane 1 was probed with anti-hepatic liapse, lanes 2–5 were probed with sera from the four different rabbits, and lanes 6–9 were probed with preimmune sera from the latter four rabbits. The arrows indicate the marker positions, in kilodaltons.

of IgG were used to make the beads: purified IgG from a rabbit injected with a synthetic peptide corresponding to amino acids 209–238 of mature rat hepatic lipase, and purified preimmune IgG from the same rabbit. After immunoprecipitation, the supernatants were assayed for esterase activity and, in addition, run on SDS-PAGE, blotted, and developed with polyclonal antibody to native rat hepatic lipase. As can be seen in **Fig. 2**, the disappearance of the fusion protein-specific band was seen only

when the anti-peptide IgG was used, and corresponded to a decrease in the observed esterase activity.

Anti-fusion protein antibodies

Gel-purified fusion protein was used to raise antiserum in rabbits. Four rabbits were injected and boosted, and serum was collected and assayed for binding to native rat hepatic lipase and inhibition of activity. As can be seen (**Fig. 3**), serum from each of the injected rabbits bound to a protein with the same M_r as native hepatic lipase, while preimmune serum did not bind. Despite antigen and lipase recognition by all four antiserum, only one of the antisera inactivated native hepatic lipase (**Fig. 4**).

Isolation of CHO cells producing rat hepatic lipase

The vector pMTSV40polyA·Bam is a derivative of pHSI (20, 33, 34) that contains a metallothionein promoter, the SV40 enhancer, and the 3' untranslated region of the human growth hormone cDNA (**Fig. 5**) [This vector is also known as pMThGHSV40 2, and has been described in more detail (34)]. The expression of foreign DNA cloned into the BamH I site is under the control of the human metallothionein II (hMTII) gene promoter and is thus inducible with zinc. In the expression construct D71.A-32, the rat hepatic lipase cDNA insert contained the entire coding region and 3' untranslated region, as well as the 66-base (22 amino acid) signal peptide and 15 bases of 5' untranslated region. This construct was cotransfected into CHO cells with the selectable marker pSV2neo, and a pool of G418-resistant cells was selected and used to isolate lipase-producing cell lines. Since rat hepatic lipase is a secreted protein (35), the medium of induced cells was assayed for the presence of
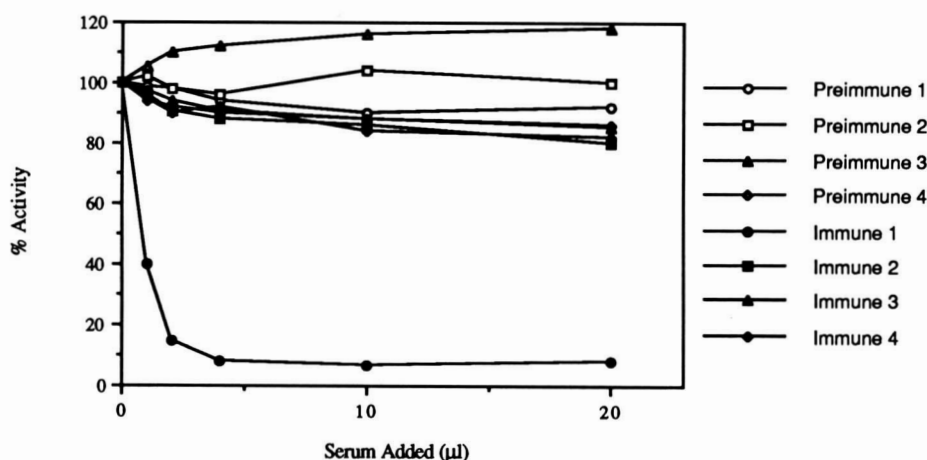
Fig. 4. Inhibition of rat hepatic lipase by antisera to bacterial fusion protein. Purified lipase was incubated for 1 h with immune or preimmune serum in a total volume of 100 μl and assayed in the triolein emulsion described in Materials and Methods. Sera from four different rabbits were used. The 100% value refers to lipase incubated without antiserum.
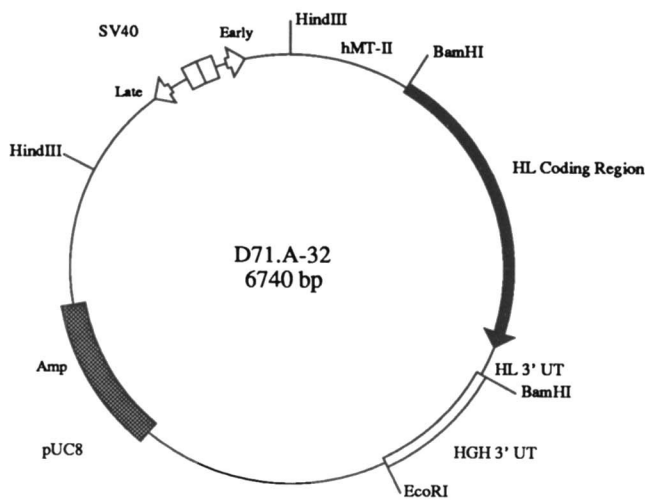
Fig. 5. Structure of the mammalian hepatic lipase expression plasmid D71.A-32. A BamH I fragment containing the rat hepatic lipase cDNA (HL) was inserted into the vector pMTSV40polyA·Bam as described in the text. Features of this vector include most of pUC8, the human metallothionein II promoter (hMT-II), and the human growth hormone 3' untranslated region (HGH). Arrows indicate directions of transcription.

the enzyme by protein dot blotting and immunoscreening, and individual cells were cloned and expanded. The cell line D71.A1 secreted the highest levels of triglyceride lipase activity and was chosen for further analysis.

### RNA blot analysis of lipase-producing CHO cells

Northern blot analysis of D71.A1 cells and untransfected CHO cells (**Fig. 6**) showed that a single hybridizing band was seen in the former and none in the latter. There was substantially more hepatic lipase-specific mRNA in the D71.A1 cells than in rat liver, as shown by the difference in the amount hybridized. The difference in mRNA levels was even more pronounced than is apparent in Fig. 6, since the rat liver RNA was selected on oligo dT-cellulose and thus enriched for the mRNA, while the D71.A1 RNA was total cellular RNA. These data also indicate that the hepatic lipase-specific band in the D71.A1 cells was slightly larger than the corresponding band in rat liver mRNA. It is probable that this is the result of use of the polyadenylation site from the human growth hormone cDNA, which is present in the vector and should yield an mRNA that is approximately 150 bases larger than RNA that used the hepatic lipase polyadenylation site. Also shown in Fig. 6 are RNA samples from induced sense- and antisense-transfected pools. These are RNAs from CHO cells that have been transfected with pMTSV40polyA·Bam containing the hepatic lipase cDNA in either the coding (sense) orientation or the opposite (antisense) orientation, with respect to the metallothionein promoter. These samples were extracted from the total transfection pools after 2 weeks of G418 selection, and the results indicate that a substantial

amount of lipase-specific RNA is readily seen in the total pool. In addition, the results suggest that either the antisense construct is not transcribed or the RNA is rapidly degraded.

Characterization of lipase produced in CHO cells

Western blot analysis of medium from induced D71.A1 cells showed that the cells produced a protein that was immunologically indistinguishable from, and electrophoretically identical to, native hepatic lipase. Neither nontransfected CHO cells nor cells transfected with the vector alone secreted proteins that were detected by antibodies to the native protein (**Fig. 7**). In addition, we screened medium from a pool of G418-resistant cells that had been cotransfected with pSV2neo and a DNA construct containing the hepatic lipase cDNA in the antisense orientation. This pool, in contrast to a pool from the above sense-orientation transfection, did not exhibit either salt-resistant lipase activity (data not shown) or reactive bands on an immunoblot (Fig. 7).

The lipase secreted from D71.A1 cells focused at an isoelectric point of 4.9, as has been observed for native rat hepatic lipase (35). The recombinant lipase bound to heparin-Sepharose, hydroxyapatite and DEAE-Sephacel,
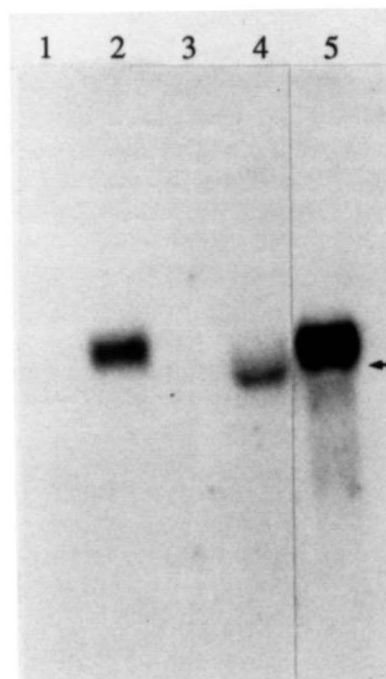
Fig. 6. Northern blot analysis of transfected CHO cells. Ten μg of each total cellular RNA was run on a denaturing agarose gel, transferred to a nylon filter, and hybridized to a rat hepatic lipase cDNA probe as described (14). RNA was purified from the following sources; lane 1, untransfected CHO cells; lane 2, sense-transfected pool; lane 3, antisense-transfected pool; lane 4, rat liver poly A + (10 μg); lane 5, D71.A1 cells. The arrow indicates the position of 18S RNA.
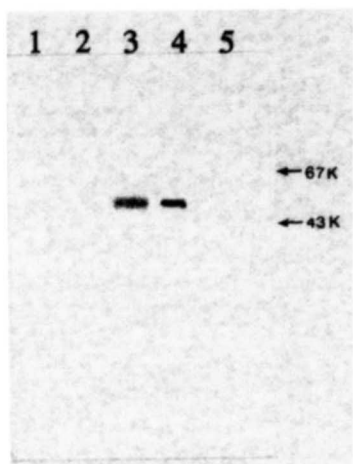
Fig. 7. Western blot analysis of medium from transfected cells. Cells were grown to confluence and induced as described, and 12 μl of each conditioned medium was run on 8% SDS-PAGE, transferred, and developed with antibody to native rat hepatic lipase. The following cell lines or samples were used: lane 1, nontransfected CHO cells; lane 2, CHO cells transfected with vector alone; lane 3, D71.A1 cells; lane 4, native 2, CHO cells transfected with vector alone; lane 3, D71.A1 cells; lane 4, native rat hepatic lipase (50 ng); lane 5, antisense-transfected cells. Marker sizes (in kilodaltons) are indicated by the arrows.

again properties that have been demonstrated for the native hepatic lipase (36). Other such properties exhibited by the recombinant lipase include an alkaline pH optimum, lipolytic activity in the presence of 1 M NaCl, and inactivation by 0.5 mM sodium dodecyl sulfate (29, 37).

Recombinant lipase was partially purified as described in Methods. After heparin-Sepharose and DEAE-Trisacryl M column chromatography steps, SDS-PAGE analysis showed a major band with the same relative mobility as rat hepatic lipase (**Fig. 8**). This species also has the same relative mobility as the immunoreactive material seen in Fig. 7 (data not shown). Faint minor bands were observed, suggesting that further purification would be necessary to isolate homogeneous lipase.

A variety of substrates was used to characterize the purified D71.A1 lipase. Both native rat lipase and the recombinant protein hydrolyzed triglycerides, monoglycerides, phospholipids, and water-soluble esters (data not shown). The substrates 1,2-di[1-^{14}C]palmitoyl-sn-3-glycerophosphocholine and 1-palmitoyl,2-[1-^{14}C]palmitoyl-sn-3-glycerophosphocholine were used as described (26) for determination of the positional specificity of the phospholipase activity. When the former (labeled at both the sn-1 and sn-2 positions) was used, ^{14}C-labeled fatty acid was released, while use of the latter (labeled at only the sn-2 position) showed no release of isotope (data not shown). The phospholipase activity was thus confined to the sn-1 position, confirming the identity of the recombinant protein as a phospholipase A$_1$. The activities of both the

recombinant CHO protein and native hepatic lipase were examined as a function of triolein concentration in the range 0.1–2.0 mM, and the apparent K_m and V_{max} for each protein were determined by Lineweaver-Burk analysis (**Table 1**).

Production of lipase

An ELISA (M. Komaromy, unpublished results) was used to quantitate the amount of immunoreactive protein produced in the roller culture system. A single 850 cm^2 roller bottle contained approximately 3 × 10^9 cells at the start of induction, and, under ideal conditions, produced approximately 80 μg of recombinant lipase per day. The specific activity of the crude lipase suggested that most of this material was intact, active lipase. Previous work using a similar vector to produce apolipoprotein A-I described a yield of 7 mg per bottle under similar conditions of growth and induction (20). It is probable that this large difference in yield is a reflection of the relative instability of hepatic lipase.

The stability of the secreted lipase was enhanced by the presence of heparin in the culture medium, as was previously noted for the enzyme secreted from hepatocytes (38). Without heparin, the lipase activity in the medium achieved a maximum with 2 h, while, in the presence of 10 μg/ml heparin, lipase activity increased linearly until 20 h post-induction. The addition of more heparin did not further increase the stability of the activity. The effects of various protease inhibitors on the yield of lipase were analyzed, and it was found that the addition of aprotinin (to 0.05 μg/ml), leupeptin (to 50 μg/ml), pepstatin A (to 5 μg/ml), or antipain (to 10 μg/ml) alone or in combination, increased the yield of active lipase by approximately 25%. The yield of purified recombinant lipase, after the protocol described in Methods, was esti-
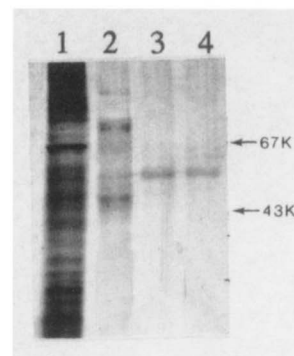
Fig. 8. Purification of CHO-derived recombinant lipase. Samples were run on 8% SDS-PAGE and silver stained as described in the text. Lane 1, ammonium sulfate precipitate; lane 2, heparin-Sepharose eluate; lane 3, DEAE-Trisacryl M eluate; lane 4, purified native rat hepatic lipase (0.2 μg). Arrows indicate marker sizes in kilodaltons.

TABLE 1. Michaelis constants and reaction velocities for native and recombinant proteins

Protein	Substrate	V_{max}	K_m
			mM
Native rat hepatic lipase	triolein	59,200	0.78
Native rat hepatic lipase	PNPB	415	1.32
CHO-derived lipase	triolein	66,800	0.85
CHO-derived lipase	PNPB	467	1.43
Bacterial fusion protein	PNPB	43	1.91

The values given for triolein substrate are apparent values.
V_{max} is given in μmol of product released per mg of protein per h. Reaction conditions are given in the text.

mated to be approximately 30%. Using a roller culture system, milligram quantities of recombinant protein can thus be purified with a relatively modest effort.

Characterization of esterase activities of bacterial fusion protein and CHO-produced lipase

Further experiments were undertaken to characterize the esterase activities of the fusion protein and hepatic lipase. The K_m and V_{max} were measured (Table 1), and the specific activity of the fusion protein esterase was shown to be lower than the esterase activity of either native hepatic lipase or the CHO-produced enzyme (**Fig. 9A**). The esterase activity of all three proteins exhibited time-dependence (Fig. 9B). The serine esterase inhibitor phenylmethylsulfonyl fluoride (PMSF) decreased the fusion protein esterase activity by approximately 55% (**Fig. 10**). Similar effects were observed with both the native and CHO-produced lipases, suggesting that serine residues are involved in the catalysis (39).

The mixed-substrate (12, 40) method was used to determine whether the same active site is used for the esterase and lipase activities of native rat hepatic lipase. Triolein lipase assays were run in the presence of different concentrations of PNPB, and a double reciprocal plot of the rate of the reactions (**Fig. 11**) showed that PNPB behaved as a competitive inhibitor, indicating that the triglyceride lipase and the esterase activities use the same active site.

DISCUSSION

The elucidation of the role that hepatic lipase plays in lipoprotein metabolism has been hampered largely because the enzyme is relatively unstable (36). We have established a derivative of the well-characterized CHO cell line that serves as a continuous source of large quantities of secreted enzyme. Cells are induced, in the absence of serum, by the addition of zinc to the culture medium, and lipase-containing conditioned medium can be collected daily. The use of serum-free medium facili-

tates purification, since serum adds a major source of contaminating protein. In addition, cell growth is slowed considerably in the absence of serum. After approximately 10–12 days of growth in induction medium, the cell mass only doubles, and the cells do not begin to die until day 14-16. The yield of the lipase is constant until this time; thus, the medium can be tapped for up to 2 weeks. In contrast, in the presence of serum, growth continues more quickly, and cells begin to die 3–4 days after achieving confluence, diminishing the overall yield of lipase. A substantial purification of the protein is straightforward and involves only two rapid chromatographic steps. This contributes to an increased yield of the enzyme, since the lipolytic activity is not stable during the preparation.

The electrophoretic mobility on SDS-PAGE is identical to that of the native lipase, even though the enzyme produced in the CHO cells is not correctly glycosylated. CHO cells produce only a β-galactoside α-2,3
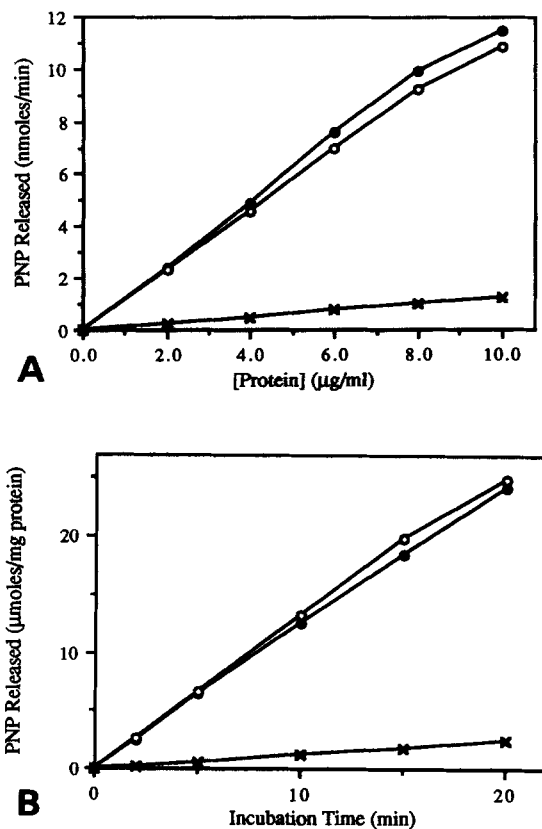

Fig. 9. A: Dependence of the esterase reaction velocity on protein concentration. The substrate was PNPB at a concentration of 1 mM. Reactions were run using the indicated amounts of protein for 10 min as described in Methods. B: Time course for the hydrolysis of PNPB. Reactions used 10 μg of lipase or fusion protein in a 1-ml reaction, with the same assay system as above. In each graph, the native rat hepatic lipase is represented by the open circles, the CHO-derived recombinant lipase is represented by the closed circles, and the fusion protein is represented by the crosses.
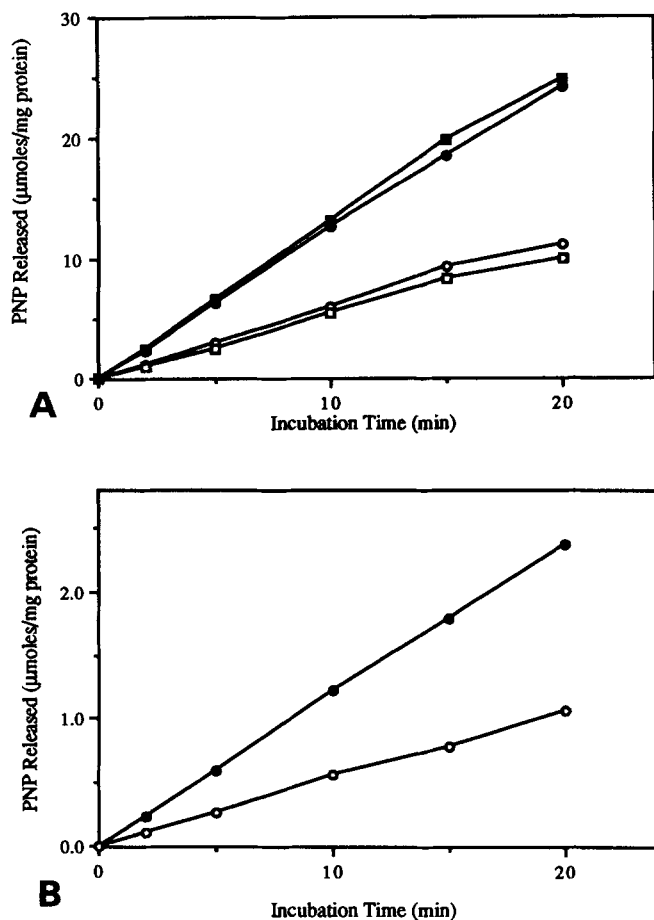
Fig. 10. A: PMSF inhibition of esterase activity of rat hepatic lipase and CHO-produced recombinant lipase. Ten μg of each enzyme was incubated with 1 mM PMSF in a complete esterase assay mix (see Methods), minus substrate, for 15 min at 37°C. Ten μl of 10 mM PNPB was added to each reaction and the absorbance at 400 mn was monitored for the indicated times. Open and closed squares refer to native rat hepatic lipase with and without PMSF added, while open and closed circles refer to native rat hepatic lipase with and without PMSF added, while open and closed circles refer to CHO-produced lipase with and without PMSF. B: PMSF inhibition of esterase activity of fusion protein. Reactions were done as above, with (open circles) and without (closed circles) PMSF.

sialyltransferase, instead of the β-galactoside α-2,6 sialyltransferase produced in hepatocytes (41, 42), so that the conformation of the sialic acid in the mature lipase is not correct in the recombinant protein. We have confirmed this (M. Komaromy, unpublished results) by demonstrating that the lectin from elderberry bark, which is specific for sialic acid attached to terminal galactose in the α-2,6 conformation (43), binds to native rat hepatic lipase and does not bind to the recombinant lipase. Conversely, the lectin from *Maackia amurensis*, which binds preferentially to sialic acid attached to terminal galactose in the α-2,3 conformation (44), binds to the recombinant lipase and not to the native lipase. After treatment with neuraminidase, the native and recombinant proteins are

indistinguishable. The results presented here indicate that the conformation of the sialic acids is not important for any of the biochemical properties that were measured, including kinetic constants, molecular weight, and binding characteristics.

The CHO-derived recombinant enzyme retains all of the known lipolytic activities of the native enzyme. These include the hydrolysis of a wide variety of triglycerides, as well as phospholipase A_1 activity and hydrolysis of small soluble substrates (esterase activity). The kinetic constants for the CHO protein and native rat hepatic lipase are in good agreement with each other and with previous results obtained using the native lipase (12, 13). It therefore appears that the CHO-derived recombinant enzyme is functionally identical to the native lipase.

It is noteworthy that this is not the case with the protein derived from a bacterial source. We have produced the entire mature hepatic lipase coding region as a fusion protein with seven non-lipase amino acids at the N-terminus. The fusion protein was expressed as an insoluble intracellular protein that could be purified by preparative gel
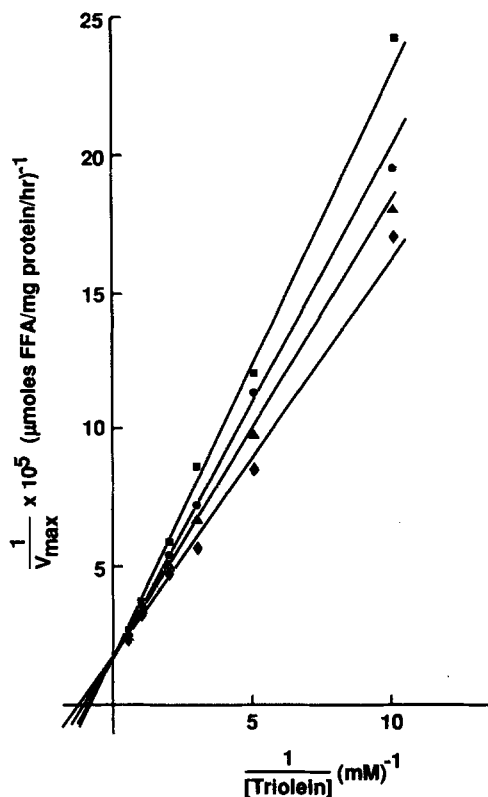
Fig. 11. Lineweaver-Burk plot of the rate of hydrolysis of triolein in the presence of different concentrations of *p*-nitrophenyl butyrate. Triolein assays (see Methods) were run without PNPB (diamonds) or in the presence of 0.1 mM (triangles), 0.2 mM (circles), or 0.5 mM (squares) *p*-nitrophenyl-butyrate. Assays were run in triplicate, and blank (enzyme-free) reactions were extracted to determine background and quenching.

electrophoresis. Since this protein was produced in *E. coli*, it was not glycosylated and was presumably not folded correctly. It was functionally active as an esterase when assayed on soluble substrates, but was not active as a lipase when a triglyceride substrate was used. The esterase activity of the fusion protein in crude lysates was only about twofold above background, but it is probable that most of the fusion protein in these lysates was contained in inclusion bodies and was therefore inactive in our assay (45). Immunoprecipitation of purified fusion protein with an anti-peptide polyclonal antibody also resulted in removal of the esterase activity, suggesting that the observed activity was not due to a bacterial protein.

The V_{max} for the fusion esterase was substantially different from the values for the CHO and native lipases. We suggest that this was probably due to either enzyme damage sustained during the isolation of the bacterial protein, which would have resulted in a lower apparent specific activity, or to the presence of the extra amino acids of the fusion moiety. It is also possible that the lack of glycosylation had an effect. In order to determine whether protein damage could have occurred during gel electrophoresis and electroelution, we have subjected pure CHO-derived lipase to the same procedures and measured the esterase specific activity. We have found that approximately 90% of the esterase activity could be recovered (M. Komaromy, unpublished data), suggesting that this phase of the isolation is not responsible for the loss. (We also note that, in this experiment, the triglyceride lipase activity was totally eliminated by this procedure, which supports the hypothesis that tertiary structure is necessary for lipid binding). It is still possible that irreversible damage occurred prior to electorphoresis. It is also possible that the fusion protein was not pure, since the one-dimensional electrophoresis analysis would not reveal comigrating proteins. It is unlikely that a large perentage of the electroeluted protein consists of non-fusion contaminants, since no band with the same M_r was seen in extracts from cells that did not contain the fusion construct. It is not likely that low-level impurities inhibited esterase activity, since extracts from non-fusion hosts were not inhibitory.

We have also produced another insoluble fusion protein that used a different fusion moiety, the *trpE* gene product from *E. coli*. The non-hepatic lipase portion of this fusion protein is 37 kDa, which represents a substantial portion of the total protein size of 90 kDa. We therefore chose to focus the biochemical studies on the smaller β-galactosidase fusion and thus presumably the simpler system. Interestingly though, the esterase activity of the purified *trpE* fusion was of comparable magnitude to that of the β-galactosidase fusion, again suggesting that the esterase activity is an intrinsic property of the hepatic lipase portion of the fusion.

The identification of the fusion protein as the source of the observed esterase activity is based on three experiments. First, as described, electroeluted protein from the 52 kDa region of SDS-PAGE gels of lysates from vector-containing bacteria had no detectable esterase activity. Second, immunoprecipitation of the fusion protein with an anti-peptide antibody also removed the esterase activity from the supernatant. It is unlikely that the anti-peptide antibody precipitated a bacterial esterase, particularly since no reactive protein in the 52 kDa region of a Western blot could be detected with this antibody (see Fig. 1). Third, a completely different fusion protein, with a different electrophoretic mobility, displayed a similar esterase activity.

Little information is available concerning the mechanism of action of hepatic lipase. The protein sequence established that it is a member of a group including lipoprotein lipase (14, 46), which has been proposed to use a serine-histidine reaction mechanism (47). The two enzymes have several strongly conserved regions (14), including a number of serine and histidine residues. A hepatic lipase serine residue important in catalysis has been identified by site-directed mutagenesis (48). Our results showing inhibition of the esterase and lipolytic activities of native lipase with phenylmethylsulfonyl fluoride further imply that hepatic lipase is acting as a serine esterase.

We suggest that the substrate specificity of the fusion protein implies that interfacial binding is mediated by either the tertiary structure of the protein or the carbohydrate modifications, while at least one catalytic site can apparently be formed from local amino acid sequence. Work with lipoprotein lipase has suggested that glycosylation is necessary for enzymatic activity (49). In contrast, results obtained using hepatic lipase (M. Komaromy, unpublished results, and 50, 51) suggest that some carbohydrate modifications are necessary for secretion of lipase from hepatocytes, but complete processing is not necessary for enzyme activity. Evidence using in vitro mutagenesis to alter the two glycosylated asparagine residues has shown that active lipase is produced in *Xenopus* oocytes (52). These experiments did not address the subject of substrate specificity, since only triglyceride lipase activity was assayed; however, the results suggest that it is not likely that the lack of glycosylation is responsible for our observed loss of triglyceride lipase activity.

It has previously been postulated that interface recognition and catalysis are localized to different sites in the lipase molecule. Several groups have reported findings that suggest that mild proteolysis differentially affects the esterase activity and the lipolytic activities of hepatic lipase (35, 53-55). Similar results have been obtained for bovine hormone-sensitive lipase (56) and for human gastric lipase (57). A mutant human lipoprotein lipase has

been identified which is apparently defective in lipase activity, but is able to hydrolyze tributyrin (58). Other work with porcine pancreatic lipase (59) has shown that modification of a presumed interfacially active serine with diethyl-p-nitrophenylphosphate inactivates enzyme activity against lipid substrates but not against p-nitrophenyl acetate, again suggesting that two sites are involved. Our findings support the two-site hypothesis, since the fusion protein effectively uncouples the two processes. It is interesting to note that, in the work of Bernbäck and Bläckberg (57), it was shown that deletion of a tetrapeptide at the N-terminus of human gastric lipase resulted in the preferential loss of lipid binding and lipase activity, while activity against soluble substrates was retained.

We emphasize that these experiments did not directly address the question of the mechanism of the loss of triglyceride lipase activity; however, it is possible that the N-terminus of hepatic lipase is involved in interface binding, or that this region mediates folding that is necessary for lipid binding. The loss of lipase activity from recombinant CHO lipase isolated from SDS-PAGE sugests that denaturation of the enzyme, regardless of the amino acid sequence of the N-terminus, is the primary defect. It is possible that the addition of sequences to the N-terminus, as in the bacterial fusion protein, could disrupt the structure needed for proper folding, and could explain our observations concerning loss of lipolytic activity and retention of esterase activity. ▪

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