The amino acid sequences of the carboxyl termini of human and mouse hepatic lipase influence cell surface association

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Abstract **Human hepatic lipase (hHL) mainly exists cell surface bound, whereas mouse HL (mHL) circulates in the blood stream. Studies have suggested that the carboxyl terminus of HL mediates cell surface binding. We prepared recombinant hHL, mHL, and chimeric proteins (hHLmt and mHLht) in which the carboxyl terminal 70 amino acids of hHL were exchanged with the corresponding sequence from mHL. The hHL, mHL, and hHLmt proteins were catalytically active using triolein and tributyrin as substrates. In transfected cells, the majority of hHLs bound to the cell surface, with only 4% of total extracellular hHL released into heparin-free media, whereas under the same conditions, 61% of total extracellular mHLs were released. Like mHL, hHLmt showed decreased cell surface binding, with 68% of total extracellular hHLmt released. To determine the precise amino acid residues involved in cell surface binding, we prepared a truncated hHL mutant (hHL_{471}) by deleting the carboxyl terminal five residues (KRKIR). The hHL_{471} also retained hydrolytic activity with triolein and tributyrin, and showed decreased cell surface binding, with 40% of total extracellular protein released into the heparin-free media. These data suggest that the determinants of cell surface binding exist within the carboxyl terminal 70 amino acids of hHL, of which the last five residues play an important role.**—Brown, R. J., J. R. Schultz, K. W. S. Ko, J. S. Hill, T. A. Ramsamy, A. L. White, D. L. Sparks, and Z. Yao. **The amino acid sequences of the carboxyl termini of human and mouse hepatic lipase influence cell surface association.** ***J. Lipid Res.*** **2003.** 44: **1306–1314.**

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Mature human hepatic lipase (hHL) contains 476 amino acids, and its apparent molecular mass varies from 55 to 69 kDa (1–3), presumably owing to variation in the magnitude of glycosylation at its four *N*-linked glycosylation sites. hHL is a member of a superfamily of lipases and phospholipases (EC 3.1.1.3) that share the GxSxG motif at the active site and a catalytic Asp-His-Ser charge relay triad found in a typical serine hydrolase (4). Known members of this superfamily include lipoprotein lipase (LPL), endothelial lipase, pancreatic lipase, and HL (1–3, 5–7). The active hHL is a homodimer (8) with broad substrate specificity and is involved in the metabolism of HDLs and triacylglycerol (TG)-rich lipoproteins (9–13).

A series of studies have been conducted to compare heparin binding, enzyme activity, and substrate specificities between HL and LPL (14–18). The amino acid sequences of HL and LPL show close sequence homology to that of pancreatic lipase. Thus, based on the known structure of pancreatic lipase showing a two-domain structure, HL and LPL have been modeled into two domains with distinct functions. The active sites of HL and LPL reside within the respective N-terminal domains. Relative to TG hydrolysis, HL displays higher phospholipase activity than LPL. The substrate specificity of HL and LPL is governed by a 22-amino acid loop ("lid") within the N-terminal domain of the respective lipases (15). The activity of LPL requires the cofactor apolipoprotein C-II (apoC-II) and is sensitive to high salt (i.e., 1 M NaCl), whereas the activity of HL is independent of apoC-II and remains active at 1 M NaCl. Studies with a chimeric protein composed of amino acid sequences derived from human LPL and hHL have shown that the catalytic properties and high-salt sensitivity

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Abbreviations: HBD, heparin binding domain; hHL, human hepatic lipase; HSPG, heparan sulfate proteoglycan; LPL, lipoprotein lipase; LRP, LDL receptor-related protein; mHL, mouse hepatic lipase; TG, triacylglycerol.

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of LPL are determined by the N-terminal domain (17), whereas the apoC-II activation of LPL probably involves both the N-terminal (16, 18) and carboxyl terminal domains of LPL (16). Studies with chimeric proteins have also suggested that the carboxyl terminal domains of HL and LPL play a role in determining their heparin affinity. The affinity of HL and LPL toward heparin can be estimated from the concentration of NaCl required to elute the lipases from immobilized heparin: 1.1 M NaCl to elute LPL and 0.75 M NaCl to elute HL (18). The heparin affinity of LPL is thus higher than that of HL. For chimeric proteins in which the carboxyl terminal sequences of human LPL were substituted with either rat (18) or hHL (17) sequences, reduced heparin binding affinity of the chimeric proteins was observed. From these studies, it has been suggested that the major determinant of heparin binding resides within the carboxyl terminal domain of hHL.

The majority of hHL activity and mass is found in the liver. Immunocytochemistry studies have revealed that hHL is located on the sublumenal extracellular matrix component of endothelium, the microvillar surface of hepatocytes in the space of Disse, interhepatocyte space, and lumenal surface of sinusoidal endothelium (19). Infusing heparin in vivo increases HL activity in the serum by 1,000-fold, suggesting an interaction of hHL with heparan sulfate proteoglycans (HSPGs) (20). In contrast to that of hHL (21), the major proportion of mouse HL (mHL) mass and activity (60–70% of total) circulates in the plasma (20). The low affinity of mHL to the cell surface could be attributable to the lack of glycosaminoglycans specific for HL binding that are present in humans but not in mice. Alternatively, the low affinity of mHL could be due to the lack of amino acid sequence elements that are present in hHL but not in mHL. Two pieces of evidence suggest that the determinants of HSPG binding lie within the amino acid sequence of HL. First, infusion of hHL into mice resulted in nearly a 100% association of hHL mass and activity with the cell surface (20). Second, mHL has a lower affinity for heparin versus hHL, as demonstrated by its earlier elution from heparin-Sepharose; 0.7–0.8 M NaCl was required to elute hHL, and 0.48 M NaCl for mHL (20).

The interaction of heparin with proteins containing HSPG binding motifs is presumably mediated by an electrostatic interaction (22) that can be interrupted by increasing salt concentrations. In addition to electrostatic forces, it is thought that a characteristic steric fit is also essential for specific binding to heparin (23–25). Common structural motifs were identified when sequences of a number of heparin binding proteins were compared (22); they are xBBxBx, xBxxBBBx, and xBBxxBBBxxBBx, respectively (B: basic amino acids). In the case of antithrombin III and apoE, a distinct distribution of two basic amino acids located about 20 Å apart (xBxxxxxxxxxxxxBx in α-helix and xBxxxxxxBx in β-strand) has been suggested to be critical in HSPG binding (26).

In the current study, we have tested the hypothesis that the carboxyl terminal region of hHL confers high-affinity cell surface binding activity through interaction with HSPG using chimeric HL proteins. Our data support the notion that the difference in cell surface association between hHL and mHL is caused by the divergence in the carboxyl terminal amino acids between the two proteins.

EXPERIMENTAL PROCEDURES

Materials

Triolein, tributyrin, fatty acid-free BSA, heparin, and fetal bovine serum (FBS) were purchased from Sigma. Dulbecco's modified Eagle medium (DMEM), Ham's F12 medium, and G418 were purchased from Gibco BRL. The Hi-Trap heparin-Sepharose columns, HRP-conjugated goat anti-mouse IgG antibody, HRP-conjugated goat anti-rabbit IgG antibody, and [^{35}S]methionine/cysteine were purchased from Amersham Pharmacia Biotech. [^{3}H]triolein was purchased from Dupont. [^{14}C]tributyrin was purchased from American Radiolabeled Chemicals, Inc. Restriction endonucleases were purchased from New England Biolabs. A polyclonal anti-hHL antibody (27) was a generous gift of Dr. Ann White (University of Texas Southwestern Medical Center). The monoclonal anti-hHL antibody XHL3-6 (28) was a gift of Dr. André Bensadoun (Cornell University). A polyclonal anti-rat HL antibody was a gift of Dr. Howard Wong (University of California, Los Angeles). The hHL was isolated from postheparin human plasma as described previously (29).

Preparation of HL expression plasmids

The hHL cDNA was excised from the pLiv10.hHL vector (provided by Dr. John Taylor at The Gladstone Institute) (30) by digestion with *Kpn*I and *Hind*III, and the *Kpn*I-*Hind*III fragment was inserted into the polylinker region of the pCMV5 expression vector (31) to create pCMV5.hHL. Similarly, the mHL cDNA was excised from the pSP64.mHL vector (provided by Dr. Hans Will at Hamburg University) (32) by digestion with *Hind*III and *Xba*I, and the *Hind*III-*Xba*I fragment was inserted into pCMV5 to create pCMV5.mHL. To create the chimeric construct hHLmt encoding amino acids 1−406 of hHL and amino acids 409−488 of mHL, the respective hHL and mHL cDNAs were excised from pCMV5.hHL and pCMV5.mHL by digestion with *Kpn*I and *Xba*I, and subcloned into pBluescript (pBlue.hHL and pBlue.mHL). A *Pflm*I-*Xba*I fragment was excised from pBlue.hHL and replaced with a *Pflm*I-*Xba*I fragment from pBlue.mHL, generating an in-frame chimeric HL construct (pBlue.hHLmt). The *Kpn*I-*Xba*I fragment was excised from pBlue.hHLmt and inserted into pCMV5 to create pCMV5.hHLmt. A chimeric construct encoding the amino acids 1−408 of mHL and the amino acids 407−476 of hHL, designated pCVM5.mHLht, was similarly constructed. To generate the plasmid encoding hHL_{471}, forward (ACGTAAGCTTGCCACCATGGACACAAGTCCCCTGTGT) and reverse (ACGTGGATCCTCATCTGATCTTTCGCTATGATGT) PCR primers were designed such that the carboxyl terminal five residues of wild-type hHL were eliminated. The PCR product was inserted into pCMV5 using the *Hind*III and *Bam*HI restriction sites that were encoded within the primers (underlined). All wild-type and mutant-HL constructs were sequence verified to ensure no errors were generated.

Generation of stable Chinese hamster ovary cell lines expressing recombinant HL

Chinese hamster ovary (CHO) 13-5-1 cells deficient in LDL receptor-related protein (LRP) expression (33) were cultured in Ham's F-12 medium containing 10% FBS. Cell lines were gener-

ated by cotransfecting 10 μg of the corresponding pCMV5 plasmid with 0.10 μg pSV2*neo* using the calcium precipitation method (34). Stable cell lines were selected with 500 μg/ml G418 and screened for HL expression by immunoblot analysis (details are described in figure legends).

Semi-purification of recombinant HL by heparin-Sepharose chromatography

Stably transfected cells were grown to confluency (T175 flasks), washed three times with phosphate buffered saline (PBS), and incubated overnight at 37°C with Ham's F-12 medium containing 1% FBS, 500 μg/ml G418, and 10 U/ml heparin. Media (up to 400 ml) were collected, centrifuged at 1,200 rpm for 10 min at 4°C to remove any cell debris, and the supernatant was adjusted with glycerol to a final concentration of 20%. The media was cooled to 4°C, then applied to a Hi-Trap heparin-Sepharose column at 4°C preequilibrated with 90% Buffer A (10 mM sodium phosphate, 20% glycerol, pH 7.2) and 10% Buffer B (10 mM sodium phosphate, 1.5 M sodium chloride, 20% glycerol, pH 7.2). Following reequilibration of the column, 33% Buffer A and 67% Buffer B (final NaCl concentration of 1.0 M) was used to elute bound proteins. The fractions (1 ml) were collected, and those containing protein (as detected using the absorbance at 280 nm) were stored at −80°C. In other experiments, confluent cells (T175 flasks) were washed three times with PBS and incubated with 20 ml serum-free media containing 100 U/ml heparin. Following 4 h incubation at 37°C, the media were treated as above and applied to a Hi-Trap heparin-Sepharose column at 4°C preequilibrated with 90% Buffer A and 10% Buffer B. Following reequilibration of the column, a gradient of Buffer A and Buffer B (NaCl concentration from 0.15−1.5 M) was used to elute the bound proteins. The fractions (1 ml each) were collected and stored at −80°C.

Measurement of HL activity

The activity of HL obtained by chromatography was determined using a [^{3}H]triolein emulsion (35) and [^{14}C]tributyrin (36), as previously described. The protein content of HL samples was determined using a modified Lowry assay (37).

Heparin treatment of cells

Stably transfected cells (100 mm dishes) were grown to confluency, washed three times with PBS, and incubated with either 4 ml serum-free Ham's F-12 medium supplemented with G418 or serum-free media containing 100 U/ml heparin. After 4 h incubation at 37°C, the media were collected, centrifuged at 1,200 rpm for 10 min to remove any cell debris, and the HL proteins in the supernatant were concentrated with fumed silica (50 mg) by overnight incubation at 4°C (38). The absorbed HL proteins were eluted from fumed silica with 200 μl of lysis/gel-loading buffer (38.5 mM Tris-HCL, 0.1% EDTA, 2% SDS, 6 M urea, 0.1% dithiothreitol, 0.05% reduced glutathione, 0.001% bromophenol blue) by occasional mixing at 90°C for 20 min, then stored at −80°C until needed for immunoblot analysis.

Pulse-chase analysis

Confluent cells (60 mm dishes) were washed three times with PBS and pulse-labeled with [^{35}S]methionine/cysteine (200 μCi/ml) for 2 h in 2 ml methionine/cysteine- and serum-free DMEM. Media were replaced and cells were chased for 2 or 4 h in 2 ml serum-free media ±100 U/ml heparin. The media were collected at the end of chase, and an aliquot (1 ml) was mixed with 5 μl of the anti-hHL polyclonal antibody overnight at 4°C. The cells were lysed with RIPA buffer (50 mM Tris-HCL, pH 8.0, 1 mM EDTA, 1% Triton X-100, 1% deoxycholic acid, 1 mM dithiothreitol, 150 mM NaCl, 0.015% phenylmethylsulfonyl fluoride, 0.1% SDS), and cell-associated HL was likewise immunoprecipitated from the cell lysates. The antibody-HL complexes were adsorbed to Protein A and washed six times with PBS (for medium samples) or RIPA buffer (for cell samples). The HL was eluted from Protein A with 200 μl of gel loading buffer at 100°C for 10 min, separated by electrophoresis on 10% polyacrylamide gel containing 0.1% SDS (SDS-PAGE), and analyzed by fluorography.

Statistical analyses

Data where statistical values were provided were analyzed using the paired *t*-test. The error bars on data are ±SD.

RESULTS

Expression of chimeric HL proteins

The amino acid sequence of the carboxyl terminus of hHL differs significantly from that of mHL. Not only does hHL have 10 fewer carboxyl terminal amino acid residues than mHL, but also the putative heparin binding domains (HBDs) (22, 26) found in hHL are absent in mHL (**Fig. 1A**). Furthermore, the amino acids in mHL corresponding to the six basic amino acid residues (R or K) located within the putative HBD of hHL (underlined in Fig. 1A) were found to be either acidic or neutral. Notably, four of the basic amino acid residues are present in the carboxyl terminus of hHL. The sequence divergence between hHL and mHL led us to postulate that the carboxyl terminal amino acids govern cell surface association.

To test this hypothesis, we prepared the chimeric proteins hHLmt and mHLht, in which the carboxyl terminal regions of hHL and mHL, respectively, were exchanged. We chose a convenient in-frame *PflmI* restriction endonuclease site within the hHL and mHL cDNAs that allowed us to swap 70 amino acids of hHL with the corresponding 80 amino acids derived from the carboxyl terminus of mHL. To more closely examine the amino acid residues involved in heparin binding, we prepared an hHL mutant (designated hHL_{471}) in which the carboxyl terminal five residues (KRKIR) were deleted. Expression of hHL, mHL, hHLmt, and hHL_{471} was achieved in CHO 13-5-1 cells that are deficient in LRP expression (33). The use of LRP-null cells circumvented complications of potential interaction between HL and LRP (38, 39). Immunoblots of whole-cell extracts from representative hHL, mHL, hHLmt, and hHL_{471} stable cells are shown in Fig. 1B. The doublet of hHL and hHL_{471} (53 kDa and 66 kDa), similar to what was seen for rat HL in primary hepatocytes (40), represented differently glycosylated species. Likewise, the doublet of hHLmt (57 kDa and 72 kDa) was seen in the whole-cell extracts. The molecular mass of mHL was observed to be 55 kDa. Expression of hHL, mHL, hHLmt, and hHL_{471} was also achieved in COS-7 cells, human epithelial kidney 293 cells, and CHO-K1 cells (data not shown). The chimeric protein mHLht transiently expressed in COS-7 cells was unstable and showed multiple degradation products in addition to the full-length protein (Fig. 1C). For this reason, mHLht was not further studied in the following experiments.

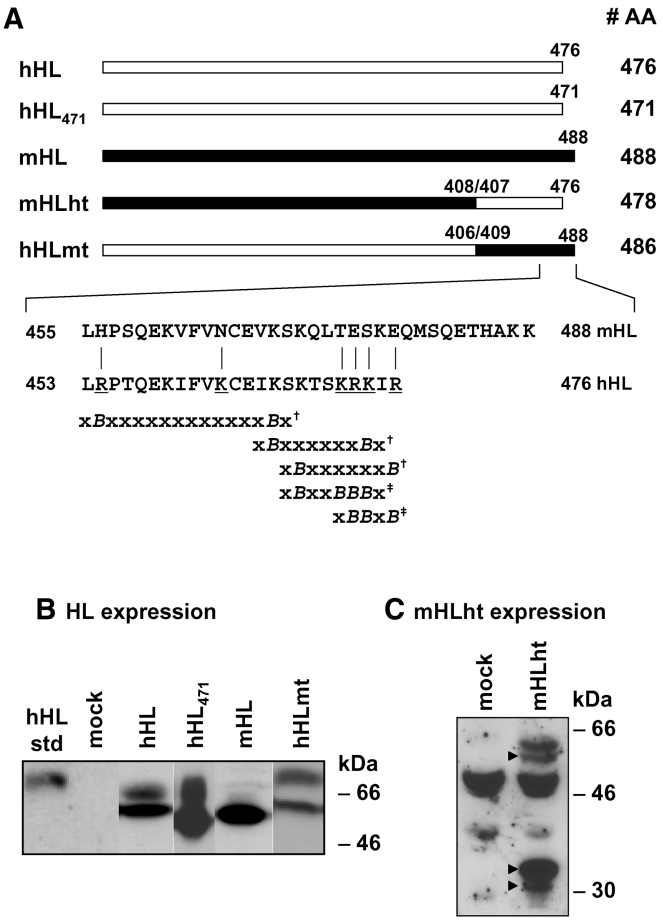

Fig. 1. Structure and transient expression of recombinant hepatic lipase (HL). A: Schematic diagrams of human HL (hHL), hHL_{471}, mouse HL (mHL), mHLht, and hHLmt. The numbers indicate the amino acid positions of the corresponding proteins. The amino acid length of each protein is indicated under "# AA." The carboxyl terminal 33 amino acids of mHL are aligned with the carboxyl terminal 23 amino acids of hHL. Six basic residues (underlined) of hHL are found to be either neutral or acidic in mHL. Shown at the bottom are the basic amino acids of hHL that align with previously described heparin binding domain (HBD) consensus sequences (†ref. 26; ‡ref. 22). B: Immunoblots of HL proteins from whole-cell extracts of stably transfected Chinese hamster ovary (CHO) 13-5-1 cells. The cells were lysed, and an aliquot of the sample (10 μg of cell protein) was resolved by SDS-PAGE. Following transfer to nitrocellulose membranes, the HL proteins were probed using either XHL3-6 (for hHL, hHL_{471}, and hHLmt) or anti-rat HL (for mHL) and visualized by chemiluminescence. C: Expression of mHLht in COS-7 cells. Cells were transfected with mHLht plasmid (10 μg of DNA). Forty-eight hours after transfection, cells were lysed and an aliquot of cell lysate was resolved by SDS-PAGE, and mHLht was visualized by immunoblotting using the anti-rat HL antibody. Degradation products of mHLht are indicated by arrowheads. A 50 kDa protein was detected with the anti-rat HL antibody in both mock and mHLht-transfected COS-7 cells; the identity of this protein is unknown.

Enzymatic activity of recombinant HL

To ascertain whether or not the recombinant HL proteins were catalytically active, we semi-purified the hHL, mHL, hHLmt, and hHL_{471} proteins from conditioned media (in the presence of heparin) by heparin-Sepharose affinity chromatography. All four of the recombinant HL proteins showed catalytic activity toward the short-chained triglyceride tributyrin (**Fig. 2A**) and the long-chained triglyceride triolein (Fig. 2C). No salt-insensitive hydrolytic activity was detected from control mock-transfected cells. The rates of butyrate production and oleate production per microgram of semi-purified hHL, mHL, hHLmt, and hHL_{471} were assessed at different concentrations of tributyrin (Fig. 2A) and triolein (Fig. 2C). The apparent K_m and V_{max} values for the semi-purified HLs (**Table 1**) were obtained from Lineweaver-Burk plots (Fig. 2B, D) using the data shown in Figs. 2A and C, respectively. The apparent K_m values for hHL, hHLmt, and hHL_{471} were comparable, being 0.63 ± 0.17 mM, 0.59 ± 0.13 mM, and 0.57 ± 0.11 mM tributyrin, respectively. These data suggest that the mutant HL proteins had catalytic properties similar to those of hHL when using tributyrin as a substrate. In contrast, mHL has an apparent K_m of 2.83 ± 0.66 mM tributyrin ($P < 0.02$ vs. other HL proteins). Compared with tributyrin, the apparent K_m data using triolein for hHL, hHLmt, and mHL were similar, being 0.57 ± 0.16 mM, 0.74 ± 0.14 mM, and 0.63 ± 0.12 mM triolein, respectively. The apparent K_m value for hHL_{471} was 0.78 ± 0.07 mM. Although the apparent K_m for hHL_{471} was statistically significant versus hHL ($P = 0.02$), it was not significant to the other HL proteins. Thus, these

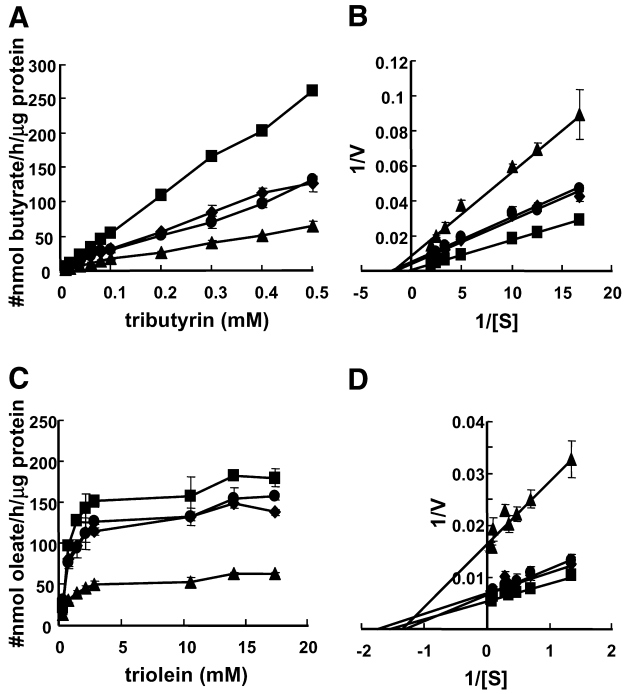

Fig. 2. Enzyme kinetics of semi-purified recombinant HL proteins. Semi-purified hHL (5 μl of 0.346 mg/ml; diamonds), mHL (5 μl of 0.369 mg/ml; squares), hHLmt (5 μl of 0.427 mg/ml; triangles), and hHL_{471} (5 μl of 0.167 mg/ml; circles) were assayed for (A) tributyrin hydrolysis and (B) triolein hydrolysis, as previously described (35, 36). Data were obtained using a range of tributyrin concentrations from 0.01 to 0.50 mM, and triolein concentrations from 0.35 to 17.3 mM. Lineweaver-Burk double-reciprocal plots of data obtained from A and C were generated and are shown in B and D, respectively. Apparent K_m and V_{max} values determined from these plots, and their statistical analyses, are shown in Table 1.

TABLE 1. Apparent K_m and V_{max} values of the recombinant hepatic lipases

HL Species	Tributyrin (n = 3)		Triolein (n = 3)	
	K_m	V_{max}	K_m	V_{max}
	mM tributyrin	*nmol butyrate/h/μg*	*mM triolein*	*nmol oleate/h/μg*
hHL	0.63 ± 0.17	260 ± 46[a]	0.57 ± 0.16	137 ± 4[a]
mHL	2.83 ± 0.66[a]	1663 ± 391[a]	0.63 ± 0.12	179 ± 13[a]
hHLmt	0.59 ± 0.13	118 ± 16[a]	0.74 ± 0.14	60 ± 2[a]
hHL_{471}	0.57 ± 0.11	218 ± 36[b]	0.78 ± 0.07[c]	152 ± 5[a]

hHL, human hepatic lipase; mHL, mouse hepatic lipase.
[a] $P < 0.02$ versus other HLs.
[b] $P < 0.05$ versus other HLs.
[c] $P = 0.02$ versus only hHL.

data suggest that the four HL proteins had a similar catalytic activity when using triolein as a substrate, and that the catalytic site of the chimeric lipase was not grossly affected by the carboxyl terminal amino acid swap. We cannot at this time explain the observed differences in apparent K_m values for mHL versus hHL with the two different substrates, and it is a unique feature of mHL that may warrant further investigation.

Cell surface association of recombinant HL

We demonstrated that hHL has a strong cell surface affinity. In the absence of heparin during a 4 h incubation period, very little hHL (66 kDa form) was detected in media by immunoblot analysis, regardless of the level of hHL expression among different clones (**Fig. 3A**). In contrast, a spontaneous release of mHL into heparin-free media was observed in all stable transfectants that expressed different levels of the mHL protein (Fig. 3B). Thus, there is a striking difference in cell surface binding between hHL and mHL. Similar to the observations with mHL, a spontaneous release of hHLmt (72 kDa form; Fig. 3C) and hHL_{471} (66 kDa form; Fig. 3D) into heparin-free media was also observed in all stable transfectants regardless of the level of expression.

These data suggest strongly that one or more elements within the carboxyl terminal 70 amino acid residues of hHL are necessary for cell surface binding. On average (calculated from densitometry data obtained from at least four individual stable cell lines), release of hHL into heparin-free media was only 4% of the total hHL released by heparin over 4 h (**Fig. 4**). In contrast, release of mHL and hHLmt in the absence of heparin was 61% and 68%, respectively, of the corresponding total heparin-releasable proteins. Thus, mHL and hHLmt exhibited >20-fold reduced affinity toward the cell surface as compared with hHL in transfected CHO 13-5-1 cells. Intermediate between hHL and mHL, 40% of total extracellular hHL_{471} was released into heparin-free media. The percentages of mHL and the mutant HL proteins into heparin-free media were statistically significant versus hHL ($P < 0.001$).

To determine cell surface association of the recombinant HLs, we metabolically labeled them in stable cell lines and monitored their release into media in the absence or presence of heparin. A polyclonal antibody that reacted with native hHL (27) was able to immunoprecipitate mHL and hHLmt from media in the presence of heparin (**Fig. 5A**). Preliminary pulse-chase experiments with hHL-transfected cells showed that the recombinant hHL was recovered almost completely from the cells and media during chase. Semi-quantification of immunoblots by

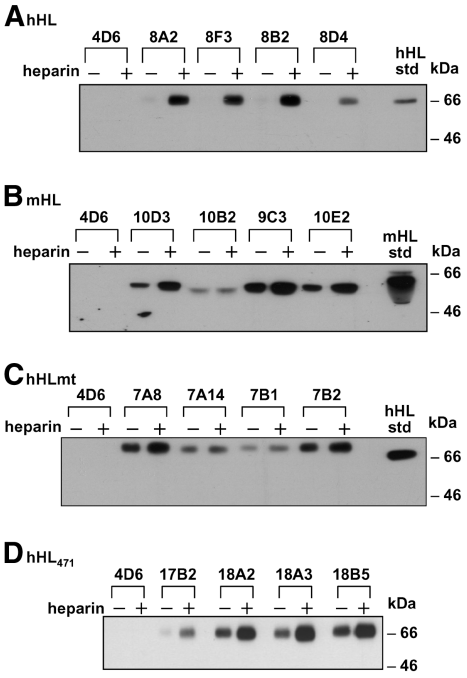

Fig. 3. Analysis of HL secretion in the absence or presence of heparin. Four different clones of (A) hHL-, (B) mHL-, (C) hHLmt-, and (D) hHL_{471}-expressing cells were incubated with or without heparin for 4 h. Media HL was analyzed by immunoblot analysis using XHL3-6 (hHL, hHLmt, and hHL_{471}) or anti-rat HL (mHL). Clone 4D6 is the neomycin-resistant mock-transfected control cell line.

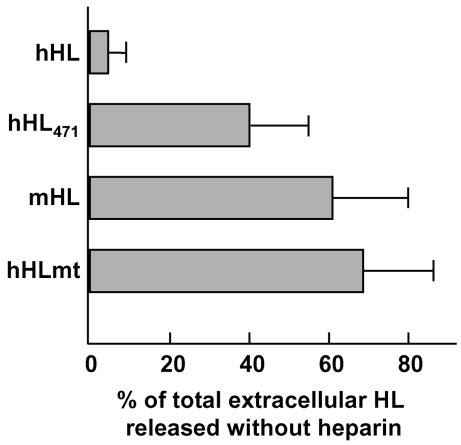

Fig. 4. Semi-quantitative analysis of HL secretion. The intensities of medium HL protein secreted in the absence or presence of heparin (from Fig. 3) were quantified by scanning densitometry of the immunoblots. Data are presented as HL released into heparin-free medium as a percentage of that released into medium containing heparin (i.e., total extracellular HL). $P < 0.001$ for mHL, hHLmt, and hHL_{471} versus hHL.

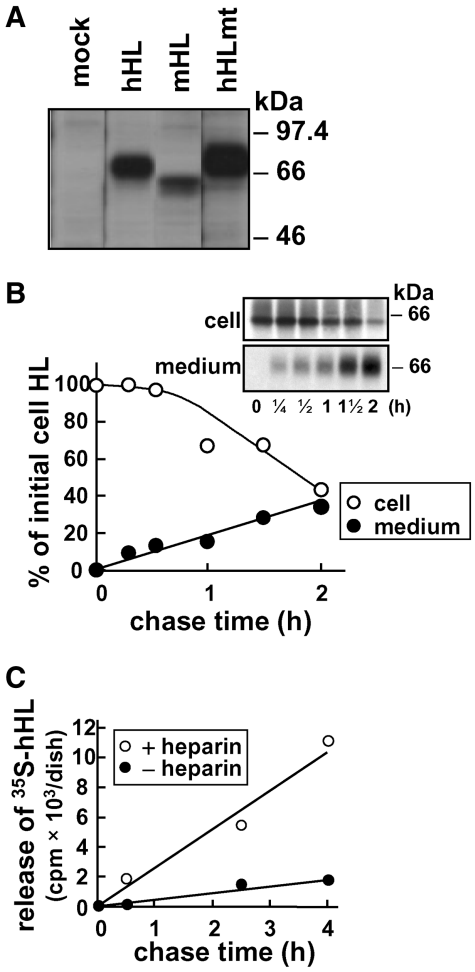

Fig. 5. Pulse-chase analysis of HL secretion. A: Human HL-, mHL-, and hHLmt-expressing cells were pulse-labeled with [^{35}S]methionine/cysteine for 2 h, and chased for 2 h in the presence of 100 U/ml heparin. The HL proteins from media were immunoprecipitated using the polyclonal anti-hHL antibody, resolved by SDS-PAGE, and analyzed by fluorography. B: hHL-expressing cells were pulse-labeled as above and chased for 2 h in the presence of heparin. hHL was immunoprecipitated from the cells and media, respectively, resolved by SDS-PAGE, and analyzed by fluorography (insets). The radioactivity associated with cell and medium hHL was semi-quantified by scanning densitometry, and data are presented as the percentage of initial cell HL (i.e., cell HL at the zero time of chase). C: The hHL-expressing cells (cell line 8A2) were pulse-labeled for 2 h and chased for 4 h with or without heparin. Proteins were resolved by SDS-PAGE, and the radioactivity associated with the medium hHL was excised from the gel and quantified. The rates of hHL release with or without heparin were determined for each time point, and the mean rates are shown in Table 2. Similar pulse-chase data (not shown) for mHL, hHLmt, and hHL_{471} were assessed, and their rates are also shown in Table 2.

scanning densitometry showed that the release of newly synthesized hHL (66 kDa form) into media was linear for up to 2 h, and ~40% of the initially labeled hHL (53 kDa form) from cells was recovered from the medium as the 66 kDa form at the end of chase (Fig. 5B).

Having validated the efficacy of the antibody for immunoprecipitation, we performed quantitative pulse-chase experiments to determine the effect of heparin treatment on the release of hHL (cell line 8A2), mHL (9C3), hHLmt (7A15), and hHL_{471} (17C3) into the chase medium over a 4 h period. We determined the rates of HL release in the absence or presence of heparin into media at three different time points (0.5 h, 2.5 h, and 4 h), and summarized the data in **Table 2**. The release of HL proteins into media is linear, as demonstrated with the hHL pulse-chase data (Fig. 5C). The ratio of the rates for HL release in the absence versus presence of heparin provides the percentage of total extracellular HL released without heparin. The values for hHL, mHL, hHLmt, and hHL_{471} are 12%, 74%, 63%, and 44%, respectively. These data obtained by pulse-chase agree with the densitometry data obtained from multiple cell lines (Fig. 4).

Heparin-Sepharose affinity of recombinant HL proteins

Conditioned media (in the presence of heparin) collected from hHL, mHL, hHLmt, and hHL_{471} cells were subjected to heparin-Sepharose affinity chromatography. Proteins were eluted into 1 ml fractions using a 0.15 M to 1.50 M NaCl gradient, and the eluted proteins were detected by immunoblotting. The hHL was eluted as one peak at 0.76 M NaCl (fraction #34; **Fig. 6A**), whereas the mHL was eluted at lower concentrations of NaCl (Fig. 6B). The eluted mHL from the heparin-Sepharose column exhibited a molecular mass above 220 kDa by SDS-PAGE, suggesting the formation of tetramers during chromatography. The hHLmt chimeric protein had a weaker affinity to heparin-Sepharose compared with hHL, with the peak immunoreactivity eluting at 0.62 M NaCl (fraction #26; Fig. 6C). Thus, substituting the carboxyl terminal 70 amino acids of hHL with mouse sequences decreased the heparin binding to a level comparable to that of mHL. The hHL_{471} mutant was eluted from the heparin-Sepharose column at 0.69 M NaCl (fraction #30; Fig. 6D). Thus, deletion of the carboxyl terminal five residues of hHL partially reduced the concentration of NaCl required to elute hHL from heparin-Sepharose. We also determined that HL immunoreactivity correlated with HL activity (Fig. 6E). In a separate experiment, we subjected conditioned media (400 ml in the presence of heparin) from hHL-expressing cells to heparin-Sepharose chromatography and eluted proteins using an NaCl gradient as above. In this experiment, the peak activity using triolein was found at fraction #28 (0.68 M NaCl, 3.3 μmol oleate/h/fraction), and this peak activity also corresponded with peak immunoreactivity (Fig. 6E, inset).

TABLE 2. Rates of [^{35}S]HL release from cells into media

HL Species	No Heparin	100 U/ml Heparin
	cpm ^{35}S/h	*cpm ^{35}S/h*
hHL[a]	177 ± 155	1428 ± 367
mHL[a]	4947 ± 2366	6623 ± 2995
hHLmt[a]	3865 ± 918	6169 ± 333
hHL_{471}[a]	1984 ± 844	4555 ± 1229

[a] $P < 0.03$ between heparin versus no heparin for each HL. n = 3 for each condition.

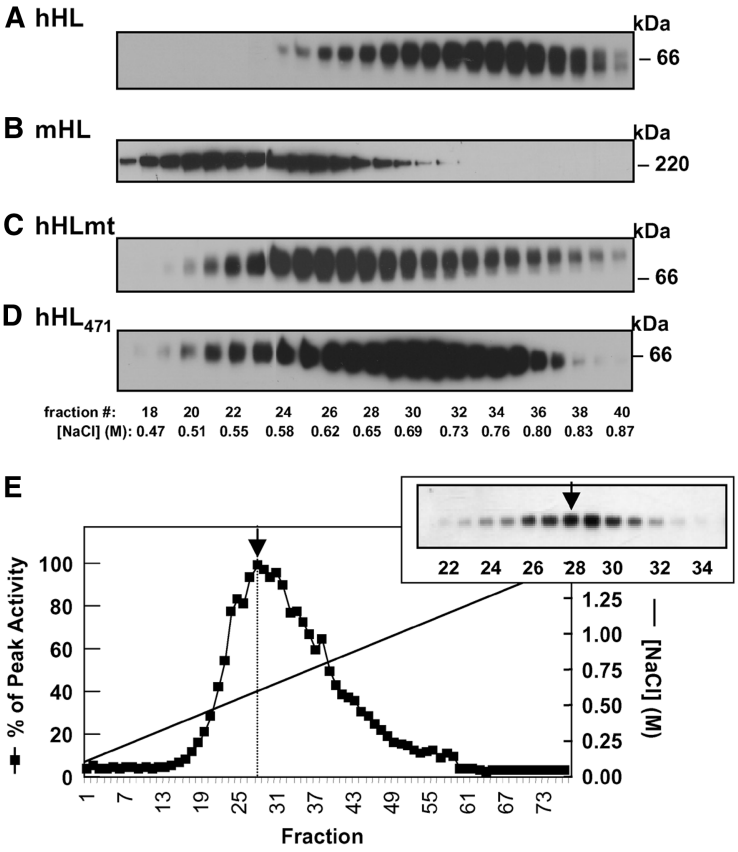

Fig. 6. Heparin-Sepharose chromatography of HL. Conditioned media (40 ml) from (A) hHL, (B) mHL, (C) hHLmt, and (D) hHL_{471} stable cells were collected and subjected to heparin-Sepharose chromatography. Proteins were eluted using a 0.15 M to 1.5 M NaCl gradient, and fractions were resolved by SDS-PAGE and detected by immunoblotting using the antibody XHL3-6 (hHL, hHLmt, and hHL_{471}) or the anti-rat HL antibody (mHL). E: Conditioned media (400 ml) from hHL stable cells were collected and subjected to heparin-Sepharose chromatography in a separate experiment from A–D. Proteins were eluted using a 0.15 M to 1.5 M NaCl gradient, and fractions were assayed for triolein hydrolysis and assessed by immunoblot analysis (insert) using the XHL3-6 antibody. The peak activity and immunoreactivity are indicated by arrows.

DISCUSSION

By using a "loss-of-function" approach, we created two hHL mutants, namely hHLmt and hHL_{471}, that lost part of the cell surface binding function. The present results suggest that at least one determinant of binding to the cell surface, presumably through HSPG, exists within the carboxyl terminal 70 amino acids of hHL, of which the last five residues contribute significantly to the binding. Alignment analysis of the primary amino acid sequences shows divergence at the carboxyl termini between hHL and mHL, particularly among the last 23 amino acids of hHL, which contain several basic amino acids that are not found in mHL (Fig. 1A). Substituting the carboxyl terminal sequence of hHL with that of mHL decreased the cell surface association of hHL by more than 20-fold (Fig. 4). This result is not limited to CHO cells; in the absence of heparin, transfected rat McA-RH7777 hepatoma cells expressing hHLmt released the lipase into media, whereas hHL expressed in these cells was tightly associated with the cell surface (R. J. Brown and Z. Yao, unpublished observations). Thus, our results provide the first in vitro evidence that the difference in cell surface association between hHL and mHL is attributable to divergence in the carboxyl terminal sequence, most likely in the basic amino acid residues.

It is generally considered that an electrostatic interaction between the positively charged amino acid residues and the negatively charged sulfate groups of HSPG is important for protein binding to heparin. However, specific positioning of the positively charged residues within a structural motif is perhaps also important. One of the proposed HBD sequences, xBxxxxxxBx, has been modeled to exist as β-strands in some proteins and as α-helices in others. In either case, the two basic residues are spaced 20 Å apart (26). Recent studies with human vitronectin show that residues 343–356, which are involved in heparin binding, are arranged in the xBBxBx sequence (41), which is thought to assume a β-sheet conformation. It should be noted that the carboxyl terminal 10 amino acids of hHL have both the xBBxBx and xBxxxxxxBx sequence (Fig. 1A). Thus, the cell surface binding motif of hHL may reside at this region in a β-strand conformation. What remains to be determined is the critical residue(s) within the KRKIR motif of hHL that confer to HSPG binding. In addition to electrostatic contacts, protein-heparin interactions involve other forces. Studies with fibroblast growth factor receptor, for example, have indicated that its interaction with heparin requires substantial van der Waals contact (42). An understanding of HSPG binding motifs within hHL awaits detailed structural analysis of the protein.

Although our study suggests that the carboxyl terminal basic amino acid residues are the major factors for HL cell surface association, it by no means implies that these are the sole determinants. In fact, mHL exhibits weak cell surface association. Approximately one-third of total releasable mHL and hHLmt remained associated to the cell surface in the absence of heparin (Fig. 4). These results suggest that sequences other than the carboxyl terminal

region must contribute to the remaining overall affinity of mHL. A recent study reported that six basic amino acid residues located in two clusters (Cluster 1: Lys^{297}, Lys^{298}, and Arg^{300}; Cluster 4: Lys^{436} and Arg^{443}) of rat HL were involved in heparin binding (43). These two clusters of basic amino acid residues have the xBBxBx and xBxxxxxxBx arrangement, respectively, that are characteristic of the putative HBD (22, 26). Alignment analysis revealed that these two clusters of the rat sequences were conserved in both hHL and mHL. Hence, it is likely that these residues may constitute the weak binding affinity components of hHL and mHL.

While the present studies derived from the "loss-of-function" approach are suggestive, they do not allow one to draw a definitive conclusion that the carboxyl terminus of hHL possesses the HBD. We attempted a "gain-of-function" approach by creating the mutant mHLht in which the carboxyl terminal sequences of the mHL were substituted with hHL sequences. Unfortunately, this chimeric protein was extremely unstable and was degraded in transfected cells with no detectable secretion (Fig. 1C). Thus, the "gain-of-function" of mHL in heparin binding by introducing the human sequences was not demonstrated in this study.

The physiological significance of cell surface binding by hHL is unclear. The functional role of hHL has been implicated in multiple processes in lipoprotein metabolism, such as selective cholesteryl ester uptake (44) and remnant lipoprotein clearance (38, 39, 45). In the development of atherosclerosis, the activity of HL has been considered as both pro-atherogenic and anti-atherogenic (46, 47). The development of an expression system for a catalytically active but HSPG binding-deficient hHL will allow us to examine the possibility that HL cell surface association plays an important physiological role, along the line of studies performed on human LPL (48).

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